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The Molecular Basis for the Pharmacokinetics and Pharmacodynamics of Curcumin and Its Metabolites in Relation to Cancer[S]

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ABBREVIATIONS: AA, arachidonic acid; ABC, ATP-binding cassette; $ABTS^{\bullet+}$, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation; AP-1, activator protein 1; ATM, ataxia-telangiectasia mutated; ATR, ATM and RAD3-related; BAG3, Bcl-2-associated athanogene 3; BCRP, breast cancer resistance protein; bFGF, basic fibroblast growth factor; 53BP1, tumor suppressor p53-binding protein 1; BRCA1, breast cancer type 1 susceptibility protein; Cdk, cyclin-dependent kinase; CKI, cyclin kinase inhibitor; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; ^{3}Cur, triplet state curcumin; CXCL, chemokine (C-X-C motif) ligand; DcytB, duodenal cytochrome B; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DSC, differential scanning calorimetry; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPR, electron paramagnetic resonance; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; ESIPT, excited state intramolecular proton transfer; FasL, Fas ligand; FDA, Food and Drug Administration; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; FOXP3, regulatory T-forkhead box P3; FT-IR, Fourier transform infrared spectroscopy; GADD153, growth arrest and DNA damage-inducible gene 153; GSH, reduced glutathione; GSK-3β, glycogen synthase kinase-3 β; GST, glutathione S-transferase; H_2O_2, hydrogen peroxide; HAT, hydrogen atom transfer; HB-EGF, heparin-binding epidermal growth factor-like growth factor; HER, human ErbB; HIMEC, human intestinal microvascular EC; HIV, human immunodeficiency virus; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory concentration; ICT, intramolecular charge transfer; IL, interleukin; IL-1R, IL-1 receptor; iNOS, inducible nitric-oxide synthase; IRAK, IL-1 receptor-associated kinase; JNK, c-Jun N-terminal kinase; k, rate constant; k_{deg}, degradation rate constant; LC_{50}, 50% lethal concentration; log P, partition coefficient; MAPK, mitogen-activated protein kinase; MDM2, murine double minute 2; MEK, mitogen-activated protein kinase/extracellular signal-regulated protein kinase kinase; miR, microRNA; MMP, matrix metalloproteinase; mPGES-1, microsomal prostaglandin E_2 synthase-1; MRP, multidrug resistance protein; MS, mass spectrometry; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBT, nitroblue tetrazolium; NDP, 3,3′-(1,4-naphthylene)-dipropionate; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; NMR, nuclear magnetic resonance spectroscopy; $^1O_2$, singlet oxygen; PG, prostaglandin; P-gp, P-glycoprotein; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; pRB, retinoblastoma protein; PTEN, phosphatase and tensin homolog; ε_r, relative permittivity; RB, retinoblastoma; ROS, reactive oxygen species; Δ_S, Stokes shift; SET, single electron transfer; SOD, superoxide dismutase; SPLET, sequential proton loss electron transfer; SRC-1, steroid receptor coactivator-1; SrcK, c-Src tyrosine kinase; SULT, sulfotransferase; SUMO, small ubiquitin-related modifiers; Tbsp, thrombospondin; TEMP, 2,2,6,6-tetramethyl piperidine; TEMPO, 2,2,6,6-tetramethyl piperidine-O_2 adduct; TGF, transforming growth factor; TNF-α, tumor necrosis factor α; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T-cell lineage cell; TrxR, thioredoxin reductase; UGT, uridine diphosphate-glucuronosyltransferase; uPA, urokinase-type plasminogen activator; VDR, vitamin D receptor; VDRE, vitamin D response element; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; WT1, Wilm's tumor-associated gene; X, halogen.

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***Abstract*——This review addresses the oncopharmacological properties of curcumin at the molecular level. First, the interactions between curcumin and its molecular targets are addressed on the basis of curcumin's distinct chemical properties, which include H-bond donating and accepting capacity of the *β*-dicarbonyl moiety and the phenylic hydroxyl groups, H-bond accepting capacity of the methoxy ethers, multivalent metal and nonmetal cation binding properties, high partition coefficient, rotamerization around multiple C–C bonds, and the ability to act as a Michael acceptor. Next, the in vitro chemical stability of curcumin is elaborated in the context of its susceptibility to photochemical and chemical modification and degradation (e.g., alkaline hydrolysis). Specific modification and degradatory pathways are provided, which mainly entail radical-based intermediates, and the in vitro catabolites are identified. The implications of curcumin's (photo) chemical instability are addressed in light of pharmaceutical curcumin preparations, the use of curcumin analogues, and implementation of nanoparticulate drug delivery systems. Furthermore, the pharmacokinetics of curcumin and its most important degradation products are detailed in light of curcumin's poor bioavailability. Particular emphasis is placed on xenobiotic phase I and II metabolism as well as excretion of curcumin in the intestines (first pass), the liver (second pass), and other organs in addition to the pharmacokinetics of curcumin metabolites and their systemic clearance. Lastly, a summary is provided of the clinical pharmacodynamics of curcumin followed by a detailed account of curcumin's direct molecular targets, whereby the phenotypical/biological changes induced in cancer cells upon completion of the curcumin-triggered signaling cascade(s) are addressed in the framework of the hallmarks of cancer. The direct molecular targets include the ErbB family of receptors, protein kinase C, enzymes involved in prostaglandin synthesis, vitamin D receptor, and DNA.**

I. Introduction

Carcinogenesis, cancer cell proliferation, and metastasis encompass a myriad of complex signaling pathways governed by multitudinous intracellular and extracellular (bio)molecules and ions that are collectively responsible for forging the cancer phenotype. Typically, malignant cellular transformation is driven by congenital or acquired genetic in- or transactivation of endogenous signaling pathways. These pathways are tightly regulated in healthy cells but become derailed (hypo- or hyperactivated) in neoplasms. In addition, after a neoplastic state has materialized, the cancer phenotype is constantly subjected to molecular changes in response to an inductive micro-environment, further altering the activity and involvement of different signaling pathways.

To fight cancer effectively, therapeutic strategies should take the dynamic nature of the cancer phenotype into account. The most ideal chemopreventive and chemotherapeutic agents therefore comprise those that

affect multiple molecular targets, preferably in the different phenotypic manifestations of the cancer cell, with minimal toxicity for healthy cells. In view of the latter, the majority of synthetic chemotherapeutics (e.g., alkylating agents, topoisomerase inhibitors, and antimetabolites), plant alkaloids (e.g., vinca alkaloids and taxanes), as well as cytotoxic antibiotics (e.g., anthracyclines) targets rapidly dividing and metabolically hyperactive cancer cells at a limited number of molecular loci. Although these compounds are notably lethal to cancer cells, the inadvertent uptake by non-cancerous cells triggers their death also, accounting for the chemotherapy-induced sequelae that patients often experience.

The use of a multitargeted approach to cancer chemotherapy (Lee et al., 2011b) may therefore be instrumental in curtailing therapy-related side effects while preserving therapeutic efficacy. Such a multitargeted modality comprises either a lower dose cocktail of highly toxic chemotherapeutic agents [referred to as metronomic dosing (Hanahan et al., 2000)] or a single, mildly to nontoxic compound that targets multiple components of vital signaling pathways in predominantly cancer cells. Certain phytochemicals are particularly eligible for the single compound type modality inasmuch as they are pleiotropic modulators of manifold signal transduction pathways and exhibit relatively low toxicity in non-cancerous cells (Khan et al., 2008; Lee et al., 2011b). Unfortunately, the currently Food and Drug Administration (FDA)-approved chemotherapeutic phytochemicals such as paclitaxel, docetaxel, vinblastine, and vincristine have only a singular molecular target (tubulin), whereas other phytochemical candidates such as (–)-epigallocatechin gallate and delphinidin have multiple molecular targets (Ermakova et al., 2006; Li et al., 2007; He et al., 2008; Kang et al., 2008; Shim et al., 2008, 2010; Hwang et al., 2009; Ozbay and Nahta, 2011) and in vitro cytostatic/cytotoxic potency (Bin Hafeez et al., 2008; Hsieh and Wu, 2009; Philips et al., 2009; Qiao et al., 2009; Yun et al., 2009; Cvorovic et al., 2010; Das et al., 2010) but lack an advanced clinical development trajectory and regulatory approval status.

One phytochemical that fully meets the criteria of the single compound-type modality is curcumin [(1*E*,6*E*)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione/diferuloylmethane], a polyphenolic compound derived from the rhizome of the *Curcuma longa*. Curcumin inhibits multiple vital pathways in cancer cells (Kunnumakkara et al., 2008) that affect all hallmarks of cancer (Hanahan and Weinberg, 2011), rendering curcumin a chemopreventive, oncostatic, and antimetastatic agent. Interestingly, curcumin appears to be toxic to cancer cells but cytoprotective to healthy cells (Dinkova-Kostova and Talalay, 2008; Bisht et al., 2010), owing in part to its strong antioxidative capacity (Priyadarsini et al., 2003). Numerous studies in humans have established that oral consumption of high doses of curcumin, up to 12,000 mg/day, is tolerable and safe (Shoba et al., 1998; Cheng et al., 2001; Sharma et al., 2004; Lao et al., 2006), and several clinical phase I and II trials have been conducted with curcumin for the treatment of multiple types of cancer (Cheng et al., 2001; Anand et al., 2008; Goel et al., 2008; Hatcher et al., 2008; Shehzad et al., 2010; Gupta et al., 2013). Moreover, curcumin has been shown to alleviate senescence (Sikora et al., 2010) and various forms of neurodegenerative (Jomova et al., 2010), infectious (Zhao et al., 2011), and autoimmune disease (Bright, 2007). The medical disciplines and research fields in which curcumin has been studied exemplify the breadth of medical and biotechnological potential of this phytochemical, with cancer clearly leading the way.

This review focuses on the oncopharmacological properties of curcumin in the context of the hallmarks of cancer. First, the relationship between curcumin structure and its ability to undergo pleiotropic intermolecular bonding and chemical stability and solubility are addressed to provide a backdrop against which information on the pharmacokinetics and pharmacodynamics of curcumin as well as its in vitro and in vivo metabolites is presented. Following a brief summary of the cancer hallmarks, the *direct* molecular targets of curcumin are identified, and a detailed account is given of the molecular pathways that are affected as a result of curcumin binding. Subsequently, the phenotypical/biological changes induced in cancer cells upon completion of the curcumin-triggered signaling cascade are addressed in the framework of the hallmarks of cancer. Whereas previous reviews have typically provided lists of (bio)molecules that are directly and indirectly affected by curcumin (Johnson and Mukhtar, 2007; Goel et al., 2008; Gupta et al., 2011; Zhou et al., 2011), this review focuses exclusively on *direct* curcumin-ligand binding as a starting point for explaining the downstream cellular effects pertinent to cancer biology and treatment. The molecular targets were selected on the criteria that (1) the curcumin-ligand interaction affects multiple cancer hallmarks, (2) curcumin binds selectively to multiple targets in a single pathway, and/or (3) curcumin binding imposes imminent lethality on the cell (e.g., by inducing interference with vital transcriptional activity and subsequent cell death). The molecular targets addressed in this review are therefore the ErbB family of receptors, protein kinase C, enzymes involved in prostaglandin synthesis, vitamin D receptor, and DNA.

Readers should note that, although it is our opinion that all sections presented in this review are imperative to fully appreciate curcumin as a pharmaceutical compound, we understand that not everyone may be equally interested in all parts. Accordingly, the sections

were divided into chemical properties, pharmacokinetics, and pharmacodynamics to facilitate selective reading and written such that each section can be understood in a stand-alone manner.

II. Chemical Properties of Curcumin

Information on the structure, solubility, and stability of curcumin is imperative for the proper interpretation of its pharmacokinetic and pharmacodynamic properties. The majority of curcumin-related data have been generated using cell-based assays that, particularly in the case of this compound, are not necessarily representative of the in vivo situation.

A. Curcumin Structure: Implications on Intermolecular Interactions

The generic structures of the turmeric-derived curcuminoids are provided in Fig. 1A, which, in order of their relative abundance in the root, comprise curcumin, demethoxycurcumin, *bis*-demethoxycurcumin, and cyclocurcumin. Curcumin (R_1 and R_2 = OCH_3) is an amphipathic molecule with polar central and flanking regions that are separated by a lipophilic methine segment (Fig. 1B). Curcumin possesses seven distinct chemical properties that facilitate intermolecular interactions and thus association with its biomolecular targets. These include (1) H-bond donating and accepting capacity of the β-dicarbonyl moiety, (2) H-bond accepting and donating capacity of the phenylic hydroxyl residues, (3) H-bond accepting capacity of the ether residue in the methoxy groups, (4) multivalent metal and nonmetal cation binding properties, (5) high partition coefficient (log P), (6) rotamerization around multiple C–C bonds, and (7) behavior as a Michael reaction acceptor. The interactions that are based on H-bonding with specific functional groups of curcumin are summarized in sections II.A.1 through II.A.3, and the hydrophobic interactions are addressed in section II.A.5. A full overview of the curcumin-target molecule interactions is provided in Supplemental Table 1, which summarizes additional molecular targets for which the interacting protein or nucleic acid residues were identified by docking studies or site-directed mutagenesis but not in relation to curcumin's specific functional groups.

1. H-bond Donating and Accepting Capacity of the β-Dicarbonyl Moiety. Curcumin's β-dicarbonyl moiety deviates in some respects from the typical behavior of β-diketones in solution, including acetylacetone (H_3C–CO–CH_2–CO–CH_3), the central structure of curcumin. β-Diketones other than curcumin generally prevail in the diketo tautomeric form in solutions with high relative permittivity (ε_r) or polarity [e.g., water and dimethylsulfoxide (DMSO)] and in the enol tautomeric form in solutions with low ε_r [e.g., (cyclo)hexane and carbon tetrachloride] (Emsley, 1984). Antithetically, the β-diketone of curcumin is proposed to exist predominantly as an enol (Fig. 1A) in both aqueous solution and in organic solvents with a lower ε_r than water, particularly in polar protic solvents (e.g., alcohols) and polar aprotic solvents (e.g., acetonitrile, DMSO) (Fig. 1B). The tendency of the keto↔enol equilibrium to shift toward the enol tautomer in these solvent systems has been confirmed experimentally by Fourier transform infrared spectroscopy (FT-IR) (Kolev et al., 2005), nuclear magnetic resonance spectroscopy (NMR) (Roughley and Whiting, 1973; Unterhalt, 1980; Gorman et al., 1994; Khopde et al., 2000), fluorescence spectroscopy (Khopde et al., 2000; Nardo et al., 2008), and absorption spectroscopy (Khopde et al., 2000; Nardo et al., 2008) as well as computationally (Kolev et al., 2005; Balasubramanian, 2006; Payton et al., 2007; Galano et al., 2009). In nonpolar solvents, the steady-state absorption spectra of curcumin in cyclohexane (Nardo et al., 2008), toluene (Chignell et al., 1994), benzene (Khopde et al., 2000), and others (Tønnesen et al., 1995) exhibit a concrete red band or shoulder, which has been ascribed to the diketo tautomer (Khopde et al., 2000; Nardo et al., 2008, 2009). Curcumin hence adopts both the enol and diketo tautomeric form in nonpolar environments (Balasubramanian, 2006), albeit the diketo tautomer is quantitatively exiguous relative to the enol tautomer (Kolev et al., 2005), and exclusively the enol tautomeric form in solvents with increasing ε_r (Fig. 1B). This is underscored by the fact that the red shoulder in the absorption spectrum of curcumin dissolved in water-dioxane, a slightly nonpolar but water-miscible solvent, disappears progressively at increasing water:dioxane ratios (Ortica and Rodgers, 2001).

Accordingly, the conformation of the β-diketone is highly dependent on the chemical environment that in turn dictates intermolecular bonding behavior. In nonpolar solvents, curcumin primarily exists as a closed *cis*-enol tautomer, where the proton is believed to be symmetrical (Nardo et al., 2008) in an electron-delocalized system (Emsley, 1984; Balasubramanian, 2006) (Fig. 1B). The enolic proton and carbonyl oxygen are therefore unavailable for H-bonding to other molecules. The intramolecular H-bonding is perturbed in weakly as well as strongly H-bonding solvents by the polar solvent molecules, as a result of which the enol tautomer adopts an open conformation (Emsley, 1984; Toullec, 1990; Khopde et al., 2000; Nardo et al., 2008), thereby abrogating the symmetry of the semiaromatic ring (Nardo et al., 2008, 2009) (Fig. 1B). In the open conformation, the valence electrons of the carbonyl and enolic oxygen act as H-bond acceptors and the enolic proton serves as an H-bond donor, possessing charges of −0.73, −0.70, and 0.50 in water (Fig. 1C), respectively (Balasubramanian, 2006). The interaction between curcumin's β-carbonyls and molecules with increasing ε_r is reflected by the generally larger Stokes

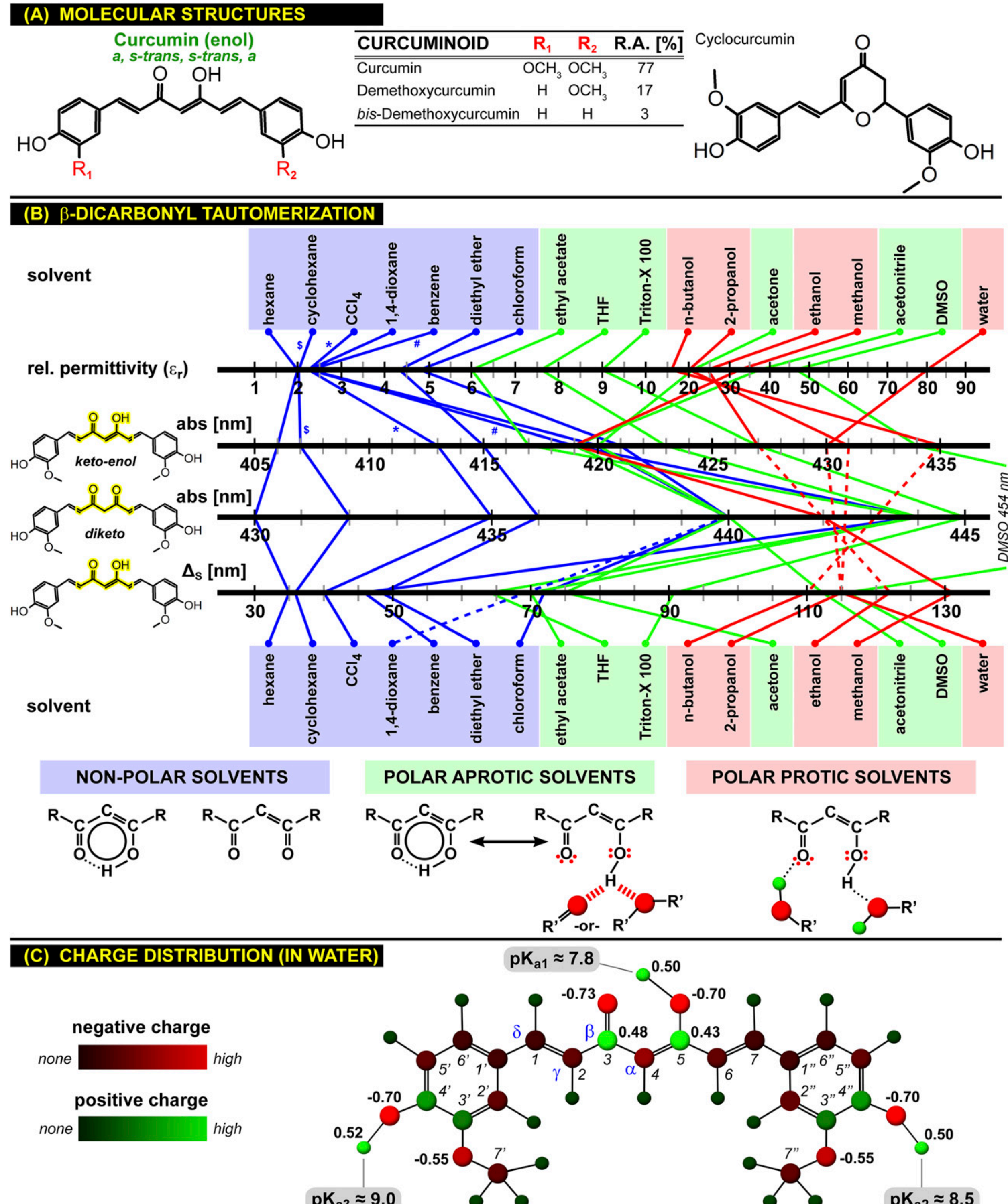


Fig. 1. A, Molecular structures of curcuminoids, which comprise 3–5% of turmeric (*Curcuma longa*). The left scheme depicts the generic structure of the most abundant curcuminoids, which are further specified in the table [R.A. = relative abundance, data from Goel et al. (2008)]. Only the structure of the most energetically stable enol conformer of curcumin ($R_1 = R_2 = OCH_3$) is portrayed (Balasubramanian, 2006), where the R_1 and R_2 moieties have adopted the *a, s-trans, s-trans, a* orientation [*a* (and *b*) represents the orientation of the OCH_3 moiety around the C–phenyl bond for R_1 and R_2, and the *trans* and *cis* notations refer to the $–C_\delta–C_\gamma–C_\beta–$ segment of the methine bridge] (Kolev et al., 2005). It should be noted that the optimized structure depends on the computational program and input variables used, as the *a, s-trans, s-trans, b* enol (Payton et al., 2007; Galano et al., 2009; Agnihotri and Mishra, 2011) and the *a, s-trans, s-trans, b* enol (Kolev et al., 2005) have also been proposed as the most stable conformer. As opposed to the planar conformation of enolic curcumin, the less abundant and less stable diketo form of curcumin is nonplanar, whereby the ketones are oriented *anti* relative to each other (Kolev et al., 2005; Balasubramanian, 2006; Agnihotri and Mishra, 2011). The least abundant curcuminoid, cyclocurcumin (right scheme), is the most recently isolated and characterized species (Kiuchi et al., 1993). B, A diagram of the β-dicarbonyl tautomer properties in nonpolar solvents (blue), polar aprotic solvents (green), and polar protic solvents (red) is presented in the upper portion. Per solvent, the relative permittivity (ε_r), the corresponding absorption maximum of enolic curcumin (top structure) and diketonic curcumin (middle structure), as well as the Stokes shift

shifts (Δ_S) with increasing solvent polarity (Fig. 1B). Accordingly, under physiological circumstances the open enol tautomer can interact with water molecules in, e.g., plasma and cytoplasm, or with polar amino acid residues in target or carrier proteins, either directly or indirectly via water molecules.

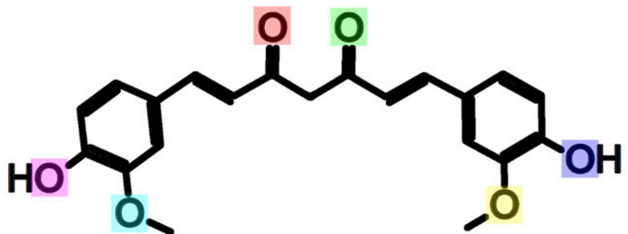
A summary of the H-bond-mediated interactions between curcumin's β-diketone moiety and amino acid residues in different proteins is provided in Fig. 2 for the H-bond accepting carbonyls and in Fig. 3 for the H-bond donating enolic proton. Some of the proteins that curcumin interacts with through the β-diketone moiety that may bear relevance to cancer include DNA methyltransferase 1 (Yoo and Medina-Franco, 2011), aldose reductase 1 and 2 (Muthenna et al., 2009), lipoxygenase (Katsori et al., 2011), 20S proteasome (Milacic et al., 2008), tubulin (Li et al., 2012), cyclooxygenase (COX)-2 (Selvam et al., 2005) (sections III. E.1.e and III.E.7), glycogen synthase kinase-3 β (GSK-3β) (Bustanji et al., 2009) (section III.E.1.b.ii), and glyoxalase I (Liu et al., 2010).

2. H-bond Accepting and Donating Capacity of the Phenylic Hydroxyl Groups. As the centrally positioned β-diketone moiety, the flanking hydroxyl groups impart H-bond accepting and donating capabilities on the molecule, expanding the number of possible interaction sites that account for curcumin's pleiotropic binding behavior. The intermolecular interactions facilitated by the phenylic hydroxyl groups are summarized in Fig. 2 for the H-bond accepting phenylic oxygens and in Fig. 3 for the H-bond donating phenylic protons. In addition to most of the protein targets mentioned in the preceding section, histone deacetylase 8 (Bora-Tatar et al., 2009) and protein kinase C (PKC)δ (Majhi et al., 2010; Mamidi et al., 2012) (section III.E.6) have been identified as cancer-pertinent proteins with which curcumin associates as a result of phenylic hydroxyl group-mediated H-bonding.

3. H-bond Accepting Capacity of the Methoxy Groups. The third functional group that is capable of mediating intermolecular interactions is the methoxy group, which can only act as an H-bond acceptor through the valence electrons of the ether. The intermolecular interactions facilitated by the methoxy ether are summarized in Fig. 2. No additional protein targets than those previously described in sections II.A.1 and II.A.2 were found to bind curcumin through the methoxy groups. Interestingly, docking studies with diketonic curcumin, i.e., the physiologically less abundant isoform (section II.A.1), revealed H-bonding with 14 amino acids of seven different proteins but no interactions for enolic curcumin (Figs. 2 and 3).

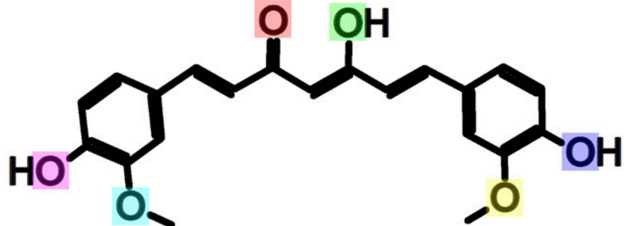
4. Multivalent Metal and Nonmetal Cation Binding. Molecules containing a β-diketone are known to form stable complexes with multivalent metal cations (Skopenko et al., 2004). For example, acetylacetone, curcumin's central moiety (section II.A.1), can complex metals at both oxygen atoms (in case of the diketo form), the ketone oxygen (in case of the keto-enol form), the olefin (C_α=C_β), or the C_α, which has a partial negative charge (Fig. 1C) (Cotton and Wilkinson, 1980). Accordingly, curcumin forms chelates with metals such as boron (Mohri et al., 2003; Rao and Aggarwal, 2008), copper (Baum and Ng, 2004; Barik et al., 2007; Zebib et al., 2010; Addicoat et al., 2011), aluminum (Jiang et al., 2011), magnesium (Zebib et al., 2010), zinc (Zebib et al., 2010), lead (Daniel et al., 2004), cadmium (Daniel et al., 2004), and ferrous (Fe^{2+}) and ferric (Fe^{3+}) iron (Tønnesen and Greenhill, 1992; Borsari et al., 2002; Baum and Ng, 2004; Bernabe-Pineda et al., 2004b; Ak and Gulcin, 2008; Dairam et al., 2008), but also with metal oxides such as vanadyl (Thompson et al., 2004) and nonmetals such as selenium (Zebib et al., 2010). Although the majority of experimental data indicate that the metal cations bind the oxygens of the β-diketo moiety (Borsari et al., 2002; Zebib et al., 2010; Jiang

(Δ_S, bottom structure) of enolic curcumin are plotted on the axes, all connected by a color-coded single trace. The dotted portion of the traces indicates that the respective axis is not crossed, either because no absorption maximum (i.e., no diketo form) exists for curcumin in the respective solvents (red dotted lines) or that the data have not been measured (Δ_S for curcumin in 1,4-dioxane, blue dotted lines). The traces of cyclohexane ($), carbon tetrachloride (CCl_4) (*), and benzene (#) are symbol-tagged for clarity. The absorption maximum of the diketo form is characterized by a red shoulder in the main absorption band of curcumin (Ortica and Rodgers, 2001). The absorption maximum of diketonic curcumin in DMSO at 454 nm was truncated. The Δ_S was calculated for the fluorescence emission maximum versus the absorption maximum of enolic curcumin. Data assembled from Tønnesen et al. (1995), Khopde et al. (2000), Ortica and Rodgers (2001) or measured (absorption and fluorescence emission spectra of freshly prepared curcumin in pH-unadjusted MilliQ water). The ε_r of Triton-X 100 was obtained from Asami (2007). For more detailed information on the spectroscopic properties of curcumin in different solvent systems the readers are referred to Priyadarsini (2009). In the bottom portion of the panel, the proposed molecular configurations of the β-dicarbonyl moiety are provided per type of solvent. The (••••) indicates hydrogen bonding and the (IIII) designates charge-charge interactions or H-bonding between curcumin and H-bond accepting solvent molecules (e.g., DMSO). The color of the atoms of the solvent molecules corresponds to partial positive charge (green) and partial negative charge (red). As is evident from the traces (dotted lines), the provided conformation under polar protic solvents applies to all listed solvents except for methanol, in which curcumin also exists as a diketo tautomer. The conformations were adapted from Nardo et al. (2008). C, Charge distribution (Balasubramanian, 2006), numbering (carbon designations are given in blue), and pK_a values of curcumin in water (Tønnesen and Karlsen, 1985b). Other published [calculated] pK_a values for curcumin in water include pK_{a2} = 8.55, pK_{a3} = 10.41 (Jovanovic et al., 1999) and pK_{a1} = [8.38] and [8.34], pK_{a2} = [9.88] and [9.75], and pK_{a3} = [10.51] and [10.10] (Bernabe-Pineda et al., 2004a). At pH = 7.4, approximately 29% of the first proton is dissociated, as opposed to 5% at pH = 6.5 (Jaruga et al., 1998). In the presence of methanol, the pK_a values are higher, namely pK_{a1} = 8.54, pK_{a2} = 9.30, and pK_{a3} = 10.69 [in water: methanol (1:1) mixtures] (Borsari et al., 2002). For more detailed information on acidity constants, see Priyadarsini (2009).



CURCUMIN (DIKETO FORM)
H-BOND ACCEPTING OXYGENS

Gln1226, side-chain amino proton, DNA methyltransferase 1 (human) [1] Trp111, aldose reductase 2 (human) [2] Lys263, aldose reductase 1 (human) [2] His513, lipoxygenase (soybean) [3] Gly20, FtsZ (*Escherichia coli*) [4]	Gly21, FtsZ (*Escherichia coli*) [4] Gly22, FtsZ (*Bacillus subtilis*) [4] Thr1, 20S proteasome β5 CT-like subunit, origin unspecified [5] Gln11, tubulin chain A (bovine) [6]
Lys1462, side-chain amino proton, DNA methyltransferase 1 (human) [1] His110, aldose reductase 2 (human) [2] Ser215, aldose reductase 1 (human) [2]	Gln267, PfATP6 (*Plasmodium falciparum*) [7] Gly109, FtsZ (*Escherichia coli*) [4] Gly21, FtsZ (*Bacillus subtilis*) [4] Lys254, tubulin chain B (bovine) [6]
Arg1311, 2 guanidino protons, DNA methyltransferase 1 (human) [1] Tyr50, aldose reductase 1 (human) [2] Lys80, aldose reductase 1 (human) [2]	Ala985, PfATP6 (*Plasmodium falciparum*) [7] Tyr100, amidic carbonyl, histone deacetylase 8 (human) [8] Asp1, monomeric amyloid beta peptide $(A\beta)_{9\text{-}40}$ (human) [9] Ser26, hexameric amyloid beta peptide $(A\beta)_{9\text{-}40}$ (human) [9]
His1458, imidazole amino proton, DNA methyltransferase 1 (human) [1] Thr266, aldose reductase 1 (human) [2] Arg269, aldose reductase 1 (human) [2] Ile1041, PfATP6 (*Plasmodium falciparum*) [7]	Leu1040, PfATP6 (*Plasmodium falciparum*) [7] Asp29, side-chain carbonyl, histone deacetylase 8 (human) [8] Met65, via H_2O bridge, glyoxalase I (human) [10]
Leu300, aldose reductase 2 (human) [2] Leu301, aldose reductase 2 (human) [2] Thr132, FtsZ (*Escherichia coli*) [4] Asn165, FtsZ (*Escherichia coli*) [4] Thr133, FtsZ (*Bacillus subtilis*) [4]	Asn166, FtsZ (*Bacillus subtilis*) [4] Gln257, amidic proton, protein kinase C δ (homology modeled) [11] Thr242, amidic proton, protein kinase C δ C1b subdomain [12]
Trp20, aldose reductase 2 (human) [2] Thr19, aldose reductase 2 (human) [2] Lys263, aldose reductase 1 (human) [2] Gly72, FtsZ (*Bacillus subtilis*) [4]	Tyr111, side-chain hydroxyl proton, histone deacetylase 8 (human) [8] Lys28, side-chain amino proton, hexameric amyloid beta peptide $(A\beta)_{9\text{-}40}$ (human) [9]



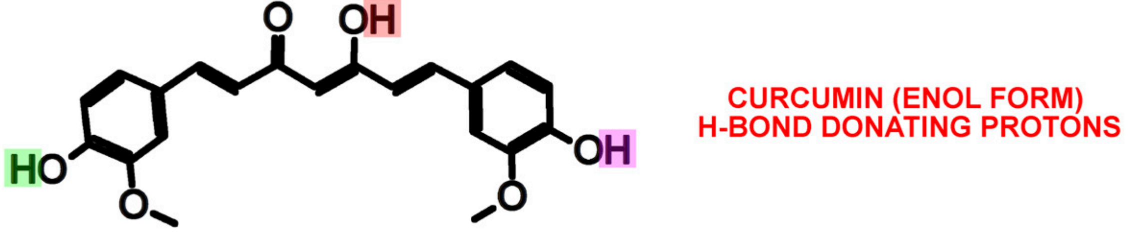
CURCUMIN (ENOL FORM)
H-BOND ACCEPTING OXYGENS

Gln267, PfATP6 (*Plasmodium falciparum*) [7] Arg120, cyclooxygenase 2 (human) [14]	Val135, amidic proton, glycogen synthase kinase-3 β (human) [15]
Glu172, via H_2O bridge, glyoxalase I (human) [10]	Ile62, amidic carbonyl via 2 H_2O bridges, glycogen synthase kinase-3 β (human) [15]
Arg141, guanidino proton, glycogen synthase kinase-3 β (human) [15]	
Ile1041, PfATP6 (*Plasmodium falciparum*) [7] Leu1040, PfATP6 (*Plasmodium falciparum*) [7]	Lys85, side-chain amino proton, glycogen synthase kinase-3 β (human) [15]
None reported	
None reported	

Fig. 2. Summary of curcumin-protein interactions that are facilitated through the hydrogen bond-accepting oxygens of diketonic curcumin (top) and enolic curcumin (bottom). The oxygen atom (structure) and the corresponding amino acids that it interacts with (table) have been color-coded. Interactions are based on docking studies and/or site-directed mutagenesis experiments. Readers should note that curcumin-protein interactions are, in most instances, not confined to a single moiety but encompass bonding between multiple functional groups and amino acids, sometimes enforced by hydrophobic interactions (not indicated here). The source of the (modeled) protein is indicated in parentheses. The references were combined for Figs. 2 and 3. References used in Fig. 2: [1] (Yoo and Medina-Franco, 2011); [2] (Muthenna et al., 2009); [3] (Katsori et al., 2011); [4] (Kaur et al., 2010); [5] (Milacic et al., 2008); [6] (Li et al., 2012); [7] (Ji and Shen, 2009); [8] (Bora-Tatar et al., 2009); [9] (Ngo and Li, 2012); [10] (Liu et al., 2010); [11] (Majhi et al., 2010); [12] (Mamidi et al., 2012); [14] (Selvam et al., 2005); [15] (Bustanji et al., 2009). The data set has been included in Supplemental Table 1.

CURCUMIN (DIKETO FORM)
H-BOND DONATING PROTONS

Glu1265, side-chain carbonyl, DNA methyltransferase 1 (human) [1] Gln184, aldose reductase 1 (human) [2] Leu251, amidic carbonyl, protein kinase C δ (homology modeled) [11] Ser96, 20S proteasome β5 CT-like subunit, origin unspecified [5]	Leu251, amidic carbonyl, protein kinase C δ C1b subdomain [12] Lys28, hexameric amyloid beta peptide $(A\beta)_{9\text{-}40}$ (human) [9] Phe108, amidic carbonyl, nonameric amyloid beta peptide $(A\beta)_{9\text{-}40}$ (human) [9]
Leu1454, amidic carbonyl, DNA methyltransferase 1 (human) [1] Ile260, aldose reductase 2 (human) [2]	Ser264, aldose reductase 1 (human) [2] Gly254, protein kinase C θ C1b subdomain (human) [13]



Glu172, glyoxalase I (human) [10]	Val135, amidic carbonyl, glycogen synthase kinase-3 β (human) [15]
None reported	
Met179, amidic carbonyl, glyoxalase I (human) [10]	

Fig. 3. Summary of curcumin-protein interactions that are facilitated through the hydrogen bond-donating protons of diketonic curcumin (top) and enolic curcumin (bottom). The proton (structure) and the corresponding amino acids that it interacts with (table) have been color coded. Interactions are based on docking studies and/or site-directed mutagenesis experiments. Readers should note that curcumin-protein interactions are, in most instances, not confined to a single moiety but encompass bonding between multiple functional groups and amino acids, sometimes enforced by hydrophobic interactions (not indicated here). The references were combined for Figs. 2 and 3. References used in Fig. 3: [1] (Yoo and Medina-Franco, 2011); [2] (Muthenna et al., 2009); [5] (Milacic et al., 2008); [9] (Ngo and Li, 2012); [10] (Liu et al., 2010); [11] (Majhi et al., 2010); [12] (Mamidi et al., 2012); [13] (Das et al., 2011); [15] (Bustanji et al., 2009). The data set has been included in Supplemental Table 1.

et al., 2011), some have proposed that metal chelation may also be facilitated by the valence electrons of the methoxyphenyl oxygens (Ak and Gulcin, 2008; Jiang et al., 2011) based on the negative charge of these oxygens (Fig. 1C) and the metal chelating activity of β-diketone-lacking (poly)phenolics such as quercetin (Fiorucci et al., 2007) and catechol (Borsari et al., 2002).

Aside from its ability to chelate free metal cations, curcumin may use the metal atoms of metalloproteins as a means to interact with these proteins. Metal-mediated interactions between curcumin and its target molecules have been described for several different classes of metalloproteins, including glyoxalase I (Liu et al., 2010; Yuan et al., 2011), thioredoxin reductase (TrxR) (Singh and Misra, 2009), and HIV-1 integrase (Vajragupta et al., 2004). Molecular docking studies revealed that curcumin coordinated with the zinc atom in the catalytic site of glyoxalase I through a carbonyl oxygen (Liu et al., 2010; Yuan et al., 2011), whereby the binding was further stabilized by H-bonding between the phenylic hydroxyl group and a single lysine (Yuan et al., 2011) or multiple amino acid residues and hydrophobic interactions (Liu et al., 2010). With respect to TrxR, docking studies showed an important interaction between the selenium atom of a selenocysteine residue (Sec498) in the enzyme's active site and the π-electrons of one of the methine bridge alkenes (Singh and Misra, 2009). Experimental studies have confirmed that curcumin in fact binds to the redox-active residues Cys496 and Sec497 in rat TrxR (Fang et al., 2005), which corresponds to the docking results with the human protein (Singh and Misra, 2009). The binding of curcumin in the active site is mediated by a Michael reaction (Fang et al., 2005), as addressed in section II.A.7, and is further strengthened by H-bonding and hydrophobic interactions (Singh and Misra, 2009). Finally, the catalytic pocket of HIV-1 integrase contains a magnesium atom to which both carbonyl oxygens of curcumin's diketo moiety coordinated (Vajragupta et al., 2005). The bonding distances between the oxygens and the magnesium were considerably shorter (1.7 and 2.1 Å) than the H-bonding

distances between curcumin and several amino acid residues (≥2.7 Å), indicating that the metal-diketone interaction is quite significant in the binding of curcumin to HIV-1 integrase.

Inasmuch as approximately one-half of all proteins contain a metal (Thomson and Gray, 1998) and an estimated 25–33% of proteins require metals to function properly (Waldron and Robinson, 2009), the binding of curcumin to metalloproteins may constitute an important chemical and biological phenomenon, provided that the metals are accessible to curcumin (e.g., in the catalytic site of enzymes).

5. High Partition Coefficient (log P). Despite the polarity of the flanking functional groups and the central dicarbonyl moiety, curcumin overall is rather lipophilic. This is evidenced by its poor solubility in aqueous solvent and good solubility in organic solvents (section II.B) as well as by its log P value, which is a measure of the extent to which a solute prefers the lipophilic phase (typically octanol) over the aqueous phase in a biphasic, immiscible solvent system. The log P of curcumin has been reported in the range of 2.3 (Jankun et al., 2006) to 2.6 (Fujisawa et al., 2004; Tomren et al., 2007). This relatively high degree of lipophilicity, which is attributable to the methine-rich segments that connect the polar regions (Balasubramanian, 2006), has several important implications on intermolecular interactions.

a. Biochemical implications of curcumin's lipophilicity. The first important implication is that curcumin's methines can interact with hydrophobic amino acids in substrate binding sites of proteins. For example, molecular docking studies have confirmed hydrophobic interactions between curcumin and alanine and tyrosine in human immunoglobulin G (Liu et al., 2008). Such interactions were also ascertained between the phenylic rings of curcumin and valine in human NADPH-dependent aldo-keto reductase, using site-directed mutagenesis (Matsunaga et al., 2009). Furthermore, the heptanoid region of curcumin, docked to COX-1, was found to be surrounded by numerous hydrophobic amino acids, including leucine, isoleucine, alanine, glycine, and valine (Selvam et al., 2005). Additional specific examples are provided in Supplemental Table 1, altogether indicating that the electrostatic bonding between curcumin and polar residues, as detailed in sections II.A.1 to II.A.4, is stabilized by these hydrophobic interactions. As a result, a greater net bonding strength is achieved between curcumin and the molecular target than would have been the case in the absence of curcumin's lipophilic features. Alternatively, the association with a target molecule could rely predominantly on hydrophobic interactions, as proposed for superficial hydrophobic clefts in albumin (Kragh-Hansen, 1981; Zsila et al., 2003).

Second, the relatively high partition coefficient of curcumin is indicative of the molecule's capacity to interact with biomembranes (Jaruga et al., 1998), which is reflected by the intercalation of curcumin into model membranes (liposomes) composed of saturated (dipalmitoylphosphatidyl choline or dimyristoylphosphatidyl choline) (Barry et al., 2009; Perez-Lara et al., 2010), monounsaturated (dioleoylphosphatidyl choline) (Ingolfsson et al., 2007; Hung et al., 2008; Sun et al., 2008), or egg yolk phosphatidyl cholines (Kunwar et al., 2006; Karewicz et al., 2011). The association of curcumin with model membranes occurs at relatively high partition constants, i.e., in the range of $2.5 \times 10^4\ M^{-1}$ (Kunwar et al., 2006) to $4.3 \times 10^4\ M^{-1}$ (Karewicz et al., 2011) for egg yolk phosphatidylcholine liposomes and $2.4 \times 10^4\ M^{-1}$ for dioleoylphosphatidyl choline liposomes (Hung et al., 2008), indicating that curcumin can be taken up by cells by direct intercalation into the cell membrane.

b. Curcumin-biomembrane interactions. The kinetics of these interactions (Sun et al., 2008) correspond to those of other amphipathic drugs and peptides (Sheetz and Singer, 1974; Lee et al., 2004) and entail a well-documented biphasic process (Banerjee et al., 1985; Huang and Wu, 1991; Heller et al., 1998). In the first phase, curcumin adsorbs to the outer membrane leaflet at the water-membrane interface in a low-energy binding state, which is associated with membrane thinning due to the lateral displacement of primarily polar phospholipid head groups (Sun et al., 2008). Within 60 seconds, a transition to a high-energy binding state occurs, whereby curcumin inserts deeper into the hydrophobic compartment of the membrane (Sun et al., 2008). No di- or multimerization of curcumin occurs during the partitioning process (Sun et al., 2008), even when saturation levels are reached at a curcumin:lipid molar ratio of ~0.1 (Sun et al., 2008; Perez-Lara et al., 2010).

The exact intermolecular interactions and corollary molecular conformation of curcumin in either membrane binding state are, however, elusive. On the basis of data obtained with differential scanning calorimetry (DSC), X-ray diffraction, ^2H NMR, and FT-IR spectroscopy it has been proposed that curcumin inserts into bilayers (Barry et al., 2009; Perez-Lara et al., 2010) with its main axis parallel to the bilayer normal (Perez-Lara et al., 2010). Theoretically, a membrane core-crossing conformation is possible, inasmuch as curcumin in planar conformation has a maximum length of roughly 22 C-atoms, whereas the acyl chain regions of dioleoylphosphatidyl choline- and dipalmitoylphosphatidyl choline-based bilayers have a length of 36 and 32 C-atoms, respectively. FT-IR analysis of the phospholipid PO_2^- and fatty acid ester C=O vibrational modes revealed that curcumin does not interact with these moieties (Perez-Lara et al., 2010), indicating that curcumin preferably localizes in the hydrocarbon-rich domain of the lipid bilayer, which is in agreement with the thermographic data obtained by DSC (Barry et al., 2009; Perez-Lara et al., 2010). Fluorescence quenching experiments established that liposome-encapsulated curcumin fluorescence is quenched more extensively by membrane-penetrating quenchers

(acrylamide) than by less membrane-permeant quenchers (iodine) (Kunwar et al., 2006). Similar assays with brominated carboxylic acid derivatives (2- or 16-bromohexadecanoic acid or 11-bromoundecanoic acid), which more or less behave as component phospholipids in model membranes, corroborated that curcumin fluorescence is most intensely quenched by the bromine at $C_{\Delta 11}$ (Karewicz et al., 2011), i.e., in a very hydrophobic region of the bilayer (Bemporad et al., 2005). Accordingly, the lipophilic methines of curcumin most likely facilitate hydrophobic interactions with the acyl chains of phospholipids in cell and subcellular membranes, as was shown for erythrocytes (Jaruga et al., 1998), whereby the polar flanking regions may undergo H-bonding with water molecules embedded in the more distal portions of the membrane relative to the bilayer center (Bemporad et al., 2005). Under physiological conditions, the β-diketones, methoxy ethers, and hydroxyl groups may also be prone to electrostatic interactions with polar transmembrane protein residues, as suggested by Ingolfsson et al. (2007).

It should be noted that some discrepancies exist regarding the planar, membrane core-crossing conformation of curcumin. Curcumin's electrons are delocalized over the entire molecule (Balasubramanian, 2006; Nardo et al., 2009), accounting for the lower transition energies of the π-electrons and therefore a considerably red-shifted absorption maximum (Kunwar et al., 2006; Ingolfsson et al., 2007; Hung et al., 2008; Karewicz et al., 2011) compared with feruloyl methane (Tønnesen et al., 1995), the exact half of the curcumin molecule. If indeed curcumin inserts into the membrane in a planar configuration parallel to the bilayer normal, then curcumin fluorescence should have been quenched by 16-bromohexadecanoic acid as well, i.e., in the most hydrophobic region of the membrane, in addition to the abovementioned quenching by 11-bromoundecanoic acid (Karewicz et al., 2011). In an electron-delocalized system such as that of curcumin, bromine-mediated quenching should be indiscriminate along the full length of the delocalized system, pleading against curcumin adopting a membrane core-crossing orientation. Moreover, such an orientation would place the highly polar central region of curcumin, where even the β-carbons and the C_α bear a slightly positive and negative charge, respectively (Balasubramanian, 2006), in the most hydrophobic part of the membrane (Bemporad et al., 2005), leading to thermodynamic instability. The adoption of the closed *cis*-enol conformation or diketo tautomerization, as has been described for curcumin in solvents (Khopde et al., 2000; Kolev et al., 2005; Balasubramanian, 2006; Nardo et al., 2008, 2009) that are chemically analogous to the bilayer core (Kragh-Hansen, 1981), or hydration of the β-diketone moiety by comigration of water molecules (Bemporad et al., 2005) might ameliorate the thermodynamic instability, but would not resolve it.

It is hence plausible that curcumin inserts deeper into the bilayer in the high-energy binding state but remains intertwined in the acyl chains of a single membrane leaflet, as illustrated in Barry et al. (2009), in a more perpendicular orientation relative to the bilayer normal. A perpendicular orientation would not be in conflict with earlier experimental data (Barry et al., 2009; Perez-Lara et al., 2010) inasmuch as the phase transition temperature of the lipid bilayer, including the pretransition of saturated phosphatidylcholines (DSC), would be affected in a fashion similar to the parallel orientation. The same applies to X-ray diffraction and vibrational (FT-IR spectroscopy) patterns as well as proton resonances (^{2}H NMR) of the phospholipid methylenes in the presence of curcumin, which attest to interactions with curcumin but do not unequivocally reveal its orientation. Curcumin may also undergo rotamerization (section II.A.6), whereby for instance the polar moieties are oriented toward the more hydrophilic membrane region and the lipophilic segments toward the membrane core to achieve a thermodynamic optimum, by, e.g., adopting the *a,s-trans,s-trans,a* or *a,s-cis, s-trans,a* conformations in Fig. 4 or by adopting a non-planar trans-diketo conformation as in Scheme 2 in Kolev et al. (2005).

6. Rotamerization. In addition to the numerous H-bond donating and accepting sites (sections II.A.1 to II. A.3), one of the most favorable features of curcumin in regard to intermolecular interactions is its ability to undergo rotamerization at multiple locations in the methine bridge. As shown in Fig. 4, enolic curcumin contains five sites where the molecule can rotate about a C–C bond, whereas diketonic curcumin has six rotameric sites. With respect to enolic curcumin, rotation about the $-C_\alpha-C_\beta-$ bond may be impaired due to resonance stabilization in the keto-enol moiety, depending on the chemical environment (section II.A.1 and Fig. 1B). However, in polar, strongly H-bonding solvents (or in cells), curcumin mainly comprises the open enol tautomer (Emsley, 1984; Khopde et al., 2000; Nardo et al., 2008) and could therefore undergo *cis*-to-*trans* isomerization around the C_α (Toullec, 1990). Density functional theory calculations on the structure of curcumin, where only four rotational axes were assumed (excluding the $-C_\alpha-C_\beta-$ bond), yielded 24 possible rotamers of enolic curcumin, of which the nine most energetically favorable structures are presented in Fig. 4 (Kolev et al., 2005).

Curcumin's rotameric capabilities considerably expand the versatility of intermolecular bonding inasmuch as both flanks can adopt the most suitable conformation to maximize the number of H-bonds between curcumin and its molecular target. This conformational flexibility is most evident from the docking studies that were used to construct Figs. 2 and 3, which revealed that curcumin tends to adopt an entirely different configuration for each molecular target. For instance, curcumin bends only slightly and retains most of its planar structure when interacting with the minor groove of duplexed oligonucleotides (Zsila et al., 2004; Koonammackal et al.,

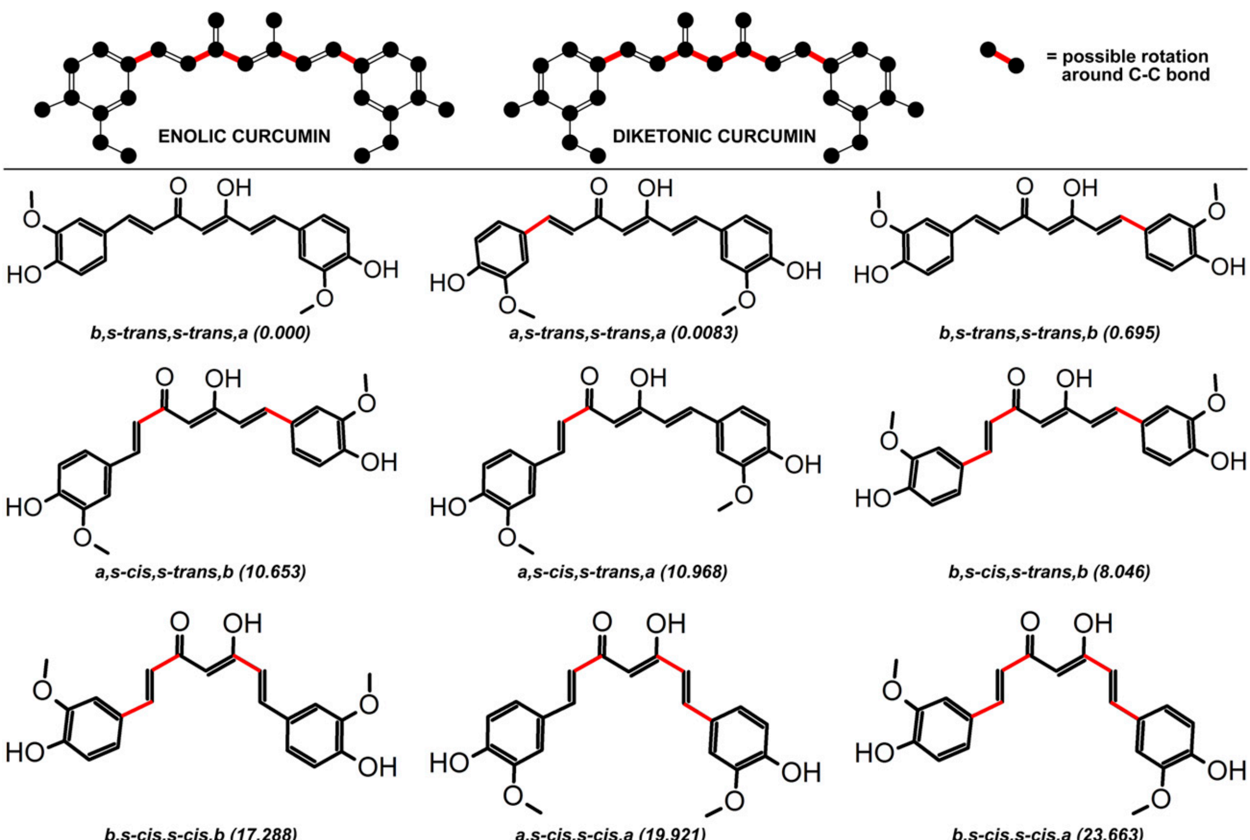


Fig. 4. Scheme of possible C−C bond rotation sites (indicated in red) in enolic and diketonic curcumin (top). The bottom panel shows several rotameric species of enolic curcumin, where *a* and *b* represent the orientation of the OCH_3 moiety around the C–phenyl bond for R_1 and R_2 (Fig. 1A) and the *trans* and *cis* notations refer to the $–C_\delta–C_\gamma–C_\beta–$ segment of the methine bridge (Kolev et al., 2005). The red bonds indicate the site of rotation relative to the *b, s-trans,s-trans,a* configuration, the most stable structure according to Kolev et al. (2005). The differences in relative energy (KJ/mol) versus the most stable structure are provided in the parentheses. Structures adopted from Kolev et al. (2005).

2011), whereas it becomes entirely nonplanar and often rotates around the longitudinal axis when interacting with, e.g., the C1B domain of PKCθ (Majhi et al., 2010; Das et al., 2011), PfATP6 (Ji and Shen, 2009), aldose reductases (Muthenna et al., 2009; Katsori et al., 2011), and beta amyloid peptides (Ngo and Li, 2012). As a result, curcumin is able to associate with a plethora of biomolecules due to its structural adaptability, which is "exploited" to maximize the number of interatomic bonds between curcumin and its target.

7. Michael Acceptor Capacity. The final chemical attribute of curcumin that facilitates intermolecular interactions is the ability to act as a Michael acceptor (Dinkova-Kostova et al., 2001). A Michael reaction is an addition reaction whereby a nucleophile (i.e., molecules or ions with a lone pair of electrons such as thiols/thiolates and amines) is covalently attached to a compound containing an α,β-unsaturated carbonyl, which for β-diketones such as curcumin comprises the $–C_\delta=C_\gamma–(C_\beta=O)–$ segment (Fig. 1C). The reaction has been proposed to proceed via a carbocation intermediate $[–C_\delta{}^+–C_\gamma–(C_\beta–O^-)–]$, resulting in the binding of the nucleophilic atom (:N–R, where :N is the nucleophilic atom) to curcumin's C_δ, yielding $–(C_\delta–N–R)=C_\gamma–(C_\beta–OH)–$ (Fang et al., 2005).

Michael additions to curcumin have been described for molecular targets such as reduced glutathione (GSH) (Mathews and Rao, 1991; Awasthi et al., 2000), glutathione S-transferase (GST) (van Iersel et al., 1996, 1997), TrxR (Fang et al., 2005), interleukin (IL)-1 receptor-associated kinase (IRAK) (Jurrmann et al., 2005), the histone acetyltransferase E1A binding protein p300 (Marcu et al., 2006), calcium release-activated calcium channel protein 1 (Shin et al., 2012), and human ErbB-2 (HER2) (Jung et al., 2007), which is addressed separately in section III.E.1. The atoms through which the target molecules undergo a Michael addition include thiols (Mathews and Rao, 1991; Awasthi et al., 2000; Fang et al., 2005; Jurrmann et al., 2005) and selenols (Fang et al., 2005), both of which are mostly associated with a (seleno)cysteine residue.

The simplest and most illustrative example of a Michael reaction with curcumin is GSH, a tripeptide composed of glutamate-cysteine-glycine that is known for its different types of reactions involving nucleophiles, including its capacity to act as a Michael donor to α,β-unsaturated carbonyl compounds (Ketterer, 1988). Incubation of curcumin with increasing concentrations of GSH led to a proportional (Mathews and Rao, 1991) and time-dependent (Awasthi et al., 2000) decrease in

absorbance at ~430 nm, which is curcumin's main absorption band in polar protic solvents (Tønnesen et al., 1995) such as buffered aqueous solutions (Fig. 1B), emanating from the electron delocalization over the entire conjugated system (Balasubramanian, 1990, 1991, 2006). A Michael reaction, which abrogates the conjugation in the methine segment, is therefore consistent with the observed decrease in absorbance at this wavelength. Although the experiments were performed at or above (near-)neutral pH [at which curcumin degradation is likely to occur (section II.C.1.a)] and curcumin was shown to cause GSH degradation (Awasthi et al., 2000), the formation of curcumin-GSH adducts was confirmed by high performance liquid chromatography (HPLC) and mass spectrometry (MS) (Awasthi et al., 2000). Moreover, curcumin is a very potent inhibitor of π-class GSTs (van Iersel et al., 1996) by binding to cysteine residues, mainly Cys47 in the active site of the P1-1 subunit of the GSTs, most likely via a Michael addition (van Iersel et al., 1997), altogether indicating that curcumin interferes in cellular glutathione redox metabolism through binding multiple targets via a Michael reaction.

Furthermore, curcumin has been shown to covalently bind to Cys496 and Sec497 in the catalytic pocket of TrxR in vitro, which resulted in curcumin concentration-dependent, irreversible inhibition of enzymatic activity (Fang et al., 2005). TrxR inhibition by curcumin also proceeded in a concentration-dependent manner in HeLa cells (Fang et al., 2005). The binding of curcumin to Cys496 and Sec497 was proposed to proceed via a nucleophilic attack by the thiol and selenol, respectively, on curcumin's carbocation (C_{δ}^{+}). A similar mechanism, i.e., alkylation of thiol residues, was observed in an IRAK-overexpressing murine T-cell line (EL-4^{IRAK}) incubated with curcumin (Jurrmann et al., 2005). IRAK is recruited to the IL-1 receptor (IL-1R) upon IL-1 binding, which initiates the IL-1 signaling cascade (Bol et al., 2003). The recruitment of IRAK to IL-1R can be inhibited by numerous thiol-modifying compounds, including diamide, menadione, and phenylarsine oxide (Bol et al., 2003), as was also shown to be the case for curcumin (Jurrmann et al., 2005). In IL-1β-stimulated EL-4^{IRAK} cells, the recruitment of IRAK to IL-1R was inhibited in a curcumin concentration-dependent manner without interfering with IL-1β binding to IL-1R. When the free thiol groups of IRAK were occupied by iodo-acetyl-[^{125}I]iodotyrosine in IL-1-stimulated cells, curcumin blocked the extent of radiolabeling in a concentration-dependent manner, indicating thiol modification by curcumin. Inasmuch as the inhibition of IRAK thiols by, e.g., phenylarsine oxide, is reversible in the presence of thiol reductants such as dithiothreitol and dimercaptopropanol (Singh and Aggarwal, 1995; Friedrichs et al., 1998) but irreversible in case of curcumin, the thiol modification by curcumin does not entail a redox reaction but most likely a covalent Michael addition-mediated alkylation (Dinkova-Kostova and Talalay, 1999; Jurrmann et al., 2005).

The most compelling evidence for the Michael acceptor properties of curcumin come from studies in which a curcumin derivative that lacked the α,β-unsaturated carbonyl (i.e., tetrahydrocurcumin, Fig. 12) was employed as negative control or where site-directed mutagenesis was used to replace the target cysteine residues with a thiol-lacking amino acid. For example, radioactively labeled curcumin was shown to inhibit the acetyltransferase activity of p300 as a result of covalent binding to the protein. However, when enzyme activity assays were performed with radiolabeled tetrahydrocurcumin, no inhibition of p300 was observed (Marcu et al., 2006), confirming that the curcumin-p300 complex was the result of a Michael reaction. Similar results were obtained with ErbB-2 (section III.E.1). With respect to calcium release-activated calcium channel protein 1, which curcumin binds to and thereby inhibits Ca^{2+} flux through the Ca^{2+}-release activated Ca^{2+} channel, replacement of the reactive Cys195 with a nonreactive serine reduced the inhibitory effect of curcumin. Moreover, tetrahydrocurcumin exhibited a less potent inhibitory effect on Ca^{2+}-release activated Ca^{2+} channel, which was entirely abrogated as a result of the serine substitution (Shin et al., 2012).

These findings collectively attest to the fact that curcumin binds to target proteins containing Michael donating residues, i.e., mainly (seleno)cysteines. Inasmuch as cysteines play an important functional and catalytic role in the substrate binding site of enzymes (Saito, 1989; Carugo et al., 2003), the covalent binding of curcumin to target molecules via a Michael reaction likely constitutes an important contributory factor in curcumin's pleiotropic binding behavior and corollary biological effects.

B. Curcumin Solubility

Because of its relatively high log P value (section II.A.5), curcumin is practically insoluble in aqueous medium. The solubility of curcumin is high in polar aprotic and polar protic solvents, as reflected by its order of solubility: acetone > 2-butanone > ethyl acetate > methanol > ethanol > 1,2-dichloroethane > 2-propanol > ether > benzene > hexane. DMSO is also a commonly used solvent, which dissolves curcumin up to a concentration of 11 mg/ml (versus 1 mg/ml for ethanol). Furthermore, curcumin is soluble in some nonpolar solvents with comparable ε_r values [benzene (2.3), toluene (2.38), diethyl ether (4.3), chloroform (4.81)] but does not dissolve well in alipathic or alicyclic organic solvents such as hexane and cyclohexane, respectively (Khopde et al., 2000).

The poor aqueous solubility of curcumin bears several important implications for in vitro and in vivo research. First, curcumin should always be dissolved in solvents that are miscible in water, which include

acetone, butanone (to an extent), methanol, ethanol, 1,2-dichloroethane (up to 8.7 g/l w/w), 2-propanol, and DMSO. Of these solvents, the least toxic should preferably be used in experiments, which is best judged by their lethal 50% dose values, given here for the oral administration route in rats, unless noted otherwise (obtained from the respective material safety data sheet): acetone, 9.8 g/kg; 2-butanone, 2.7 g/kg; methanol, 5.6 g/kg; ethanol, 7.1 g/kg; 1,2-dichloroethane, 0.41 g/kg (mouse); isopropanol, 5.0 g/kg; DMSO, 18.0 g/kg. Accordingly, DMSO constitutes the most suitable solvent for in vitro and in vivo studies, although proper controls (solvent alone) should always be employed in all assays.

Second, some researchers report dissolving curcumin in slightly basic water or aqueous buffer, and several suppliers of curcumin even advocate raising the pH to improve solubility in aqueous medium. Making the conditions more alkaline, however, does not yield a tremendous increase in solubility relative to the 1.1 mM solubility at pH = 7.3 (Tønnesen, 1989b). More importantly, those who conduct experiments with curcumin should note that curcumin becomes very susceptible to degradation under alkaline conditions, i.e., a pH > 6.5 [Fig. 6B and "Solvolysis: (alkaline) hydrolysis," section II.C.1.b.i], due in part to the formation of the phenylate anion (Fig. 8 and "Chemical degradation and modification of curcumin," section II.C.1.b.i). As described in the latter section, the phenylate anion can give rise to curcumin radicals that in turn mediate degradation of the molecule, react with other curcumin radicals to form dimeric catabolites, or react with biomolecules in the cells, which may lead to different experimental outcomes. Inasmuch as protonated curcumin (i.e., at acidic pH) is even less soluble in water, preference should be given to DMSO as solvent system.

C. Curcumin Stability In Vitro

A drug must remain stable through all formulation stages and in the body after administration until a pharmacological effect has been conveyed. The stability of a drug, characterized by its chemical, physical, microbiological, therapeutic, and toxicological stability (O'Donnell and Bokser, 2005), is critical for drug safety and efficacy and can be affected by multiple factors, including oxidative/nitrosative degradation and/or modification, solvolysis, and aggregation. For curcumin, there are two major in vitro stability issues that complicate its use as a pharmaceutical, namely oxidative degradation and modification and solvolysis (Fig. 5). The oxidative degradation and modification can be categorized into photochemical processes (i.e., those induced by light absorption) and chemical processes (i.e., those induced in the absence of light).

Chemical modification and/or degradation change curcumin's chemical structure and properties and thus affect its intermolecular bonding behavior (section II.A). This in turn may drastically affect curcumin's pharmacokinetic and pharmacodynamic properties (section III), depending on whether the site interacting with the molecular target (section II.A) is chemically altered. Consequently, curcumin may entirely lose its anticancer attributes as a result of the modification/degradatory processes described in section II.C.1. Additionally, there are intracellular and in vivo stability concerns. These are alluded to in section II.C.2.a.

1. In Vitro Oxidative Degradation and Modification of Curcumin. The main mode of photochemical and chemical degradation and modification of curcumin is through oxidation, which is primarily mediated by reactive oxygen species (ROS). It is well-established that curcumin is a potent antioxidant that interacts with different types of physiologically produced oxygen-centered radicals, including hydroxyl radical (•OH) (Tønnesen, 1989a; Kunchandy and Rao, 1990; Tønnesen and Greenhill, 1992; Reddy and Lokesh, 1994; Ruby et al., 1995; Das and Das, 2002; Vajragupta et al., 2004; Biswas et al., 2005; Agnihotri and Mishra, 2011), superoxide anion ($O_2^{\bullet -}$) (Kunchandy and Rao, 1990; Reddy and Lokesh, 1994; Ruby et al., 1995; Priyadarsini, 1997; Das and Das, 2002; Vajragupta et al., 2004; Biswas et al., 2005; Ak and Gulcin, 2008; Dairam et al., 2008), and peroxyl radicals (Priyadarsini, 1997; Masuda et al., 2001; Deng et al., 2006). Curcumin also reacts with physiologically produced nitrogen-centered radicals (nitric oxide, •NO, and nitrogen dioxide radicals) (Unnikrishnan and Rao, 1995; Sreejayan and Rao, 1997; Onoda and Inano, 2000), sulfur-centered radicals (oxidized glutathione) (Khopde et al., 1999), and oxidants such as hydrogen peroxide (H_2O_2) (Tønnesen and Greenhill, 1992; Iwunze, 2004; Ak and Gulcin, 2008; Griesser et al., 2011) as well as singlet oxygen ($^1O_2$) (Tønnesen et al., 1986; Chignell et al., 1994; Subramanian et al., 1994; Das and Das, 2002). Curcumin is further capable of reacting with nonphysiological radicals such as azide radicals (Gorman et al., 1994; Priyadarsini, 1997; Khopde et al., 1999; Priyadarsini et al., 2003), 2,2-diphenyl-1-picrylhydrazyl (DPPH, a stable nitrogen-centered radical) (Venkatesan and Rao, 2000; Priyadarsini et al., 2003; Fujisawa et al., 2004; Ak and Gulcin, 2008; Feng and Liu, 2009), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cations ($ABTS^{\bullet +}$) (Venkatesan and Rao, 2000; Ak and Gulcin, 2008; Feng and Liu, 2009), dimethyl-4-phenylenediamine dihydrochloride radical cations (Ak and Gulcin, 2008), halocarbonperoxyl radicals (Priyadarsini, 1997; Khopde et al., 1999), dibromine radical anions (Khopde et al., 1999), galvinoxyl radicals (Feng and Liu, 2009), and Triton-X 100 radicals (Priyadarsini, 1997). Lastly, it has been shown to interact with nonphysiological peroxides such as *tert*-butyl hydroperoxide (Sugiyama et al., 1996).

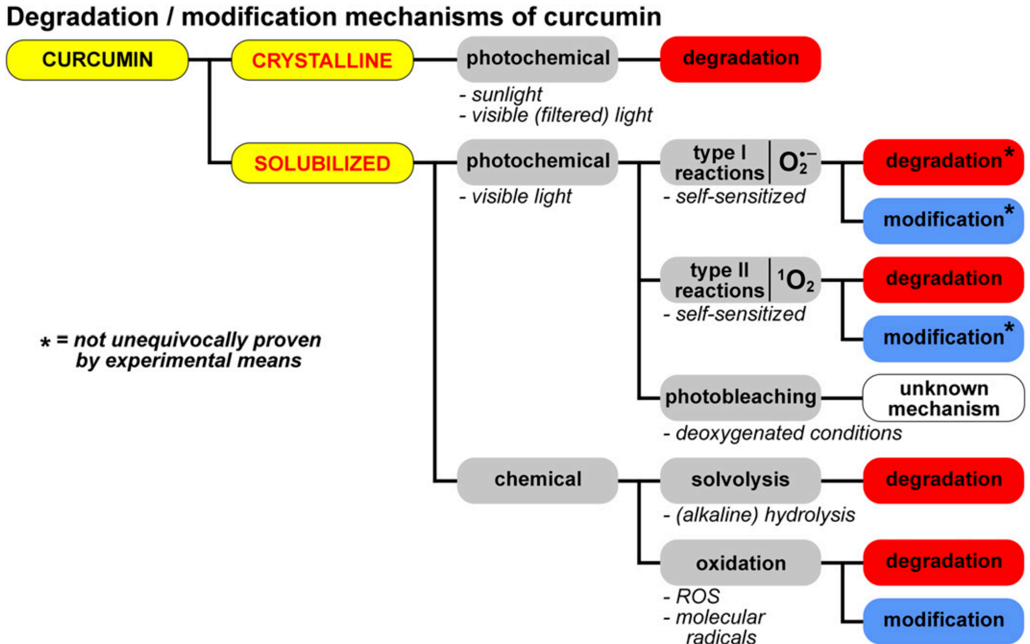


Fig. 5. Summary of the general mechanisms by which curcumin in crystalline and solubilized state is degraded and/or chemically modified. Solubilized curcumin refers to buffered aqueous solutions and/or organic solvents only. The processes are detailed in sections II.C.1.a (photochemical) and II.C.1.b (chemical).

The reactivity of radicals toward curcumin is chiefly dictated by the bond dissociation energies (Fujisawa et al., 2002) of curcumin's functional groups, being approximately 80 kcal•mol^{-1} for the phenylic O−H, $>$ 90 kcal•mol^{-1} for the central C−H (C_α, Fig. 1C), and 98 kcal•mol^{-1} for the enolic O−H (Sun et al., 2002; Wright, 2002). Accordingly, radicals predominantly attack the phenylic hydroxyl group (Barclay et al., 2000; Sun et al., 2002; Wright, 2002; Priyadarsini et al., 2003; Chen et al., 2006c; Feng and Liu, 2009; Agnihotri and Mishra, 2011), either by hydrogen atom transfer (HAT; $H_3R + R'^{\bullet} \rightarrow H_2R^{\bullet} + HR'$, where H_3R represents fully protonated curcumin) or by single electron transfer (SET) from curcumin to the radical ($H_3R + R'^{\bullet} \rightarrow H_3R^{\bullet+} + R'^{-}$) (Barclay et al., 2000; Sun et al., 2002; Priyadarsini et al., 2003; Litwinienko and Ingold, 2004; Galano et al., 2009; Agnihotri and Mishra, 2011). HAT can also occur at the methines, with the C_α as the most frequently proposed location (Sugiyama et al., 1996; Jovanovic et al., 1999; Sun et al., 2002; Wright, 2002; Priyadarsini et al., 2003; Agnihotri and Mishra, 2011). Additionally, the enol moiety is subject to HAT (Sugiyama et al., 1996; Ak and Gulcin, 2008; Agnihotri and Mishra, 2011) or to ionization of the enol proton (pK_{a1} = 7.5) and subsequent electron transfer from the radical via sequential proton loss electron transfer (SPLET; $H_3R \rightarrow H_2R^- + H^+$, $H_2R^- + R'^{\bullet} \rightarrow H_2R^{\bullet} + R'^-$, $H_2R^{\bullet} \rightarrow HR^{\bullet-} + H^+$) (Litwinienko and Ingold, 2004; Galano et al., 2009), but in all instances the site of attack depends strongly on the reactivity and thus type of the radical (i.e., the bond dissociation energy of the parent compound and the redox potential), the ionization potential of abstractable substrate atoms, and the solvent (Zheng et al., 1997; Wright, 2002; Litwinienko and Ingold, 2004; Feng and Liu, 2009; Galano et al., 2009; Agnihotri and Mishra, 2011). Consequently, •OH can oxidize curcumin at almost every O−H and C−H bond (Wright, 2002; Agnihotri and Mishra, 2011), whereas less reactive radicals such as $O_2^{\bullet-}$ will mainly attack phenylic O−H bonds (Sawyer and Valentine, 1981; Jurasek and Argyropoulos, 2006), as has been reported for other (poly)phenolic compounds (Paya et al., 1992; Yen and Hsieh, 1997; Mochizuki et al., 2002; Cetinkaya et al., 2012).

Curcumin derivatives have been employed to demonstrate the importance of the type of radical in relation to the HAT/SET/SPLET site on curcumin. For certain ROS (e.g., *tert*-butyl hydroperoxide and $ABTS^{\bullet+}$), the phenylic groups and/or the alkenes in the methine segment are not required for an antioxidant effect, as was shown for dimethoxytetrahydrocurcumin (keto-enolic curcumin with both methoxyphenyls substituted by *ortho*-dimethoxybenzenes and a hepta-4-ene bridge) (Sugiyama et al., 1996) and for benzylated curcumin derivatives, where the phenylic hydroxyl groups are protected by benzyl groups (Feng and Liu, 2009). Lipid peroxidation by *tert*-butyl hydroperoxide was inhibited by 36% by dimethoxytetrahydrocurcumin compared with ~71% by curcumin and ~83% by tetrahydrocurcumin (keto-enolic curcumin with hepta-4-ene bridge) (Sugiyama et al., 1996). Similarly, the radical scavenging capacity of the benzyl-protected curcumin was equal to curcumin and tetrahydrocurcumin for $ABTS^{\bullet+}$ but nonexistent for galvinoxyl or DPPH radicals (Feng and Liu, 2009). For the latter two radical species, the phenylic groups were of paramount importance (Sreejayan and Rao, 1996; Feng and Liu,

2009). The order of antioxidative capacity of curcumin toward DPPH is tetrahydrocurcumin > hexahydrocurcumin (keto-enolic curcumin with heptane bridge) ≈ octahydrocurcumin (di-enolic curcumin heptane bridge) > curcumin > > demethoxycurcumin (R_1 = H; Fig. 1A) > >> *bis*demethoxycurcumin (R_1 = R_2 = H; Fig. 1A) (Somparn et al., 2007; Feng and Liu, 2009). With respect to galvinoxyl radicals, the order was curcumin > > octahydrocurcumin > tetrahydrocurcumin (Feng and Liu, 2009).

The HAT/SET/SPLET reactions between curcumin and radicals cause curcumin to become a radical itself, which subsequently may undergo radical delocalization over the conjugated segments (Masuda et al., 1999; Priyadarsini et al., 2003; Litwinienko and Ingold, 2004; Chen et al., 2006c; Griesser et al., 2011), intramolecular rearrangement of bonds (Tønnesen and Greenhill, 1992; Griesser et al., 2011; Gordon and Schneider, 2012), degradation (Tønnesen et al., 1986; Tønnesen and Greenhill, 1992; Chignell et al., 1994; Masuda et al., 1999; Agnihotri and Mishra, 2011), dimerization through radical coupling between curcumin radicals (Masuda et al., 1999, 2002; Fujisawa et al., 2004), or radical coupling with other molecular radicals to yield heterodimeric complexes (Masuda et al., 2001) or possibly multimeric complexes (in case of double deprotonation at the phenylic oxygens) (Priyadarsini et al., 2003). In the latter case, noncurcumin radicals may comprise, for example, lipid (cholesterol) radicals (Iuliano, 2011) co-encapsulated in liposomal formulations of curcumin (Thangapazham et al., 2008a; Chen et al., 2009a; Shi et al., 2012) or radicals of antioxidants (Suntres, 2011) added to the curcumin formulation (Oetari et al., 1996), both formed by curcumin-mediated type I or II photochemical reactions ("Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/modification of dissolved curcumin," section II.C.1.a.ii), Haber and Weiss (1932) or Fenton (1894) reactions ("Chemical degradation and modification of curcumin," section II.C.1.b.i), and/or oxidation reactions in the absence of transition metals ("Chemical degradation and modification of curcumin," section II.C.1.b.i).

With respect to degradation, cleavage of the molecule occurs at different locations in the methine bridge (Tønnesen et al., 1986; Tønnesen and Greenhill, 1992; Chignell et al., 1994; Masuda et al., 1999; Agnihotri and Mishra, 2011), yielding two nonidentical, monophenylic catabolites. The degradation of curcumin does not require alkenes [which are involved in the formation of cyclic ether (Griesser et al., 2011) and dioxetane (Masuda et al., 1999) intermediates in curcumin radicals] or the phenylic groups per se, as catabolites were found after ROS-induced degradation of dimethoxy tetrahydrocurcumin in O_2-saturated acetonitrile that were structurally analogous to the catabolites found after curcumin degradation (i.e., 3,4-dimethoxybenzoic acid, 3′,4′-dimethoxyacetophenone, and 3-(3,4-dimethoxyphenyl)-propionic acid) (Sugiyama et al., 1996).

a. Photochemical degradation and modification of curcumin. Curcumin is a chromophore that absorbs strongly in the visible wavelength range, making it susceptible to photo(oxidative) degradation and modification in daylight and artificial lighting. Photo-induced degradation of curcumin occurs irrespectively of the chemical environment, i.e., in solid state as well as in different organic solvents (Tønnesen et al., 1986; Khurana and Ho, 1988; Ansari et al., 2005), even in the absence of UV light and oxygen (Tønnesen et al., 1986). However, the composition, degradation kinetics, and relative abundance of the degradation products differ depending on the physical state of the compound and the conditions.

i. Photochemical Degradation of Solid State (Crystalline) Curcumin. In regard to the crystalline form of curcumin, the type of solvent used for extracting and purifying curcumin appears to have an effect on the degree of degradation. For example, 120-hour sunlight exposure of crystalline curcumin obtained by ethanol extraction yielded three unidentified compounds with a cumulative relative concentration of 33% in addition to vanillin (34%), ferulic aldehyde (0.5%), ferulic acid (0.5%), and vanillic acid (0.5%) (Khurana and Ho, 1988) (Fig. 6A). For sunlight-exposed, methanol-extracted curcumin in solid state, the degradation products were identical but the concentrations of vanillin, ferulic aldehyde, ferulic acid, and vanillic acid were mostly lower, namely 2.0%, 0.2%, 0.1%, and 1.5%, respectively (Khurana and Ho, 1988). The degradation of crystalline curcumin abides by second order kinetics, at least when exposed to light in the 400- to 750-nm wavelength range for 4 hours (Tønnesen et al., 1986).

ii. Photochemical Degradation and Modification of Dissolved Curcumin. The photochemical degradation of solubilized curcumin yields similar end products as light-irradiated crystalline curcumin, namely vanillin, vanillic acid, 4-vinylguaiacol, ferulic acid, and ferulic aldehyde (Fig. 6A), when dissolved in isopropanol and irradiated for 4 hours at 400-510 nm (Tønnesen et al., 1986). However, the degradation kinetics are solvent dependent. The degradation rate constants (k_{deg}) of 40 μM curcumin dissolved in different organic solvents and exposed for 4 hours to visible light (400–750 nm) conform to first-order kinetics and constitute k_{deg} = 1.4×10^{-2} h^{-1} (methanol), k_{deg} = 1.4×10^{-1} h^{-1} (ethyl acetate), k_{deg} = 2.9×10^{-1} h^{-1} (chloroform), and k_{deg} = 5.2×10^{-1} h^{-1} (acetonitrile), corresponding to half-lives of 50.2 hours, 5.1 hours, 2.4 hours, and 1.3 hours, respectively (Tønnesen et al., 1986). Visible light imposes a broader degree of degradation than UV light inasmuch as irradiation of curcumin in methanol with 254-nm light produced three unspecified degradation

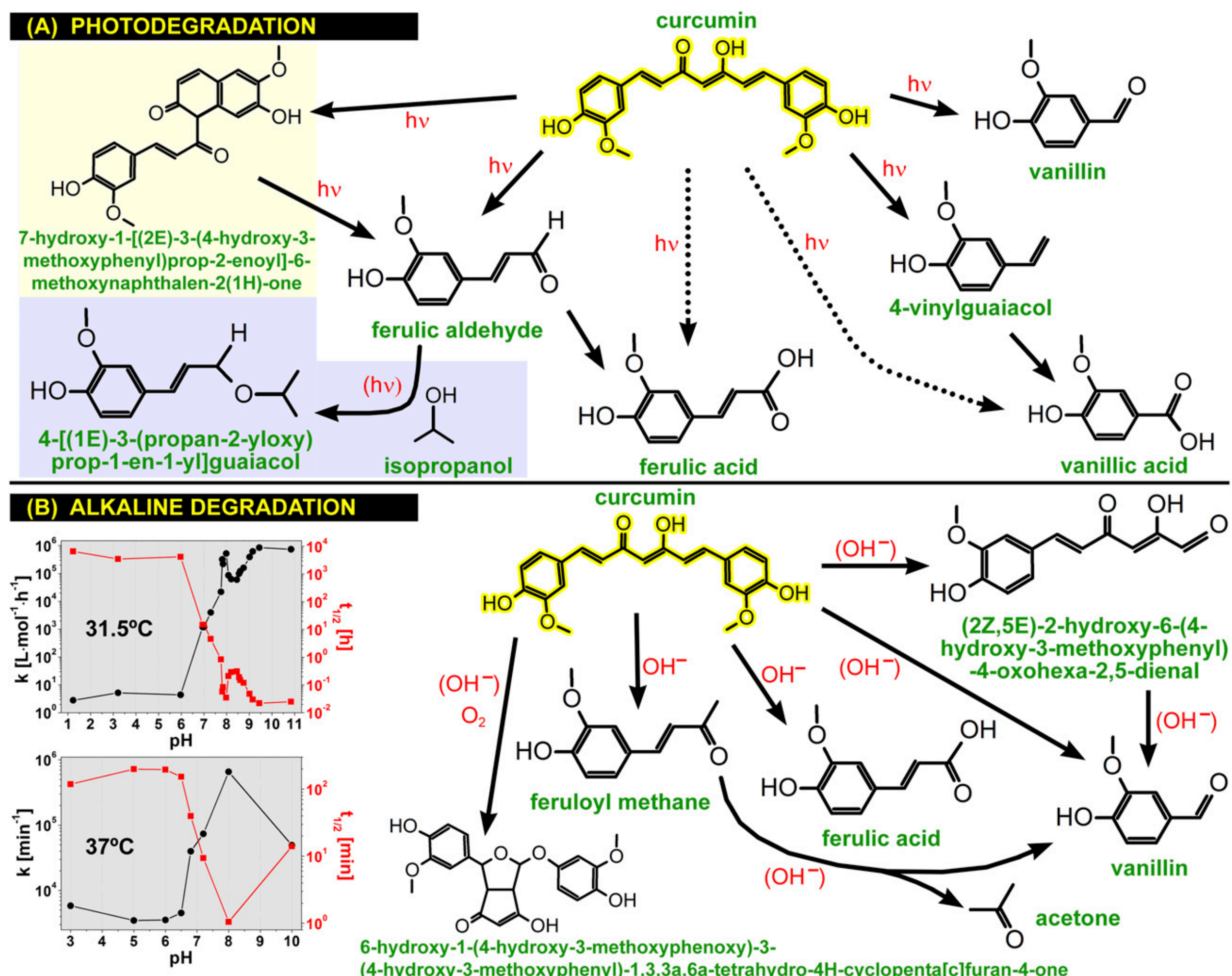


Fig. 6. A, Photo-induced degradation of curcumin by visible light and identification of the catalytic byproducts. The byproducts in the white palette are formed during irradiation of solid state curcumin as well as of curcumin dissolved in organic solvent. Under specific conditions, additional metabolites are formed in the case of solubilized curcumin, including cyclized curcumin in methanol, isopropanol, and chloroform (yellow palette) and a guaiacol derivative in isopropanol (blue palette), which requires isopropanol as a substrate in the reaction. The dotted arrows indicate that the direct formation of the respective byproducts from curcumin has not been unequivocally demonstrated, and hν signifies light. Data compiled from Tønnesen et al. (1986) and Khurana and Ho (1988). B, Degradation of curcumin dissolved in buffered aqueous solutions and identification of the byproducts. The reactions for which the OH^- is placed in parentheses indicate that the reactions were performed under alkaline conditions but may not necessarily require OH^- ions for the degradation to occur. The reaction rate constants (left y-axis) and half-times ($t_{1/2}$, right y-axis) of curcumin degradation in phosphate buffer at 31.5°C (upper panel) and 37°C (bottom panel), plotted as a function of pH, are provided on the left. For the data at 37°C, curcumin was dissolved in PBS buffer except for pH = 3 and 5 (PBS + citrate buffer) and pH = 10 (carbonate buffer). Data compiled from Tønnesen and Karlsen (1985a,b), Wang et al. (1997), Griesser et al. (2011), and Gordon and Schneider (2012).

products, whereas irradiation with daylight produced five unspecified degradation products (Ansari et al., 2005), indicating that some of the degradative reactions require the first excited or triplet state induced through the violet absorption band ("Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/modification of dissolved curcumin," section II.C.1.a.ii).

Exposure of curcumin to visible light also causes solvent-dependent structural modifications in addition to degradation. Chromatographic separation and subsequent analysis by MS and NMR revealed that curcumin subjected to irradiation with 400- to 750-nm light for 4 hours was associated with double deprotonation and cyclization at one of the two o-methoxyphenyl residues (Tønnesen et al., 1986), yielding 7-hydroxy-1-[(2E)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]-6-methoxynaphthalen-2(1H)-one (Fig. 6A). The internal cyclization occurred in isopropanol, methanol, and chloroform but not in acetonitrile and ethyl acetate (Tønnesen et al., 1986). Moreover, the photo-induced degradation products of curcumin can further react with solvent molecules under the influence of visible light irradiation, as has been shown for ferulic aldehyde in isopropanol, yielding the condensation product 4-[(1E)-3-(propan-2-yloxy)prop-1-en-1-yl]guaiacol (Fig. 6A) (Tønnesen et al., 1986).

Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/modification of dissolved curcumin. Visible light-induced curcumin fragmentation or modification of its chemical structure is caused mostly by oxidative processes through

ROS, which in this case are produced by triplet state curcumin (^{3}Cur) itself upon irradiation. The production of ROS by curcumin is mediated via type I (electron transfer to molecular oxygen (O_2): $^3Cur + O_2 \rightarrow Cur^{\bullet +} + O_2^{\bullet -}$) (Tønnesen et al., 1986; Chignell et al., 1994; Dahl et al., 1994; Shen et al., 2005) and type II (energy transfer to O_2: $^3Cur + O_2 \rightarrow Cur + {}^1O_2$) (Chignell et al., 1994; Dahl et al., 1994; Shen et al., 2005) photochemical reactions. However, the extent of $O_2^{\bullet -}$ and $^1O_2$ generation by photochemical means is probably limited (Priyadarsini, 2009) insofar as singlet curcumin predominantly undergoes nonradiative decay to the ground state via intramolecular charge transfer (ICT) (Khopde et al., 2000; Zsila et al., 2003; Shen and Ji, 2007) and subsequent excited state intramolecular proton transfer (ESIPT) (Khopde et al., 2000; Nardo et al., 2008; Priyadarsini, 2009). Intersystem crossing to the triplet state, from which the type I and II reactions proceed, constitutes a minor pathway (Priyadarsini, 2009). Nevertheless, these photochemical reactions produce sufficient ROS to cause substantial curcumin modification and degradation, as addressed in the previous section.

By virtue of its ability to generate ROS as well as scavenge radicals, curcumin is best characterized as an autodestructive photosensitizer (Bruzell et al., 2005; Koon et al., 2006).

The photogeneration of $O_2^{\bullet -}$ by curcumin has been studied by electron paramagnetic resonance (EPR) using 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) as spin trap in combination with concomitant resonant light (420 nm) irradiation (Chignell et al., 1994). Curcumin was found to generate $O_2^{\bullet -}$ in different types of solvents, including benzene, toluene, acetone, acetonitrile, DMSO, and ethanol, which could not be inhibited by the $^1O_2$ quencher strychnine (Chignell et al., 1994). Furthermore, irradiation of curcumin-encapsulating sodium dodecyl sulfate micelles with a 100-W tungsten-halogen lamp (400-nm cutoff filter) for 3 hours concurred with complete consumption of dissolved oxygen, of which ~5% was recovered after the addition of catalase (Chignell et al., 1994), an enzyme responsible for disproportionating H_2O_2 to H_2O and O_2. The H_2O_2 was most likely formed by the spontaneous disproportionation of $O_2^{\bullet -}$ (Sawyer and Valentine, 1981), which had formed as a result of curcumin irradiation. These experimental data confirm that O_2 undergoes a one-electron reduction by accepting a triplet state electron from curcumin, yielding a curcumin radical in the process (Shen et al., 2005).

The site of the type I process, and hence the location where the curcumin radical forms, appears to be the diketo moiety following excited state ICT from the methoxyphenyl group (Khopde et al., 2000; Zsila et al., 2003; Shen and Ji, 2007). Accordingly, type I reactions in curcumin produce two radical species, $O_2^{\bullet -}$ and a ketonic O-centered curcumin radical (Khopde et al., 2000; Zsila et al., 2003; Shen et al., 2005), which is most likely stabilized by the dicarbonyl moiety ($O{=}C{-}C{=}C{-}O^\bullet \leftrightarrow O{=}C{-}C^\bullet{-}C{=}O \leftrightarrow {}^\bullet O{-}C{=}C{-}C{=}O$). In turn, photogenerated $O_2^{\bullet -}$ can be scavenged by curcumin, producing a phenylic O-centered curcumin radical in the process. Both types of O-centered curcumin radicals are catalysts for the degradation and modification of curcumin.

The scavenging of $O_2^{\bullet -}$ by curcumin has been demonstrated in several studies. By use of an in vitro xanthine-xanthine oxidase system to generate $O_2^{\bullet -}$, curcumin was shown to inhibit the $O_2^{\bullet -}$-mediated reduction of ferricytochrome *c* by 25–40% at a concentration of 20–80 μM in a concentration-dependent manner (Reddy and Lokesh, 1994; Das and Das, 2002) and of nitroblue tetrazolium (NBT) by 19% at a curcumin concentration of 270 μM [this system also contained catalase and superoxide dismutase (SOD)] (Vajragupta et al., 2004). The latter results were confirmed by EPR in the same $O_2^{\bullet -}$-generating system employing DMPO as $O_2^{\bullet -}$ trap, showing that 10 mM curcumin inhibited 30% of DMPO-OOH adduct formation at a 250-mM DMPO final concentration (Vajragupta et al., 2004). Similarly, in an $O_2^{\bullet -}$-generating system based on the photosensitization of riboflavin, curcumin inhibited the $O_2^{\bullet -}$-mediated reduction of NBT by 50% at a concentration of 17 μM (Ruby et al., 1995) and by 43% at a concentration of 41 μM (Ak and Gulcin, 2008). Although the quantitative data are not in complete agreement because of slight differences in the assay method and possibly differences in the employed light source and irradiation parameters, the trend is the same, namely a curcumin-mediated decrease in the reduction of NBT. The findings hence collectively attest to the fact that curcumin is capable of reacting with $O_2^{\bullet -}$. Reactions between $O_2^{\bullet -}$ and antioxidants can proceed via SET (Buettner, 1993; Bielksi et al., 1985), but in the case of curcumin, $O_2^{\bullet -}$ is more likely to disproportionate via HAT from the phenylic hydroxyl group to yield a curcumin phenoxyl radical (Jovanovic et al., 1999; Masuda et al., 1999; Barclay et al., 2000; Sun et al., 2002; Wright, 2002; Priyadarsini et al., 2003; Litwinienko and Ingold, 2004; Chen et al., 2006c; Agnihotri and Mishra, 2011) and a hydroperoxyl anion ($O_2^{\bullet -} + ROH \rightarrow HO_2^- + RO^\bullet$) (Nakanishi et al., 2003; Lee et al., 2006; Jung and Ham, 2007), given the relatively low bond dissociation energy of $O_2^{\bullet -}$ (Jurasek and Argyropoulos, 2006). However, the disproportionation of $O_2^{\bullet -}$ in aqueous medium ($2\ O_2^{\bullet -} + 2\ H_2O \rightarrow O_2 + H_2O_2 + 2\ OH^-$) occurs at a rate constant of $< 3{\times}10^{-1}$ $L{\bullet}mol^{-1}{\bullet}s^{-1}$ (Sawyer and Valentine, 1981). The oxidation of curcumin by this route is therefore associated with a marginal phenoxyl radical yield, as $O_2^{\bullet -}$ reacts with comparable antioxidants such as ascorbic acid and α-tocopherol at considerably lower rate constants (k = 10^{-4}-10^{-5} $L{\bullet}mol^{-1}{\bullet}s^{-1}$) (Bielksi et al., 1985; Buettner, 1993). The oxidation of the phenylic hydroxyl group is largely outcompeted by the spontaneous disproportionation process.

Once a phenylic O-centered curcumin radical has been formed, however, the radical can translocate along the conjugated system to the methine carbons (Sugiyama et al., 1996; Priyadarsini et al., 2003; Fujisawa et al., 2004; Litwinienko and Ingold, 2004; Chen et al., 2006c; Griesser et al., 2011) to produce C-centered radicals. The carbonyl O-centered radical formed by triplet state electron transfer to O_2 exhibits a similar pattern, albeit the radical is only confined to the C_α and not the other methine carbons. The reactions that proceed from the C-centered curcumin radicals ultimately yield cyclized end products (Masuda et al., 2002; Fujisawa et al., 2004; Griesser et al., 2011) and degradatory catabolites (Tønnesen et al., 1986; Tønnesen and Greenhill, 1992; Chignell et al., 1994; Masuda et al., 1999; Agnihotri and Mishra, 2011), as pointed out previously (section II.C.1) and detailed in the section on chemical degradation ("Chemical degradation and modification of curcumin," section II.C.1.b.i).

Additionally, curcumin may be oxidized by H_2O_2 formed from the spontaneous disproportionation of $O_2^{\bullet-}$. H_2O_2 apparently oxidizes curcumin at the phenylic hydroxyl group, as evidenced by the decrease in the rate of curcumin oxidation from 4.4 ± 0.4 to 3.9 ± 1.0 $\mu M \bullet min^{-1}$ (11%) for 4′-methoxycurcumin (see Fig. 1C for numbering) and to 0.07 ± 0.01 $\mu M \bullet min^{-1}$ (98%) for 4′,4′′-dimethoxycurcumin in Tris-HCl buffered solution (pH = 8.0) upon addition of H_2O_2 (Griesser et al., 2011). The incidental formation of •OH from H_2O_2 is precluded in these experiments, as •OH would have easily oxidized 4′,4′′-dimethoxycurcumin. H_2O_2-mediated curcumin oxidation produces a phenoxyl radical, the fate of which has been clarified above. Corroboratively, oxidation of curcumin by H_2O_2 was reported to yield ferulic acid (Tønnesen and Greenhill, 1992), one of the identified catabolites of irradiated curcumin (Fig. 6A) produced by C−C bond cleavage.

H_2O_2 could also facilitate the production of •OH through Haber-Weiss/Fenton chemistry (Fenton, 1894; Haber and Weiss, 1932) or in the absence of redox-sensitive transition metals (Blanksby et al., 2007). The indirect formation of more reactive radicals such as •OH from $O_2^{\bullet-}$ (via H_2O_2) is not unlikely, provided that *p*-anisyl and veratryl derivatives of curcumin, which lack both phenylic hydroxyl groups (Babu and Rajasekharan, 1994), were able to scavenge $O_2^{\bullet-}$ in an isolated $O_2^{\bullet-}$-generating system (photosensitization of riboflavin) (Anto et al., 1996b). This could have only occurred via enolic O−H or C−H bonds that have higher bond dissociation energies than $O_2^{\bullet-}$, and thus by more reactive ROS than $O_2^{\bullet-}$. The scavenging of •OH by curcumin and its subsequent degradation have been proven in numerous studies (Tønnesen, 1989a; Kunchandy and Rao, 1990; Tønnesen and Greenhill, 1992; Reddy and Lokesh, 1994; Ruby et al., 1995; Das and Das, 2002), the specifics of which are further discussed in the section on chemical degradation ("Chemical degradation and modification of curcumin," section II.C.1.b.i), where this process is more prominent and experimentally better supported.

Lastly, it has been reported that the water-mediated disproportionation of $O_2^{\bullet-}$ as well as electron transfer reactions between $O_2^{\bullet-}$ and redox-sensitive transition metals yield $^1O_2$ (Khan, 1981, 1989; Corey et al., 1987a, b). $^1O_2$ production also occurs in the Haber-Weiss reaction (Khan and Kasha, 1994). Consequently, the curcumin-mediated photoproduction of the relatively innocuous $O_2^{\bullet-}$ could leverage into the generation of the highly toxic $^1O_2$ via multiple pathways, which will have profound implications on curcumin degradation and modification ("Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/modification of dissolved curcumin," section II.C.1.a.ii).

The abovementioned set of reactions, including additional $O_2^{\bullet-}$-mediated reactions as described in Sawyer et al. (1981) and Winterbourn and Kettle (2003), in all probability constitute the starting conditions from which curcumin is chemically degraded (Tønnesen et al., 1986; Ansari et al., 2005) or modified via pathways as proposed by, e.g., Tønnesen and Greenhill (1992), Masuda et al. (1999, 2002), Fujisawa et al. (2004), Agnihotri and Mishra (2011), and Griesser et al. (2011). These reactions also account for the formation of C-centered radicals as observed by Chignell et al. (1994) during irradiation of dissolved curcumin.

As opposed to $O_2^{\bullet-}$, type II reaction-derived $^1O_2$ constitutes a highly reactive ROS that attacks numerous biomolecules (e.g., fatty acids, amino acids, nucleic acids, steroids, and endogenous pigments) as well as olefins and (hetero-)aromatics, both of which are present in curcumin. The reactions typically proceed via oxygenation (H-abstraction and O-addition) or cycloaddition (forming 1,2-dioxetane or an endoperoxide).

In case of curcumin, $^1O_2$ formation as a result of irradiation with resonant visible light has been demonstrated directly by $^1O_2$ phosphorescence (Chignell et al., 1994), steady-state luminescence spectroscopy (Nardo et al., 2008), and EPR spin trapping (Chignell et al., 1994) and indirectly by absorption spectroscopy of curcumin in the presence of $^1O_2$ quenchers (Tønnesen et al., 1986; Chignell et al., 1994) as well as by density functional theory calculations (Shen et al., 2005). The $^1O_2$ yields after irradiation of curcumin with resonant visible light are strongly solvent dependent (Chignell et al., 1994), which indicates that the energy transfer from triplet state curcumin to O_2 proceeds from the β-dicarbonyl moiety. In nonpolar (toluene and benzene) and polar aprotic (acetonitrile) solvents, the quantum yield of $^1O_2$ formation is 0.11–0.12 (Chignell et al., 1994; Gorman et al., 1994), whereas in polar protic solvents such as ethanol and iso-propanol as well as in surfactant micelles (sodium dodecyl sulfate and Triton X-100) the quantum yields are approximately 10-fold lower

(Chignell et al., 1994) and fourfold lower (in methanol versus acetonitrile, Hanne Hjort Tønnesen, personal communication). This may stem from the shift in curcumin's keto-enol equilibrium toward the enol form in solvents with increasing proticity (section II.A.1). Ketones have long-lived triplet states (required for $^1O_2$ generation) in aprotic solvents (Wagner, 1976), and curcumin partially adopts a diketo conformation in aprotic solvents (Fig. 1B). The longevity of the keto triplet state is perturbed by inter- and intramolecular H-bonding in alcohols and water (Fig. 1B), accounting for the marginal $^1O_2$ generation by curcumin in these solvents and the more extensive $^1O_2$ generation in nonpolar and aprotic solvents (Chignell et al., 1994). On the other hand, deactivation of curcumin's first excited state may proceed via mechanisms other than nonradiative decay through intermolecular H-bonding with solvent molecules and may entail ESIPT that involves reketonization, as detailed in Nardo et al. (2008). ESIPT-mediated reketonization would interfere with intersystem crossing of the excited state electron to the triplet state, subsequent energy transfer to O_2, and corollary production of $^1O_2$.

On top of producing $^1O_2$, curcumin has been shown to scavenge $^1O_2$ in two different in vitro $^1O_2$-generating systems. The first system employed peroxidized 3,3′-(1,4-naphthylene)-dipropionate (NDP-O_2), which, when incubated at 37°C, yields NDP, O_2, and $^1O_2$. The addition of NDP-O_2 to plasmid DNA at 37°C caused $^1O_2$-mediated single strand breaks in the DNA (measured by agarose gel electrophoresis and densitometry), a process that was inhibited by 48% in the presence of 100 μM curcumin and by 31% in the presence of 10 μM curcumin (Subramanian et al., 1994). Moreover, the extent of protection was in the order of curcumin > demethoxycurcumin > *bis*-demethoxycurcumin (Subramanian et al., 1994), suggesting that the methoxy groups play a role in $^1O_2$ scavenging. In the second system, $^1O_2$ was generated by photosensitization of Rose Bengal and quantified by EPR spectroscopy of 2,2,6,6-tetramethyl piperidine (TEMP)-O_2 adducts (TEMPO). TEMPO formation was inhibited by 85% at a 3.12 μM curcumin concentration and by 50% at 2.75 μM curcumin concentration (Das and Das, 2002). Both studies hence provide evidence that curcumin is a $^1O_2$ scavenger.

Curcumin-mediated $^1O_2$ photoproduction and subsequent scavenging is accompanied by curcumin degradation that concurs with a reduction in $^1O_2$ phosphorescence (Chignell et al., 1994), underscoring the notion that, just as singlet state curcumin ("Chemical degradation and modification of curcumin," section II.C.1.b.i), triplet state curcumin is autodestructive. Some or all of the resulting degradation products (vanillin, vanillic acid, ferulic acid, ferulic aldehyde, 4-vinylguaiacol, and 7-hydroxy-1-[(2*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]-6-methoxynaphthalen-2(1*H*)-one, Fig. 6A) (Tønnesen et al., 1986) are photosensitizers themselves, albeit weaker than curcumin, and propagate the degradation process with continued light exposure (Chignell et al., 1994). Unfortunately, the precise $^1O_2$-mediated degradation mechanism is presently elusive, although it is known that certain hindered phenyls such as 2,6-di-*tert*-butylphenol, which is somewhat similar to the methoxyphenyl residues of curcumin, are susceptible to H-abstraction by $^1O_2$ to yield substituted phenoxyl radicals and corollary dimers (Matsuura et al., 1972). Corroboratively, the breakdown of curcumin in isopropanol by visible light (400–750 nm) could be inhibited by the addition of $^1O_2$ quenchers β-carotene and 1,4-diazabicyclo(2,2,2)octane and induced by photoactivation of methylene blue (an $^1O_2$ generator) at wavelengths not absorbed by curcumin (500–750 nm) (Tønnesen et al., 1986). Methylene blue-facilitated degradation of curcumin could in turn be inhibited by β-carotene (Tønnesen et al., 1986), altogether confirming that $^1O_2$ is responsible for the catalytic effects. Similarly, photo-induced curcumin degradation in toluene could be prevented by the addition of strychnine (Chignell et al., 1994).

The autodestructiveness of light-exposed curcumin does not necessarily require an aerated environment and can manifest itself under deoxygenated conditions, i.e., in the absence of $O_2^{\bullet-}$ or $^1O_2$. When curcumin was embedded in an oxygen-impermeable carbohydrate matrix and irradiated with broadband (240–600 nm) or filtered (400–510 nm) light, extensive photobleaching was observed, but chromatographic analysis did not reveal any of the common degradation products (Tønnesen et al., 1986). A comparable effect was evidenced for curcumin in deoxygenated toluene, where photobleaching occurred that could not have been caused by ROS (Chignell et al., 1994). However, the exact mechanism underlying these O_2-independent processes has remained at large, mainly because the fundamentals of nonoxidative photobleaching are elusive.

b. Chemical degradation. In addition to photo-induced degradation, curcumin undergoes rapid and considerable degradation in alkaline buffered aqueous solutions, cell culture medium (RPMI 1640), and human plasma (Tønnesen and Karlsen, 1985a,b; Wang et al., 1997; Griesser et al., 2011; Gordon and Schneider, 2012). The catalytic end products of curcumin in phosphate-buffered solutions equilibrated at 31.5°C (pH = 8.5) (Tønnesen and Karlsen, 1985a) or 37°C (pH = 7.2) (Wang et al., 1997) were vanillin, ferulic acid, and feruloyl methane (Fig. 6B). Incubation at 31.5°C for 5 minutes or 28 hours at pH = 8.5 yielded predominantly feruloyl methane, the further degradation of which resulted in the formation of vanillin and acetone as byproduct (Tønnesen and Karlsen, 1985a). Incubation of curcumin in phosphate-buffered solution, RPMI 1640 medium, or human plasma at 37°C for 1 hour at pH = 7.2 produced vanillin as the major

degradation product as well as (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal (Fig. 6B) (Wang et al., 1997). At longer incubation times the (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal was further degraded to vanillin (Wang et al., 1997), implying the cogeneration of (2*Z*)-2-hydroxy-4-oxopent-2-enal as catabolic byproduct, albeit this was not confirmed experimentally. Furthermore, the suspension of curcumin in Tris-HCl buffer (pH = 8) at room temperature for 30 minutes or phosphate buffer (pH = 7.5) for 2 hours yielded vanillin, ferulic acid, and feruloylmethane as minor degradation products and 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one as the major degradation product (Fig. 6B) (Griesser et al., 2011; Gordon and Schneider, 2012), indicating that internal cyclization can also occur in the absence of type I or type II photochemical reactions.

With respect to buffered aqueous solutions, the rate and rate order of the decomposition are pH and temperature dependent, respectively. The degradation rate constants and curcumin half-lives in phosphate-buffered solutions at 31.5°C (pH = 8.5) (Tønnesen and Karlsen, 1985a) or 37°C (pH = 7.2) (Wang et al., 1997) are plotted as a function of pH in Fig. 6B. It is evident that at both temperatures curcumin is relatively stable in a pH range of 1–6, i.e., acidities at which curcumin is expected to be (almost) fully protonated in the enol form (Tønnesen and Karlsen, 1985b) (Fig. 1C). At pH > 6, degradation rate constants accelerate rapidly by more than two orders of magnitude and reach a maximum around pH = 8. The degradation kinetics at 31.5°C are of a second order (Tønnesen and Karlsen, 1985b), whereas at body temperature the kinetics entail a first-order process (Wang et al., 1997). Curcumin degrades much faster at 37°C (50% in 9.4 minutes at pH = 7.2 and in 1.1 minute at pH = 8.0) (Wang et al., 1997) than at 31.5°C (50% in 4.6 hours at pH = 7.3 and in 2.1 minutes at pH = 8.0) (Tønnesen and Karlsen, 1985b).

i. Mechanisms of Chemical Degradation.

Solvolysis: (alkaline) hydrolysis. Solvolysis encompasses the degradation of a compound through a nucleophilic substitution or elimination reaction by solvent molecules. Here, only hydrolysis will be considered, a process that yields a protonated and a hydroxylated fragment of the parent molecule. The same principally applies to alkaline hydrolysis, where, in the case of β-dicarbonyl compounds, the reaction advances according to $RCOCH_2COR' + OH^- \rightarrow RCOCH_3 + R'CO_2^-$ (Rahil and Pratt, 1977).

Accordingly, ferulic acid and feruloyl methane are justifiable byproducts of hydrolytic degradation of curcumin (Fig. 7A). Hydrolysis is facilitated by OH^- ions, and the rate at which curcumin degrades increases with decreasing pOH (Fig. 6B and 7A). This premise notwithstanding, radical-induced strand cleavage at the C_α also constitutes a possible route to ferulic acid and feruloyl methane formation, as explained in the next section.

On the other hand, the formation of the other catabolites cannot be explained by hydrolytic processes, even under basic conditions. In the absence of substitutable N- or S-atoms, alkynes, specific ketone-producing reagents, ester or amide groups, or resonant light, oxo-additions to a terminal methyl group [in case of vanillin and (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal] or to an alkene chain [in case of 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one] typically cannot occur in aqueous solutions (Larson and Weber, 1994) by other processes than radical-mediated reactions.

Chemical degradation and modification of curcumin. On the basis of the previous paragraph, it is evident that ROS-mediated redox reactions not initiated by photochemical means also result in curcumin degradation and modification. The chemical degradation of curcumin in sodium phosphate/citrate buffer (pH = 8.0) coincides with the depletion of dissolved O_2 at approximately equal rates, namely 4.3 μM•min^{-1} and 4.5 μM•min^{-1}, respectively (Griesser et al., 2011). One of the recently identified catabolites of curcumin, 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta [*c*]furan-4-one (Fig. 7B) (Griesser et al., 2011; Gordon and Schneider, 2012), contains 8 O-atoms instead of the 6 O-atoms of the parent compound, altogether corroborating the role of O_2 and the potential involvement of ROS in the reactions that lead to the chemical degradation and modification of curcumin.

With the exception of feruloyl methane and ferulic acid, the cyclized, dimerized, and monomethoxyphenylic catabolites of curcumin have one distinct feature in common, namely that they are formed from a curcumin (phenoxyl, alkoxy, or C-centered) radical (Masuda et al., 1999, 2002; Fujisawa et al., 2004; Agnihotri and Mishra, 2011; Griesser et al., 2011). Curcumin "radicalization" as a precursory condition for subsequent degradation/modification is unequivocal and uncontested, but the mechanism(s) underlying the formation of a curcumin radical in the absence of ROS-generating compounds (e.g., enzymes such as xanthine oxidase), noncurcumin radicals (e.g., DPPH), and self-sensitized type I or type II photochemical reactions is partly elusive.

Both $O_2^{\bullet-}$ and •OH have been implicated as the ROS generated by curcumin in physiological buffers. In potassium phosphate buffer (pH = 8.0), 50 μM curcumin was shown to generate $O_2^{\bullet-}$ at a rate of 0.1 μM•min^{-1} over the course of 20 minutes, measured spectrophotometrically by the $O_2^{\bullet-}$-mediated reduction of cytochrome *c* (Griesser et al., 2011). The kinetics of

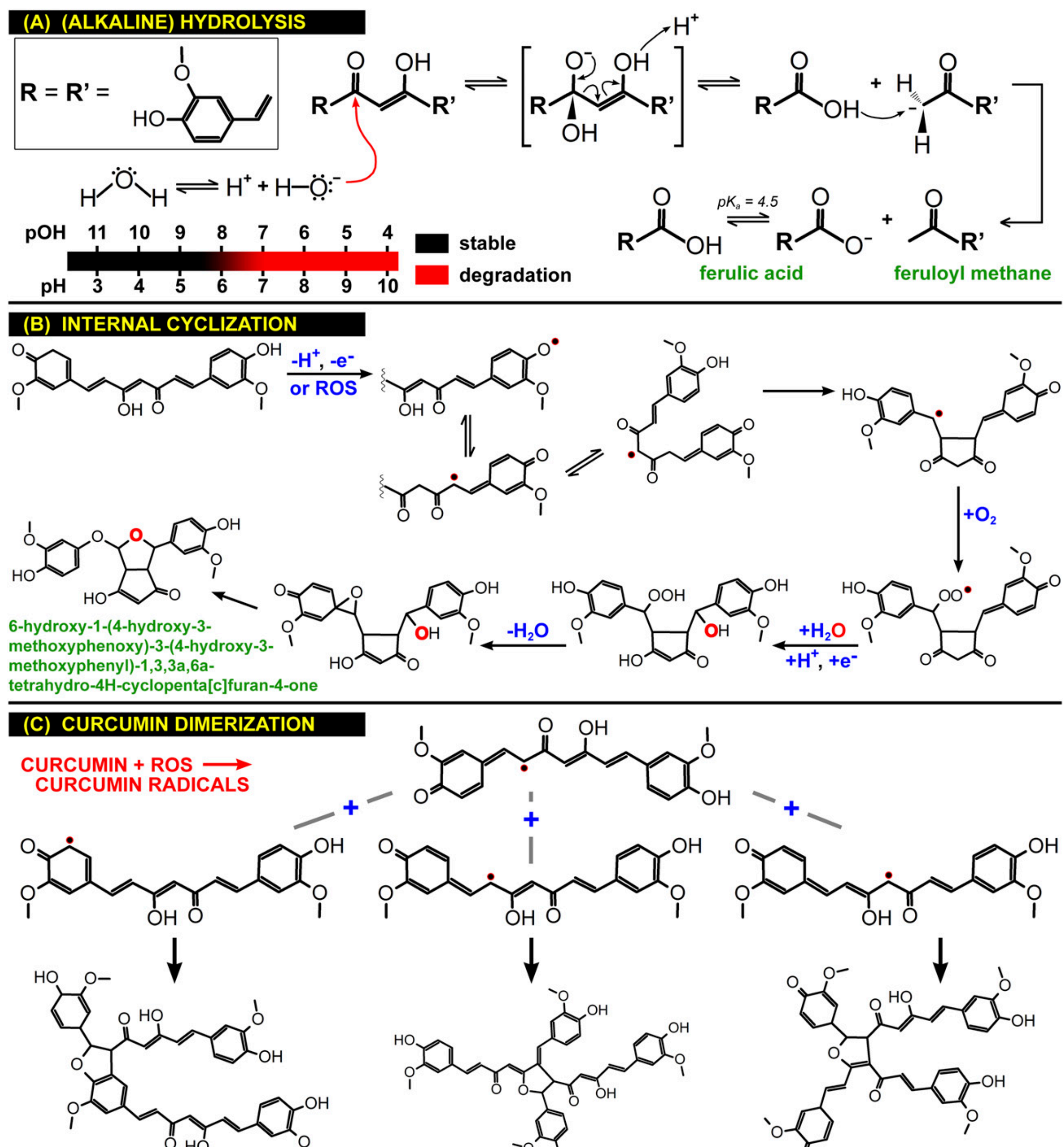


Fig. 7. A, Mechanistic pathway of (alkaline) hydrolysis of curcumin, resulting in the formation of feruloyl methane and ferulic acid. The reaction starts with a nucleophilic attack at the carbonyl carbon by a hydroxyl anion. Hydroxyl anions arise from the spontaneous dissociation of water molecules, as drawn, or are abundantly present under alkaline conditions. A pH and pOH scale was inserted to show at which values curcumin degradation occurs most prominently (black = no degradation, red = degradation). Degradation data extrapolated from Tønnesen and Karlsen (1985a,b) and Wang et al. (1997). The p*K*a of ferulic acid was deduced from pKa values of similar structures (Brown et al., 1955). B, Internal cyclization mechanism after the formation of a phenoxyl radical, either by auto-oxidation or by ROS, as proposed by Griesser et al. (2011). The reaction was carried out in radioactive water ($H_2{}^{18}O$, radioactive O in red font) and assayed by liquid chromatography-tandem-MS using an ion trap mass detector to determine the origin of the dioxo-addition. The experiments evinced that one of the added O-atoms comes from molecular oxygen consumed during the reaction and the other added O-atom is derived from the water molecules in the buffer. C, Mechanism of curcumin dimerization. The scavenging of radicals causes curcumin to become a radical itself, which then reacts with other curcumin radicals to form furan-centered curcumin dimers. The structure of the end products from these radical-radical coupling reactions is dictated by the position of the radical on the curcumin molecule. Data adapted from Masuda et al. (1999, 2002) and Fujisawa et al. (2004).

curcumin degradation and O_2 depletion were superimposable, and the reduction of cytochrome *c* was inhibited by SOD, confirming the production of specifically $O_2{}^{\bullet-}$. The authors proposed that a phenoxyl radical is formed by oxidation of the phenylate anion by O_2, whereby O_2 is reduced to $O_2{}^{\bullet-}$, forming a curcumin phenylate anion radical in the process. This mechanism most probably applies to curcumin, given the fact

that at pH = 8 the relative abundance of the phenylate anion is ~25% (pK_{a2} = 8.5, Fig. 1C) and that the formation of a phenoxyl radical in eugenol (half-curcumin minus the C_α and carbonyl) starts at pH = 9 (Fujisawa et al., 2002) (i.e., close to pK_{a2} of curcumin) in a pH-dependent pattern that is very similar to the pattern of pH-dependent degradation of curcumin (Fig. 6B). Nevertheless, this mechanism does not account for all aspects of the degradation kinetics. In the absence of additional reducing agents, redox reactions between redox couples (curcumin and ROS) proceed at stoichiometric parity (Schafer and Buettner, 2001). It is therefore peculiar that, at a ~15 μM (~25%) concentration of the phenylate anion, 45 μM (75%) of curcumin was degraded, especially when only 1 μM $O_2^{\bullet-}$ was produced during these 10-minute lasting reactions (Griesser et al., 2011). Hydrolysis as the predominant degradation mechanism is unlikely here because the amount of ferulic acid produced was marginal relative to the internally cyclized catabolite, 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one, a well-characterized curcumin radical end product (Griesser et al., 2011; Gordon and Schneider, 2012).

The arguments presented in the previous paragraph provide compelling evidence for the possibility that other mechanisms/ROS govern the chemical degradation of curcumin. Several studies have been published on the catalytic properties of curcumin in the Haber-Weiss/Fenton reactions. The Haber-Weiss reaction starts with the reduction of ferric iron to ferrous iron by $O_2^{\bullet-}$ ($Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$) followed by the oxidation of Fe^{2+} by H_2O_2 to yield •OH ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$), also referred to as the Fenton reaction. When the Haber-Weiss reaction was carried out with hypoxanthine/xanthine oxidase as a generator of $O_2^{\bullet-}$, $FeCl_3$ as a source of Fe^{3+}, and hyaluronic acid as a substrate for •OH, the oxidative degradation of hyaluronic acid (as evidenced by a decrease in viscosity) was exacerbated by the addition of curcumin (Tønnesen, 1989a). In similar experiments, the reduction in viscosity of curcumin-lacking hyaluronic acid mixtures was 68% in the presence of Fe^{3+} and 7% in the absence of Fe^{3+} after 20 minutes (Tønnesen, 1989b), which showed that hyaluronic acid was in fact degraded as a result of the iron-catalyzed Haber-Weiss reaction. A 66% decrease in viscosity was found when curcumin was added to mixtures not containing Fe^{3+} (Tønnesen, 1989b), indicating that curcumin is capable of generating •OH even when the first step of the Haber-Weiss reaction is circumvented. It should be noted that no H_2O_2 was added to these reaction mixtures, as the authors relied on the production of H_2O_2 from the spontaneous disproportionation of $O_2^{\bullet-}$ (Marklund, 1976; Tønnesen, 1989b). The inhibition of hyaluronic acid degradation by mannitol, a specific •OH scavenger (Shen et al., 1997), substantiates the formation of H_2O_2 by spontaneous disproportionation of $O_2^{\bullet-}$ in these experiments.

Subsequent studies, which focused on the Fenton component only, revealed that curcumin in aqueous solution exhibits a Jekyll and Hyde-type of ROS personality that is comparable to ascorbic acid (Miller and Aust, 1989), namely one with both antioxidative and pro-oxidative properties (Kunchandy and Rao, 1990). At higher concentrations, curcumin behaves as an antioxidant, whereas at lower concentrations it acts as a pro-oxidant. The turning point was reported to lie between a curcumin concentration of 0.61 and 2.70 μM and depends on the type of substrate used to measure •OH production/scavenging (Kunchandy and Rao, 1990). It was further demonstrated that curcumin, just as ascorbic acid (Miller and Aust, 1989), is capable of reducing transition metals such as Fe^{3+} in a concentration-dependent manner (Kunchandy and Rao, 1989; Tønnesen and Greenhill, 1992). In fact, curcumin was more effective in reducing Fe^{3+} than ascorbic acid at equimolar concentrations (Reddy and Lokesh, 1994). However, the reduction of Fe^{3+} (100 μM) by curcumin was not directly proportional to the consequent •OH-mediated degradation of deoxyribose (used as substrate for •OH in the Fenton reaction) in the presence of H_2O_2, which was directly proportional in case of ascorbic acid (Reddy and Lokesh, 1994). The extent of deoxyribose degradation occurred in the order $10 > 20 > 5 = 50 = 100 >> 0$ μM curcumin (Reddy and Lokesh, 1994), indicating that beyond a threshold •OH:curcumin ratio the Fenton reaction becomes debilitated, possibly due to curcumin oxidative degradation/modification and corollary inability to chelate and reduce transition metals. The reduction of Fe^{3+} occurs at the keto-enol moiety, i.e., curcumin's metal chelating center (section II.A.4), inasmuch as the redox reaction is unaffected in curcumin derivatives lacking both phenylic hydroxyl and methoxy groups (Tønnesen and Greenhill, 1992). This, however, does not preclude the possibility that SET reactions to transition metals do not occur from the phenylic hydroxyl group, as has been demonstrated for ferulic acid (Mellican et al., 2003). In both instances, reduction of Fe^{3+} concurs with the generation of O-centered curcumin radicals.

Based on the above, there are several possible pathways that lead to radical-mediated degradation and modification of curcumin in aqueous solution, which have been summarized in Fig. 8. Essentially, these pathways can be categorized into metal-free oxidation reactions and transition metal-catalyzed reactions, both yielding a curcumin radical. The metal-free oxidation reactions require curcumin phenylate anions for SET to molecular oxygen (Nakanishi et al., 2003; Lee et al., 2006; Jung and Ham, 2007) and are therefore both pH and O_2 dependent. At pH = pK_{a2} = 8.5, ~50% of the curcumin molecules comprise the phenylate anion tautomer, which translates to ~5% at

pH = 7.5 and ~0.5% at pH = 6.5. The fact that curcumin degradation rapidly accelerates from pH = 6.5 is hence in compliance with the increasing presence of the phenylate anion and the metal-free oxidation of this curcumin tautomer by codissolved O_2 in neutral-to-alkaline buffers. The $O_2^{\bullet -}$ that is formed in the process can in turn oxidize phenylic residues by HAT ("Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/ modification of dissolved curcumin," section II.C.1.a.ii) or spontaneously disproportionate into H_2O_2, which is also capable of oxidizing the phenylic hydroxyl group ("Mechanisms of photo-induced reactive oxygen species production by curcumin and photochemical degradation/ modification of dissolved curcumin," section II.C.1.a.ii) and possibly form •OH in the absence of redox-active metals (Blanksby et al., 2007). The resulting curcumin phenylate anion radicals can subsequently undergo radical delocalization, giving rise to radical-radical coupling-, cyclization-, and C−C bond cleavage reactions (Fig. 8). The latter reactions further deplete dissolved O_2 during the formation of 1,2-dioxetane adducts as reaction intermediates.

The metal-free oxidation reactions most likely proceed concomitantly with transition metal-catalyzed reactions, because redox-active metals such iron and copper are often present in trace amounts in aqueous buffers (Powell and Wapnir, 1994), including phosphate-buffered saline (Huang et al., 2004), cell culture medium (Huang et al., 2004), and possibly in (purified) curcumin extracts. There are two main transition metal-facilitated pathways that result in curcumin degradation or modification, of which the most ubiquitous pathway entails a redox reaction between

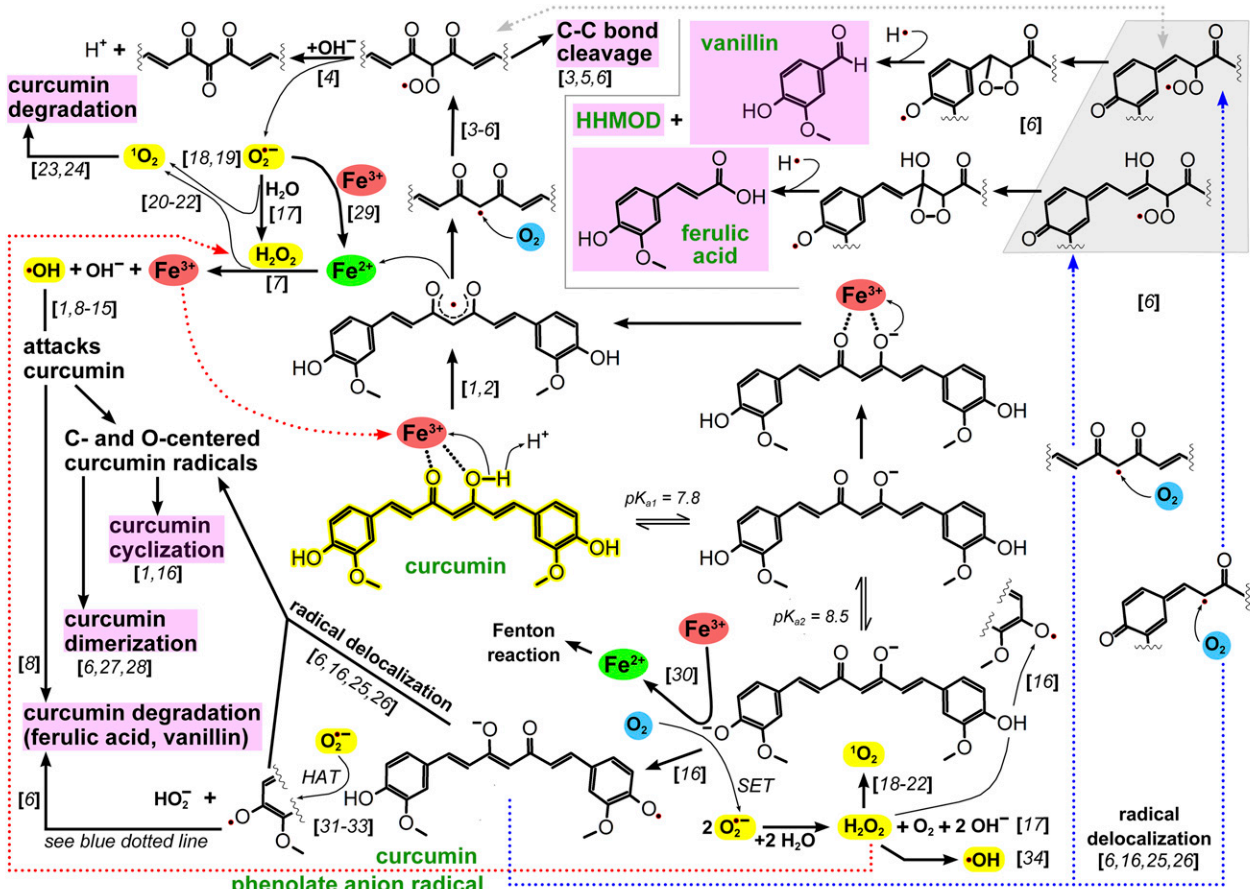


Fig. 8. Mechanistic pathways of radical-mediated degradation and modification of curcumin in aqueous solution. These degradatory pathways occur in addition to alkaline hydrolysis as presented in Fig. 7A. The parent compound is stroked in yellow. The reactions start at the parent compound and proceed according to the arrows. The end products or processes leading to the formation of end products are indicated in pink, and ROS are highlighted in yellow. The species formed from curcumin radicals after cyclization and dimerization are provided in Fig. 7, B and C. HHMOD = (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal (Fig. 6B). Iron is used as an exemplary transition metal; the redox reactions can also be catalyzed by other transition metals. The references for each step are provided in brackets: [1], (Tønnesen and Greenhill, 1992); [2], (Kunchandy and Rao, 1989); [3], (Litwinienko and Ingold, 2004); [4], (Chamulitrat et al., 1991); [5], (Sugiyama et al., 1996); [6], (Masuda et al., 1999); [7], (Prousek, 2007); [8], (Agnihotri and Mishra, 2011); [9], (Das and Das, 2002); [10], (Kunchandy and Rao, 1990); [11], (Reddy and Lokesh, 1994); [12], (Ruby et al., 1995); [13], (Tønnesen, 1989a); [14], (Vajragupta et al., 2004); [15], (Biswas et al., 2005); [16], (Griesser et al., 2011); [17], (Marklund, 1976); [18], (Khan, 1981); [19], (Corey et al., 1987b); [20], (Corey et al., 1987a); [21], (Khan, 1989); [22], (Khan and Kasha, 1994); [23], (Chignell et al., 1994); [24], (Tønnesen et al., 1986); [25], (Chen et al., 2006c); [26], (Priyadarsini et al., 2003); [27], (Fujisawa et al., 2004); [28], (Masuda et al., 2002); [29], (Haber and Weiss, 1932); [30], (Mellican et al., 2003); [31], (Jung and Ham, 2007); [32], (Lee et al., 2006); [33], (Nakanishi et al., 2003); [34], (Blanksby et al., 2007).

curcumin and the metal cation, producing a carbonyl O-centered curcumin radical (Fig. 8) that is resonance stabilized by the diketo moiety. The C_α radicals may undergo internal cyclization (Fig. 7B) or radical-radical coupling with other curcumin radicals (Fig. 7C) or peroxidation by O_2 in aerated solvents. The latter progresses to either C−C bond cleavage or ketonization under alkaline conditions to yield $O_2^{\bullet-}$, which in turn can react with curcumin as described in the previous paragraph, oxidize ferric iron to its ferrous state, or disproportionate into H_2O_2. The second pathway requires the presence of (curcumin-reduced) Fe^{2+}, whereby H_2O_2 facilitates the generation of the most reactive ROS, •OH, via the Fenton reaction. •OH subsequently abstracts a proton from any curcumin atom to produce a curcumin radical from which modified catabolites and degradation products are formed (Fig. 8). The Fenton reaction causes oxidation of ferrous iron into its ferric state, which can be reduced again by curcumin to propagate the redox cycle ($Cur + Fe^{3+} \rightarrow Cur^{\bullet} + Fe^{2+} \rightarrow Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + Cur \rightarrow$ repetition). Evidently, the Fenton reaction is highly exacerbated at neutral and basic pH, where $O_2^{\bullet-}$ and its disproportionated derivative H_2O_2 are formed more abundantly due to the tautomerization of curcumin into a phenylate anion.

c. Uncharacterized curcumin degradation products (in vitro). As addressed in section II.C.1, curcumin undergoes degradation/modification upon exposure to (alkaline) aqueous solutions, light, and oxygen (particularly when dissolved), yielding numerous catabolites (Figs. 6–8), of which some have not been characterized. The in vitro studies in which the curcumin degradation products were examined mainly employed thin layer chromatography (Tønnesen and Karlsen, 1985a; Tønnesen et al., 1986; Tønnesen and Greenhill, 1992; Masuda et al., 1999, 2001, 2002; Ansari et al., 2005) or HPLC (Tønnesen and Karlsen, 1985a,b; Tønnesen et al., 1986; Khurana and Ho, 1988; Tønnesen and Greenhill, 1992; Wang et al., 1997; Masuda et al., 1999, 2001, 2002; Griesser et al., 2011; Gordon and Schneider, 2012) to isolate the curcumin catabolites and subsequently MS (Tønnesen and Karlsen, 1985a; Tønnesen et al., 1986; Masuda et al., 1999, 2001, 2002; Griesser et al., 2011; Gordon and Schneider, 2012), gas chromatography (Tønnesen et al., 1986), gas chromotography-MS (Wang et al., 1997), or NMR (Tønnesen et al., 1986; Masuda et al., 1999, 2001, 2002; Griesser et al., 2011) to characterize the catabolites. All HPLC isolations were based on reversed phase chromatography (Tønnesen et al., 1986; Khurana and Ho, 1988; Tønnesen and Greenhill, 1992; Wang et al., 1997; Masuda et al., 1999, 2001, 2002; Griesser et al., 2011; Gordon and Schneider, 2012) with detection of the eluates by fluorescence spectroscopy (λ_{ex} = 420 nm, λ_{em} = 470 nm) (Tønnesen and Karlsen, 1985a,b) or absorption spectroscopy at predominantly single wavelengths in the UV (Khurana and Ho, 1988), such as 240 nm (Masuda et al., 2002), 280 nm (Tønnesen and Karlsen, 1985a; Tønnesen et al., 1986), 300 nm (Masuda et al., 1999, 2002), 350 nm (Tønnesen et al., 1986; Tønnesen and Greenhill, 1992), and 360 nm (Masuda et al., 1999), as well as the visible spectrum, including 420 nm (Tønnesen et al., 1986; Masuda et al., 1999, 2001) and 430 nm (Ansari et al., 2005). Variable wavelength UV/visible spectrum or diode array spectrometers were employed only in a limited number of studies to detect the eluted compounds (Wang et al., 1997; Griesser et al., 2011; Gordon and Schneider, 2012).

In the majority of these curcumin modification/degradation studies, the chromatograms revealed peaks of which the compounds were not further characterized. For example, in the study by Khurana and Ho (1988), three compounds with a relative abundance of 33% were isolated but not characterized. These compounds were not vanillin, ferulic aldehyde, ferulic acid, or vanillic acid. Similarly, the chromatograms in Tønnesen et al. (1986) display several peaks after photochemical degradation of curcumin that were not further characterized, as do chromatograms acquired after curcumin degradation in buffered solutions (Tønnesen and Karlsen, 1985a; Wang et al., 1997; Griesser et al., 2011; Gordon and Schneider, 2012) and after oxidative modification (Masuda et al., 1999, 2002). These observations demonstrate that the list of curcumin catabolites presented in the literature and summarized in section II.C.1 is incomplete.

In addition, the isolation, and with it the subsequent characterization of curcumin modification/degradation products by liquid chromatography, depends chiefly on the stationary phase to capture the catabolites, the mobile phase with which the catabolites are eluted, and the spectroscopic detection system used to identify and collect the eluted catabolites. For any given combination of these chromatographic parameters, a specific set of molecules with specific chemical properties can be isolated and characterized. Consequently, more hydrophilic catabolites [e.g., those that contain nucleophilic groups due to oxidation/(di)oxo-addition] may not be captured by the reversed phase column material or may coelute early with other catabolites of similarly low log P value, as a result of which they will not be adequately separated. Such effects are nicely exemplified in Ansari et al. (2005), where curcumin that was degraded photochemically, oxidatively (by H_2O_2), or under acidic and basic conditions yielded peaks on the silica gel thin layer chromatography with a fractionation range that was lower and higher than that of curcumin. These peak positions indicate that, in light of a chloroform:methanol (92.5:7.5) mobile phase, degradation products were formed that were respectively more hydrophobic and more hydrophilic than curcumin. Depending on the HPLC conditions, these catabolites may fall outside of the properly separated range of eluents and hence go undetected.

The use of a single wavelength detection system further restricts the spectrum of compounds that can be detected, because curcumin has an absorption maximum at >400 nm (Tønnesen et al., 1995), whereas vanillin, vanillic acid, ferulic acid, feruloyl methane absorb at 327–340 nm (Tønnesen et al., 1995; Gordon and Schneider, 2012), the methoxyphenyl as well as tetrahydrocurcumin absorb at ~276 nm (Tønnesen et al., 1995), and the methine bridge containing only the β-dicarbonyl moiety absorbs at 272 nm (Tønnesen et al., 1995). On top of that, photochemically modified catabolites such as 7-hydroxy-1-[(2*E*)-3-(4-hydroxy-3-methoxyphenyl)prop-2-enoyl]-6-methoxynaphthalen-2(1*H*)-one (Fig. 6A) (Tønnesen et al., 1986), chemically modified catabolites such as (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal (Fig. 6B) (Wang et al., 1997) and 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3a,6a-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one (Fig. 7B) (Griesser et al., 2011; Gordon and Schneider, 2012), and the dimerized derivatives (Fig. 7C) (Fujisawa et al., 2004; Masuda et al., 1999, 2002) exhibit different absorption maxima than curcumin and its monophenylic degradation products (Gordon and Schneider, 2012), the position of which depends on whether the conjugated system has been perturbed. These catabolites may therefore go undetected when a single wavelength detection system is used during HPLC. The chemically modified catabolites may also exhibit different chromatographic properties, altogether adding to the fact that not all curcumin modification/degradation products have been characterized.

2. Implications of the (Photo)Chemical Instability of Curcumin.

a. Implications on pharmaceutical preparations of curcumin. The (photo)chemical instability (Fig. 5) makes curcumin a rather "onerous" pharmaceutical and brings forth several critical implications for the posology and formulation process. The first implication is that oral curcumin formulations should be administered at relatively high doses to compensate for the inevitable loss of pharmacological potency due to (photo)chemical degradation (section II.C.1) in aqueous environments. Although curcumin is initially dissolved in solvents in which it is stable (e.g., methanol, ethanol, DMSO), it will ultimately come in contact with an aqueous environment (buffer, culture medium, blood, cytosol) that is, in most instances, equilibrated/sustained at 37°C and pH ≈ 7.4 as well as replete with enzymes that mediate its modification/degradation. Additionally, poor uptake from the gut (section III.A), uptake by non-target tissue (section III.A), and enzyme-mediated biotransformation in healthy and cancer cells (section III.C) further reduce curcumin bioavailability after oral intake. Although these issues can to an extent be counteracted by administering higher dosages, such regimens are not always well-tolerated by patients. Clinical trials have reported patient discontent and withdrawal of patients from the high dosage cohorts solely due to the discomfort they experienced when ingesting a large amount of curcumin pills (Cheng et al., 2001; Irving et al., 2013).

A second implication is that, in case of oral delivery, curcumin formulations should preferably contain crystalline curcumin or curcumin suspended in a stabilizing, physiologically compatible solvent (e.g., oils) or emulsifier. Pills prepared in an oxygen-free environment are most ideal because such formulations are minimally susceptible to oxidative degradation/modification and can be sufficiently protected from light, although it has been shown that pharmaceutical curcumin preparations in tablet form may contain numerous catabolites (Ansari et al., 2005). Nevertheless, dissolution of crystalline or otherwise chemically stable curcumin formulations in the stomach and transport through the gastrointestinal tract is not expected to have detrimental effects on curcumin stability due to the dark and acidic (Fig. 6B) environment.

Lastly, formulations designed for oral administration are associated with poor systemic bioavailability as a result of in vitro and in vivo degradation/modification and unfavorable pharmacokinetics (section III.A). Curcumin formulated in pills is therefore highly suitable for the treatment of gastrointestinal malignancies (Sharma et al., 2001; Garcea et al., 2005; Villegas et al., 2008) and for long-term chemopreventive therapy (Anto et al., 1996a; Perkins et al., 2002; Lao et al., 2006; Villegas et al., 2008), particularly in the gastrointestinal tract (Huang et al., 1994; Rao et al., 1995), but ill-suited for the treatment of cancers outside of the gastrointestinal system. For these reasons, curcumin for oral chemotherapy of nongastrointestinal cancers should at least be coadministered with uptake-enhancing agents such as piperine (Shoba et al., 1998; Singh et al., 2013) and turmerones (Yue et al., 2012) or in nanoparticle-encapsulated form (Shaikh et al., 2009) when systemic delivery by a (targeted) drug delivery system is not possible (section II.C.2.a.i). For the treatment of non-gastrointestinal cancers, curcumin is best administered via the intravenous route (Bisht and Maitra, 2009) to achieve maximal systemic concentrations and delivery to the tumor. Inasmuch as curcumin is poorly soluble in water (section II.B), either a delivery system is required to solubilize and stabilize curcumin in plasma or curcumin must be chemically modified, e.g., with more hydrophilic functional groups.

i. Curcumin Drug Delivery Systems. Numerous nanoparticulate drug delivery systems have been developed for curcumin, including lipid-based (liposomes, micelles, solid lipid particles, nanodisks) and polymer-based vehicles, nano-emulsions/gels/suspensions, and molecular complexes such as curcumin-plasma proteins, -amino acid, and -water-soluble polymers (Ghosh et al., 2011; Sun et al., 2012a). In addition to the equal

or improved pharmacodynamic efficacy compared with unencapsulated curcumin (Li et al., 2005b; Ma et al., 2008; Thangapazham et al., 2008a; Song et al., 2011), potential benefits of curcumin encapsulation include protection from degradation/modification in buffered solutions, cell culture medium, and blood and plasma, as has been described for lipid-based nanocarrier systems (Barry et al., 2009; Chen et al., 2009a; Karewicz et al., 2011), and reduced uptake by organs (Song et al., 2011), altogether leading to improved bioavailability (Maiti et al., 2007; Marczylo et al., 2007; Gupta and Dixit, 2011). However, the level of protection is strongly dependent on the type and composition of the drug vehicle, particularly in the case of liposomes (Chen et al., 2009a), and there seems to be no protection from photochemical degradation. Whereas curcumin encapsulation in surfactant (SDS and Triton X-100) micelles relayed no protective effect on the photochemical degradation rate compared with free curcumin (Chignell et al., 1994), complexation of curcumin with different cyclodextrins even accelerated its photochemical decomposition (Tønnesen and Karlsen, 1985a,b; Tomren et al., 2007).

As the in vitro and in vivo chemical stability of curcumin generally improves upon encapsulation, there are several steps during the nanocarrier preparation process where curcumin may be susceptible to (photo)chemical degradation. This is best exemplified for liposomal formulations, the most commonly used and typically nontoxic (Wang et al., 2008a) nanocarriers for curcumin, although the principles will mostly apply to other drug delivery systems as well. First, photochemical degradation of curcumin can occur in crystalline and solubilized forms of curcumin (section II.C.1.a), as a result of which the curcumin formulations should always be kept out of direct light at any stage of the formulation process, i.e., by using amber glassware or foil-covered containers. Where possible, (biocompatible) $^1O_2$ quenchers (e.g., β-carotene) and transitional metal chelators (Welch et al., 2002) should be added to the formulation at nontoxic concentrations to deter curcumin (per)oxidation and Fenton reactions (Fig. 8). Second, liposomes can be prepared by at least six different methods (Lasch et al., 2003), all of which include a stage in which lipids are mixed with an aqueous phase to facilitate vesicle self-assembly. To deter chemical degradation of curcumin during this stage, the aqueous phase should be pH-adjusted (pH < 6.5) and iso-osmolar (0.292 osmol/kg) to prevent formation of the phenolate tautomer ("Chemical degradation and modification of curcumin," section II.C.1.b.i) and osmolar gradient-driven destabilization of the liposomes under physiological conditions (Drummond et al., 1999), respectively. If these prerequisites are not met, the carriers may ultimately comprise a mixture of curcumin and curcumin catabolites, the implications of which are addressed in section II.C.2.

ii. Synthetic Curcumin Analogues. Similarly, a variety of curcumin analogues have been synthesized for the purpose of increasing water solubility, deterring degradation/modification (Ferrari et al., 2009; Liang et al., 2009; Wan et al., 2010; Abdel Aziz et al., 2012; Gagliardi et al., 2012; Kulkarni et al., 2012), and improving therapeutic and chemopreventive efficacy (Ishida et al., 2002; Ohtsu et al., 2002; Venkateswarlu et al., 2005; Tamvakopoulos et al., 2007a; Fuchs et al., 2009; Liang et al., 2009). A schematic overview of synthetic curcumin analogues that are both more stable in aqueous media at neutral pH and pharmacologically equally or more effective than curcumin in different cancer cell lines is provided in Fig. 9.

For the curcumin analogues, different preparatory prerequisites apply than for the curcumin nanocarriers, where the encapsulated curcumin is largely shielded from the elements that mediate its breakdown. The prerequisites are mainly structure-driven and comprise three main components. First, stability of synthetic curcumin analogues is achieved by eliminating the potential oxidation sites, i.e., the phenylic and enolic hydroxyl groups, which precludes the formation of radicals and consequent chemical degradation/modification as described in "Chemical degradation and modification of curcumin" in section II.C.1.b.i and Fig. 8. Corroboratively, substitution of the phenylic hydroxyl groups by methoxy groups (i.e., dimethoxycurcumin) conferred considerable stability in cultured cells for 48 hours and in mice for up to 4 hours (Tamvakopoulos et al., 2007a).

Second, attention should be given to the fact that, in cells and in vivo, curcumin undergoes enzymatic cleavage and modification by proteins it directly associates with (section III.C). To eliminate enzymatic cleavage/modification of curcumin at the β-diketo moiety, for instance, numerous monocarbonyl derivatives have been synthesized (e.g., Fig. 9) (Robinson et al., 2005; Fuchs et al., 2009; Liang et al., 2009). In vivo testing in rats evinced that the circulation half time of some of the monocarbonyl analogues was longer than that of curcumin after oral administration, suggesting an improved systemic stability (Liang et al., 2009). It should be noted, however, that these data were not corrected for other important pharmacokinetic parameters such as uptake, biotransformation, and excretion and may therefore not reflect chemical stability per se.

Third, inasmuch as intermolecular bonding between curcumin and target molecules may be perturbed by the elimination of the enolic and phenylic hydroxyl groups (section II.A), synthetic curcumin analogues should be substituted with other functional groups or atoms capable of electrostatic interactions to maximally retain the pleiotropic binding behavior of the parent compound. Accordingly, it has been proposed (Liang et al., 2009) that the benzene should be

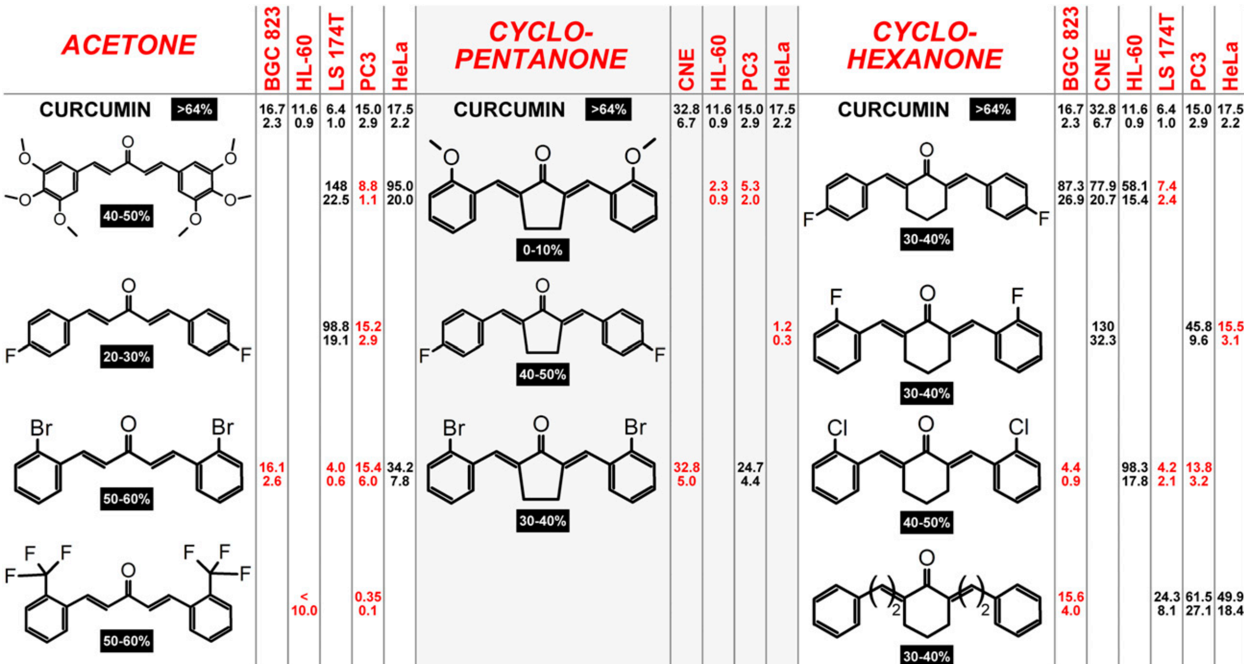


Fig. 9. Overview of synthetic monocarbonyl curcumin analogues that exhibit better stability in aqueous medium (sodium phosphate buffer containing 0.3% sodium carboxymethylcellulose, pH = 7.4) and equal or greater cytostatic potential than curcumin (indicated in the table in red). The analogues are categorized according to the substituted keto-enol linker (acetone, cyclopentanone, or cyclohexanone). The percentage degradation in the buffer solution after 75-hour incubation at 37°C is indicated in the black solid rectangles, whereas the 50% lethal concentration [LC_{50}, in μM, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay] is provided on the right of the structures per cell line: BGC 823 (human gastric adenocarcinoma), CNE (human nasopharyngeal carcinoma), HL-60 (human promyelocytic leukemia), LS 174T (human colorectal adenocarcinoma), PC3 (human prostate cancer), and HeLa (human cervix carcinoma). Data adapted from Liang et al. (2009). Dimethoxylated acetone-linked curcumin synthetics as well as different isomers of the trimethoxylated acetone-linked curcumin (top left structure) have also been shown to possess strong antiproliferative capacity in PC3 cells (2.5–4.6 μM versus 19.8 μM for curcumin), LNCaP cells (human prostate adenocarcinoma, 0.5–2.2 μM versus 19.6 μM for curcumin), MCF-7 cells (estrogen-dependent human breast carcinoma, 0.4–2.7 μM versus 21.5 μM for curcumin), and MDA-MB-231 cells (estrogen-independent human breast carcinoma, 0.6–2.4 μM versus 25.6 μM for curcumin) (Fuchs et al., 2009). Although the stability of these derivatives was not assayed in aqueous solvent, it is not expected to differ from that reported above. The di- and trimethylated acetone-linked curcumin analogues also exhibited considerably less inhibition of cell proliferation in non-cancerous cells (MCF-10A, human breast epithelial cells) than curcumin (Fuchs et al., 2009).

substituted with electron withdrawing groups (e.g., halogens, carbonyls) because the electronegativity of these groups correlates positively with cytotoxicity (Fig. 9), and/or a weak electron donating groups (e.g., alkyls, alcohols, amines) for maximum antitumor effects (Fuchs et al., 2009; Liang et al., 2009). Highly suitable electron withdrawing groups are the halogens (X = F, Cl, Br, see Fig. 9) because of their halogen bonding capacity (X•••:O) (Politzer et al., 2007) and because carbohalides are not readily oxidized by O_2 or ROS [except for •OH (Merga et al., 1996)] under physiological conditions (Yang et al., 2004a) to yield radicals or enzymatically degraded in eukaryotic organisms [humans lack the necessary enzymes (Werlen et al., 1996)]. As shown in Fig. 9 and reported by (Liang et al., 2009), the halogenated curcumin analogues can be very toxic to cancer cells, although several halogenated derivates were inactive (Ishida et al., 2002). Proper weak electron-donating groups include methoxy and alkyl peroxy ($R-O-O-CH_3$) groups, which were shown to be more cytotoxic than curcumin in various cancer cell lines (Ishida et al., 2002; Ohori et al., 2006; Mosley et al., 2007; Cen et al., 2009; Fuchs et al., 2009; Liang et al., 2009).

On a final note, whether the curcumin analogues are toxic to cells should at all times be investigated experimentally in addition to in silico and preferably in a multitude of cancer cell lines as well as in noncancer cells (control), as was done by, for instance, Fuchs et al. (2009), Liang et al. (2009), and Katsori et al. (2011). The extent of cytotoxicity is not always predictable (Robinson et al., 2005) and may vary in both healthy cells and cancer cell lines (Fuchs et al., 2009; Liang et al., 2009). Moreover, given that curcumin can inhibit cancer cells at the level of metastasis, proliferation, and viability, assays with curcumin analogues should be performed at all these levels to determine the full pharmacodynamic spectrum of the analogue. Some curcumin analogues can strongly inhibit cancer cell proliferation without imposing any lethality at that concentration [such as compound 1b in (Katsori et al., 2011)], rendering them unsuitable for cancer treatment. Similarly, structural isomers may exert entirely different pharmacodynamic effects, as for example evidenced by the curcumin analogue 1d (highly toxic at <10 μM) versus its isomer 1e (nontoxic at concentrations up to 100 μM) in Katsori et al. (2011).

III. Anti-Cancer Properties of Curcumin

A. Curcumin Pharmacokinetics and Pharmacodynamics

In section II.A the chemical basis was provided for the binding behavior of curcumin that, because of its distinct chemical properties, is capable of interacting with a plethora of molecules and thereby detrimentally affect numerous vital pathways in cancer cells (Johnson and Mukhtar, 2007; Anand et al., 2008; Gupta et al., 2011), causing their demise. In fact, curcumin is able to induce lethal effects in virtually every cancer type, at least in vitro. In support of this, a literature study was performed to determine the in vitro tumor killing capacity of curcumin per cancer type. The results, extrapolated from 137 published papers, have been summarized for the most common cancers in Fig. 10 and specified per cancer cell line for the reproductive system (Supplemental Fig. 1), digestive system (Supplemental Fig. 2), lymphatic and immune system (Supplemental Fig. 3), nervous system (Supplemental Fig. 4), as well as the pulmonary, urinary, and skeletal system and skin (Supplemental Fig. 5), whereby the in vitro 50% lethal or inhibitory curcumin concentrations (LC_{50} or IC_{50}, respectively) were plotted as a function of incubation time and cancer cell line. The complete data set containing additional information is provided in Supplemental Table 1. The actual LC_{50} values are expected to be lower than reported, inasmuch as these values are hardly ever corrected for the considerable extent of curcumin degradation in aqueous media ["Solvolysis: (alkaline) hydrolysis," section II.C.1.b.i]. Readers should also note that the data were not stratified for the method with which the LC_{50} value was determined (typically an MTT or water-soluble tetrazolium-1 assay) or the experimental procedures and conditions (e.g., medium, solvent for curcumin, degree of cell monolayer confluence, culture plate configuration, culture conditions, etc.). Although this makes valid interstudy comparison of the data difficult, some general observations and implications are worth highlighting in regard to curcumin pharmacodynamics and pharmacokinetics.

First, the LC_{50} values range mostly from 1 to 100 μM, with a mean ± SD LC_{50} value of 21 ± 17 μM when all data are clustered (n = 309). The relatively high LC_{50} values are pharmacologically problematic in light of curcumin's instability (section II.C), although the LC_{50} values may be skewed inasmuch as neither curcumin degradation nor the pharmacodynamics of the degradation products were accounted for (see also section III.C.5). Moreover, curcumin exhibits poor systemic uptake and profuse conjugation and modification (section III.C.2), altogether accounting for the submicromolar-to-low nanomolar curcumin levels found in blood after oral intake (Fig. 11 and Supplemental Table 2). Curcumin is ineffectively transported across the intestinal mucosa into the circulation (Holder et al., 1978; Wahlstrom and Blennow, 1978; Ravindranath and Chandrasekhara, 1980; Cheng et al., 2001; Sharma et al., 2001, 2004; Garcea et al., 2004, 2005; Yang et al., 2007a; Vareed et al., 2008; Villegas et al., 2008; Suresh and Srinivasan, 2010; Wahlang et al., 2011; Berginc et al., 2012). Furthermore, the curcumin molecules that bypass transport hurdles and escape biotransformation in the intestinal mucosa (Ireson et al., 2002; Hoehle et al., 2007; Wahlang et al., 2011; Berginc et al., 2012; Dempe et al., 2012), i.e., by definition the first pass effect for orally administered curcumin, and manage to reach the circulation instantaneously become susceptible to chemical modification in blood (Pan et al., 1999); uptake and biotransformation by the liver (second pass effect), kidneys, and other organs (Holder et al., 1978; Wahlstrom and Blennow, 1978; Pan et al., 1999; Asai and Miyazawa, 2000; Garcea et al., 2004; Hoehle et al., 2006; Tamvakopoulos et al., 2007b; Vareed et al., 2008; Marczylo et al., 2009), and excretion via the biliary or urinary system, albeit the latter occurs to a limited extent (Holder et al., 1978; Ravindranath and Chandrasekhara, 1980; Sharma et al., 2004; Marczylo et al., 2009; Suresh and Srinivasan, 2010). These phenomena are further elaborated in the context of biological curcumin metabolites in sections III.C.1 through III.C.3.

Accordingly, in human studies it has been shown that, at a single oral dose of 500 to 8000 mg, no curcumin was detected in serum 1, 2, and 4 hours after administration (Lao et al., 2006). In only one of the three subjects that received a 10,000-mg oral dose, serum curcumin levels reached a concentration of 66, 91, and 121 nM (baseline corrected) after 1, 2, and 4 hours, respectively. Similarly, one of the three subjects in the 12,000 mg dosage group exhibited serum curcumin levels of 81, 156, and 139 nM after 1, 2, and 4 hours, respectively. In two of the three subjects in both high-dosage groups, no curcumin was detected in serum (Lao et al., 2006). Other pharmacokinetic studies (Cheng et al., 2001; Sharma et al., 2004) in humans revealed that plasma curcumin concentrations peak at 1–2 hours after oral administration and reach a maximum of 1.77 ± 1.87 μM after an oral dose of 8000 mg (Cheng et al., 2001). Thus, plasma levels of curcumin after oral administration are so low relative to the reported LC_{50} values and are sustained for such a short period of time that they are not expected to be effective against non-gastrointestinal cancers, notwithstanding the tumoricidal efficacy of some of the curcumin degradation products and biological metabolites (sections III.B.2 and III.C.4).

For cancers outside the gastrointestinal system, the clinical data, albeit somewhat restricted because of small patient populations, generally reflect the low systemic bioavailability of orally administered

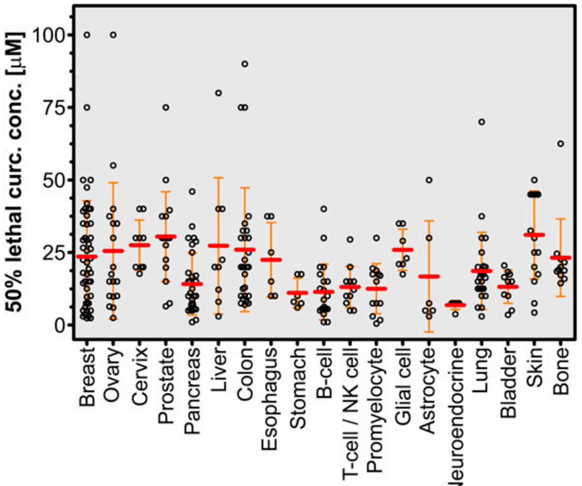


Fig. 10. The in vitro LC_{50} concentrations of curcumin (curc.) categorized per cancer type. The open circles represent mean LC_{50} values or central LC_{50} values when a range was provided (n = 309). The red horizontal lines indicate the mean LC_{50} concentration of the data set, the vertical orange lines the corresponding standard deviation. Data were included regardless of curcumin incubation time and type of cell line. All cell lines were crossreferenced against a list of known contaminated cell lines (http://www.hpacultures.org.uk/media/EDA/AB/Cross_Contaminations_v6_7.pdf). Contaminated cell lines were excluded from the plotted data sets. A complete overview is provided in Supplemental Table 1, and more detailed plots, in which the cancer cell line and curcumin incubation time are specified, are depicted separately for cancers of the reproductive system (Supplemental Fig. 1), digestive system (Supplemental Fig. 2), lymphatic and immune system (Supplemental Fig. 3), nervous system (Supplemental Fig. 4), as well as the pulmonary, urinary, and skeletal system and skin (Supplemental Fig. 5). References: Breast (Hong et al., 1999; Ramachandran and You, 1999; Shao et al., 2002; Martin-Cordero et al., 2003; Ohori et al., 2006; Poma et al., 2007; Sandur et al., 2007; Kim et al., 2008; Santel et al., 2008; Somers-Edgar et al., 2008; Hutzen et al., 2009; Kang et al., 2009; Labbozzetta et al., 2009; Lin et al., 2009; Rowe et al., 2009; Kizhakkayil et al., 2010; Quiroga et al., 2010; Yadav et al., 2010a; Altenburg et al., 2011; Fang et al., 2011; Huang et al., 2011; Somers-Edgar et al., 2011; Soung and Chung, 2011; Kunwar et al., 2012; Zong et al., 2012; Sun et al., 2012b; Yan et al., 2013), Ovary (Ohori et al., 2006; Shi et al., 2006; Lin et al., 2007; Wahl et al., 2007; Pan et al., 2008; Ganta and Amiji, 2009; Montopoli et al., 2009; Saydmohammed et al., 2010; Tan et al., 2010; Watson et al., 2010a), Cervix (Divya and Pillai, 2006; Maher et al., 2011; Lin et al., 2012), Prostate (Mukhopadhyay et al., 2001; Ohori et al., 2006; Sandur et al., 2007; Shankar et al., 2007; Shankar and Srivastava, 2007; Srivastava et al., 2007; Santel et al., 2008; Lin et al., 2009; Hilchie et al., 2010; Ide et al., 2011; Adam et al., 2012), Pancreas (Wang et al., 2006b, 2008b; Kunnumakkara et al., 2007; Lev-Ari et al., 2007; Sandur et al., 2007; Swamy et al., 2008; Hutzen et al., 2009; Li et al., 2009b; Padhye et al., 2009; Sahu et al., 2009; Ali et al., 2010; Jutooru et al., 2010), Liver (Cui et al., 2006; Ohori et al., 2006; Cao et al., 2007; Cheng et al., 2010; Chang et al., 2011; Qian et al., 2011; Strofer et al., 2011; Lin et al., 2012), Colon (Hanif et al., 1997; Collett and Campbell, 2004; Lev-Ari et al., 2005; Moon et al., 2006; Moussavi et al., 2006; Ohori et al., 2006; Cheng et al., 2007b; Gopinath and Ghosh, 2008; Patel et al., 2008; Watson et al., 2008, 2010b; Lee et al., 2009; Sandur et al., 2009; Nautiyal et al., 2011b; Lin et al., 2012), Esophagus (O'Sullivan-Coyne et al., 2009; Subramaniam et al., 2012), Stomach (Cui et al., 2006; Ohori et al., 2006; Cai et al., 2009), B-cell (Bharti et al., 2003, 2004; Liu et al., 2005; Shishodia et al., 2005; Tsvetkov et al., 2005; Uddin et al., 2005; Skommer et al., 2006; Gururajan et al., 2007; Park et al., 2008; Li et al., 2009c), T-cell and NK cell (Kim et al., 2005; Hussain et al., 2006; Sandur et al., 2007; Zhang et al., 2010a), Promyelocyte (Gautam et al., 1998; Pan et al., 2001; Atsumi et al., 2005; Cui et al., 2006; Magalska et al., 2006; Tan et al., 2006; Dikmen et al., 2010; Pesakhov et al., 2010; Saxena et al., 2010; Ng et al., 2011; Wu et al., 2011), Glial cell (Belkaid et al., 2006; Karmakar et al., 2006, 2007; Aoki et al., 2007; Huang et al., 2010; Zanotto-Filho et al., 2011, 2012), Astrocyte (Nagai et al., 2005; Santel et al., 2008), Neuroendocrine (Pisano et al., 2010), Lung (Radhakrishna and Pillai, 2004; Lev-Ari et al., 2006; Ohori et al., 2006; Sandur et al., 2007; Lin et al., 2008, 2012; Chen et al., 2009b, 2010, 2012a; Saha et al., 2010; Zhang et al., 2010b,c; Lee et al., 2011a; Wang et al., 2011b; Ye et al., 2012), Bladder (Tong et al., 2006; Hauser et al., 2007; Kamat et al., 2007; Tian et al., 2008), Skin (Bush et al., 2001; Martin-Cordero et al., 2003; Zheng et al., 2004; Ohori et al., 2006; Bill et al., 2009; Chatterjee and Pandey, 2011; Yu et al., 2012), Bone (Walters et al., 2008; Jin et al., 2009; Leow et al., 2010).

curcumin (Fig. 11). In a phase I dose-escalation study (oral administration of curcumin at 1000–12,000 mg/day for 3 months), histologic improvements in neoplastic malignancies were found in 1 of 4 patients (25%) with uterine cervical intraepithelial neoplasia, in 1 in 2 (50%) patients with recently resected urinary bladder cancer, and in 2 of 6 patients (33%) with squamous cell carcinoma in situ (Cheng et al., 2001). Poorer outcomes were obtained in a phase II trial with pancreatic adenocarcinoma patients, in which only 2 of 21 pancreatic adenocarcinoma patients (10%) that were given a daily oral dose of curcumin (8000 mg) showed a response (Dhillon et al., 2008). In the first patient, curcumin treatment caused a gradual decrease in cancer antigen 125 (also known as carbohydrate antigen 125 or mucin 16—a cancer biomarker) levels over the course of 1 year, which was accompanied by stabilization of lesion size and a reduction in the PET-CT standardized uptake value. The second patient exhibited a 73% reduction in tumor size that lasted 1 month, after which the lesions that had regressed remained small, whereas new lesions had increased in size.

The marginal systemic bioavailability gives rise to a second important consideration, namely that the cancer types targeted by curcumin chemotherapy should be scaled according to their combined degree of curcumin susceptibility and accessibility so as to roughly gauge the potential therapeutic efficacy and, with it, utility. For example, cancers of the lymphatic and immune system (Supplemental Fig. 3) appear to be more susceptible to curcumin than for instance cancers of the reproductive system (Supplemental Fig. 1), as evidenced by the generally lower LC_{50} values (notwithstanding the fact that cell lines may phenotypically differ from native cancer cells and thus respond differently to curcumin). On top of the favorable degree of susceptibility, cancers of the lymphatic and immune system, and particularly the lymphoid leukemias and other hematologic neoplasms, are more accessible for curcumin, insofar as these cancer cells are either blood-borne or arise in highly vascularized tissue (bone marrow). Consequently, malignant B-cells, T-cells, and natural killer cells are more likely to come in direct contact with gut-derived, circulating curcumin before excessive uptake/biotransformation/chemical modification has occurred. This principle of high-susceptibility–high-accessibility may also apply to lymphomas and certain bone cancers, which also consistently exhibit relatively low LC_{50} values (Supplemental Figs. 3 and 5, respectively), given that lymph nodes (Osogoe and Courtice, 1968; Webster et al., 2006; Martinez-Corral

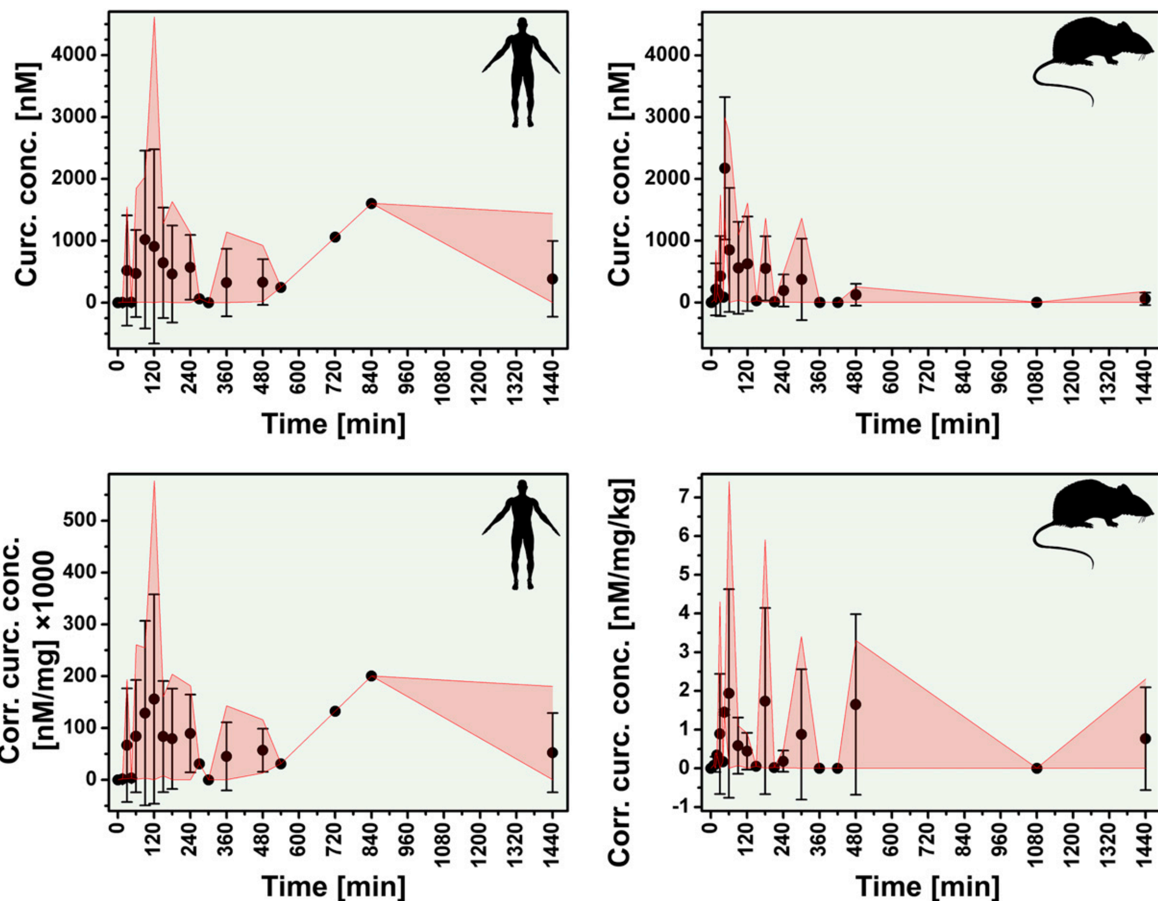


Fig. 11. Curcumin (curc.) concentrations in blood after oral administration in patients (left column) and per gavage administration in rats (right column), plotted as a function of time after administration. Data points and error bars correspond to the mean ± standard deviation curcumin concentrations, calculated from the specified or extrapolated data in the human studies (n = 10) and rat studies (n = 7) as listed in Supplemental Table 2 (contains full data set). In some instances, data points lack error bars because only one value was available for that time point. The red line connects the maximum (top) and minimum (bottom) curcumin concentrations, whereby the semi-opaque red area reflects the range of the data sets per time point. The measured curcumin blood concentrations are provided in the top row, regardless of the administered dose. In the bottom row the blood concentrations were corrected for the administered curcumin dose. For the patient data (bottom left panel), the blood concentrations were divided by the curcumin dose administered per patient. For the rat data (bottom right panel), the blood concentrations were divided by the curcumin dose administered per kg body weight. The following studies were used to compile the data: human (Shoba et al., 1998; Cheng et al., 2001; Garcea et al., 2004, 2005; Sharma et al., 2004; Anand et al., 2007; Antony et al., 2008; Dhillon et al., 2008; Cuomo et al., 2011; Kanai et al., 2011), rat (Ravindranath and Chandrasekhara, 1980; Shoba et al., 1998; Asai and Miyazawa, 2000; Maiti et al., 2007; Marczylo et al., 2007; Yang et al., 2007a).

et al., 2012) and some bone tumors (Verstraete et al., 1996; Wenger and Wold, 2000) are well perfused. By extension, the same principle holds for cancer cells undergoing metastasis, which occurs mainly via the circulation (van Zijl et al., 2011).

Even more suitable pharmacodynamic targets are malignancies of the gastrointestinal tract (Supplemental Fig. 2), where the major pharmacokinetic obstacles principally do not apply. The most prominent example is gastric cancer, a major contributor to cancer-related deaths worldwide (Jemal et al., 2011). Neoplasms typically form in the inner lining of the stomach (stage 0) and then progress to either the second and third layers (stage 1A) or the second layer and proximal lymph nodes (stage 1B). Consequently, orally ingested curcumin comes in direct contact with the venue of potential oncogenesis or malignantly transformed tissue at very high concentrations (because 100% of the orally administered curcumin is deposited directly into the stomach in intact form, rendering LC_{50} considerations obsolete) and under protective (dark, acidic) conditions. In case of gastric cancer, curcumin not only prevents malignant transformation of mucosal epithelial cells but also removes a cause of carcinogenesis. A persistent infection by *Helicobacter pylori*, the bacterial species that causes stomach ulcers, constitutes a significant risk factor for developing gastric cancer (Suganuma et al., 2012; Zabaleta, 2012) either due to chronic inflammation and/or the release of virulence factors (Hatakeyama and Higashi, 2005). Curcumin hence acts through its anti-inflammatory (Foryst-Ludwig et al., 2004; Sintara et al., 2010; Kundu et al., 2011) and bactericidal properties (Mahady et al., 2002; De et al., 2009) while favorably modulating numerous infection-induced, carcinogenesis-related pathways in mucosal epithelial cells (Foryst-Ludwig et al., 2004; Sintara et al., 2010; Kundu et al., 2011) and deterring procarcinogenic signaling in *H. pylori* (Zaidi

et al., 2009). It should be noted that results obtained in vitro and in animal models are not necessarily translatable to the clinical setting with respect to *H. pylori* infection and gastric cancer. For instance, only 1 of 17 chronic gastritis patients (6%) with confirmed *H. pylori* infection who received a turmeric tablet (700 mg of curcumin and curcumin derivatives) three times a day for 4 weeks experienced complete eradication of the infection (Koosirirat et al., 2010). Similarly, a 7-day combinatorial treatment with orally administered curcumin (30 mg), lactoferrin (100 mg), *N*-acetylcysteine (600 mg), and pantoprazole (20 mg) was able to cure the *H. pylori* infection in only 3 of 25 patients (12%), although a significant decrease in the severity of dyspeptic symptoms was observed (Di Mario et al., 2007). A phase I study in patients with intestinal metaplasia of the gastric mucosa, a condition mainly instigated by a chronic *H. pylori* infection, found that only 1 of 6 patients (17%) taking curcumin at an oral dose of 1000 mg per day for 3 months exhibited histologic improvement in the lesion (Cheng et al., 2001).

Another example where curcumin's pharmacokinetic hurdles play a minor role is colorectal cancer. Although colon cancer cell lines exhibit some of the highest LC_{50} values of all studied cancer cell lines (Supplemental Fig. 2), there is limited concern about the therapeutic efficacy of curcumin. A study in rats found that approximately 40% of per gavage administered curcumin remains in the gastrointestinal tract in chemically intact form over a period of 5 days and that the highest concentration of curcumin shifts along the gastrointestinal tract in time, i.e., stomach (15 minutes, $53 \pm 5\%$ of administered curcumin) → small intestine (30 minutes, $59 \pm 11\%$) → cecum (3–7 hours, 43–51%) → large intestine (24 hours, $5 \pm 3\%$) (Ravindranath and Chandrasekhara, 1980). These data suggest that both the stomach and the large intestine absorb a large fraction of the curcumin, enabling anticancer protection in cells of the mucosal lining of both gastrointestinal organs. In line with these findings, mice fed a curcumin-enriched diet exhibited curcumin levels that ranged from 39 ± 9 to 240 ± 69 nmol/g mucosal tissue in the small intestine (0.1 and 0.5% dietary curcumin, respectively) and from 15 ± 9 to 715 ± 448 nmol/g mucosal tissue in the colon (0.1 and 0.5% dietary curcumin, respectively) (Perkins et al., 2002). The therapeutic efficacy of the higher concentrations of orally administered curcumin was corroborated in this animal model of intestinal cancer (Perkins et al., 2002) but also in clinical trials (Sharma et al., 2001; Cruz-Correa et al., 2006; Carroll et al., 2011).

Clinical studies on the therapeutic efficacy of curcumin in relation to colorectal malignancies and premalignancies are most abundant and show the greatest promise. A recent phase IIa trial that included patients with colorectal neoplasias revealed that a 1-month-long, 4000 mg daily oral dose of curcumin reduced the number of aberrant crypt foci in 17 of 20 patients (85%) (Carroll et al., 2011). Another clinical study on familial adenomatous polyposis demonstrated that oral administration of 480 mg of curcumin three times a day for an average of 6 months led to a 60.4 and 50.9% decrease in the number and size of the polyps, respectively, in all patients ($n = 5$) when coadministered with 20 mg of quercetin (Cruz-Correa et al., 2006). Even in patients with advanced colorectal cancer refractory to standard chemotherapy, oral curcumin administration (ranging from 36 to 144 mg per day for 3–4 months) resulted in stabilization of the disease in 5 of 15 patients (33%) and yielded a 44% reduction in the plasma tumor marker carcinoembryonic antigen in one patient (Sharma et al., 2001). These effects have been attributed to curcumin-induced acceleration of apoptosis in the patients' colorectal cancer cells, characterized by elevated DNA fragmentation, increased p53 (involved in cancer cell proliferation) and Bax (pro-apoptotic) expression, and reduced Bcl-2 (anti-apoptotic) levels (He et al., 2011).

Regardless of curcumin's general anticancer effects, several peculiar pharmacodynamic and pharmacokinetic features of curcumin have surfaced in the clinical trial reports. First, a number of trials that entailed dose escalation monitoring reported positive biological effects for some of the lower curcumin concentrations but no effect for the highest concentration in the same patient cohort (Cheng et al., 2001; Sharma et al., 2001; Dhillon et al., 2008). For example, in the previously mentioned phase II trial with advanced pancreatic cancer patients, the peak plasma level of curcumin was 7 nM at 6 hours postadministration in the first patient that had remained stable for 1 year but almost sixfold higher (40 nM) in the second patient that had exhibited the tumor regression (Dhillon et al., 2008). Second, curcumin concentrations in tissue (Carroll et al., 2011) and fecal (Sharma et al., 2001) samples were occasionally higher for the lower oral curcumin dosages than for the highest oral dose given in the same patient cohort. Although plasma levels were found to be proportional to the administered dose in one clinical study (Cheng et al., 2001), a striking incongruence between oral dose and plasma concentrations was observed in a rat study (Ravindranath and Chandrasekhara, 1982), where the 10-mg and 400-mg dosages of radioactively labeled curcumin yielded similar blood concentrations at various time points after administration, both of which were higher than the blood concentrations after an 80-mg dose. Third, blood curcumin concentrations exhibit a notable variance in patients subjected to the same dosing regimen (Supplemental Table 2), indicating lack of dose linearity. For instance, vastly distinct mean blood concentrations were found in patients 2 hours after oral administration of curcumin at 2000 mg, namely 0 nM (Shoba et al., 1998), 7 nM (Anand et al., 2007), and 402 nM (Antony et al., 2008), and in

patients who had been given 8000 mg, namely 60 nM (Dhillon et al., 2008) versus 4615 nM (Cheng et al., 2001). Despite the possibility that this may be attributable to the dissimilar curcumin extraction and quantification processes, the differences also arose when the same analytical procedures were implemented. Kanai et al. (2011) observed an almost 10-fold difference in blood curcumin levels (149 versus 1118 nM) in 2 patients 4 hours after an oral dose of 8000 mg. The exact reasons for the lacking dose linearity notwithstanding, it is incontrovertible that the pharmacodynamics and pharmacokinetics of orally administered curcumin are very complex and apparently difficult to control, which is underscored by the large standard deviations associated with the corrected blood curcumin concentrations in both the human and rat studies (Fig. 11, bottom panels, respectively). Moreover, the overall clinical evidence on the in vivo anticancer properties of curcumin is currently too limited to draw any definitive conclusions as to its therapeutic efficacy.

B. Pharmacokinetics and Pharmacodynamics of Curcumin Catabolites Generated In Vitro

Given the ease, propensity, and extent of curcumin metabolism in vitro and in vivo, the question arises whether curcumin alone exerts the pharmacological effects or whether it does so in conjunction with its metabolites, as has been propagated by some (Shen and Ji, 2009, 2012). Ample evidence points to the latter, namely that some of the curcumin metabolites contribute to the documented oncostatic effects of the parent compound, and may even do so to a sizeable degree. This is not very surprising given that some of the curcumin catabolites generated in vitro retain the methoxyphenyl residue (Figs. 6 and 7) that facilitates intermolecular interactions with target molecules through the H-bond donating and accepting properties (sections II.A.2 and II.A.3), Michael acceptor sites (section II.A.7), and strong antioxidant capacity (section II.C.1). The same applies to curcumin metabolites produced in cells and living organisms, which is addressed in section III.C. Naturally, the full extent of the oncostatic effects at the account of the curcumin metabolites cannot be accurately gauged insofar as not all metabolites have been characterized (section II.C.1.b) or evaluated in pharmacodynamic context. But, despite the previously stated "onerousness" of curcumin as a pharmaceutical, the consequences of its instability and biotransformation seem to be considerably less detrimental for oncostatic pharmacodynamics than for many other pharmacological compounds, which lose biological activity after degradation/modification.

With respect to the characterized curcumin catabolites in nonbiological samples and in in vitro test systems, vanillin and ferulic acid, which are naturally occurring phenolics themselves, have most extensively been studied for their pharmacokinetic behavior and antimutagenic, anticarcinogenic, tumoricidal, and antimetastatic properties.

1. Pharmacokinetics of Curcumin Catabolites (Generated In Vitro). In vitro curcumin catabolism generally involves cleavage of the molecule in the methine bridge to a stable, substituted phenyl containing a terminal ketone (vanillin and (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal), methyl ketone (feruloyl methane), or acid (vanillic and ferulic acid) (Fig. 6). These catabolites, and particularly the acids that are deprotonated at physiological pH (Brown et al., 1955), have different physicochemical properties than curcumin (e.g., lower log P, covalent/H-bonding sites) and are therefore expected to exhibit slightly different pharmacokinetics than the parent compound. The extent to which the catabolites form in oral formulations, especially those for human use, is marginal relative to the degree to which the curcumin metabolites form in vivo (section III.C), making them biologically less relevant in regard to pharmacokinetics. Accordingly, the pharmacokinetics of the in vitro catabolites will only be briefly addressed, mainly in regard to vanillin and ferulic acid. However, it should be noted that the formation of the (photo)chemical degradation products is highly relevant in the context of in vitro research, where curcumin is added to the culture medium or physiological buffer (often at 37°C and neutral pH) and thus becomes susceptible to degradation with corollary effects on pharmacodynamics (sections III.B.2).

a. Pharmacokinetics of vanillin. Studies in rats have shown that orally administered vanillin (100 mg/kg) is absorbed in the gastrointestinal tract and reaches a ~3 μM peak plasma concentration 4 hours after administration (Beaudry et al., 2010), suggesting poor uptake, rapid clearance, and/or excessive metabolism as has been described for curcumin (section III.A and Fig. 11). Intravenously administered vanillin is almost completely cleared or metabolized within 2 hours (Beaudry et al., 2010). Correspondingly, analysis of urine retrieved from rats 24 hours after an oral dose of vanillin (100 mg/kg) revealed that vanillin is extensively cleared via the renal system in conjugated form, mainly as vanillin glucuronide and sulfate (Kirwin and Galvin, 1993). After 48 hours, 94% of the administered vanillin had accumulated in urine as vanillin (7%), vanillic acid (47%), vanillyl alcohol (19%), vanilloylglycine (19%), catechol (8%), 4-methylcatechol (2%), guaiacol (0.5%), and 4-methylguaiacol (0.6%) (Kirwin and Galvin, 1993), indicating that vanillin does not only undergo phase II xenobiotic metabolism but also extensive chemical modification, although it is not clear whether this occurs in the kidneys or before renal uptake. It is certain that the liver is able to metabolize vanillin, as experiments with liver slices demonstrated that vanillin is rapidly oxidized to vanillic acid by aldehyde oxidase and subsequently O-demethylated to

protocatechuic acid and that small amounts of vanillyl alcohol are produced as well by a different reaction mechanism (Panoutsopoulos and Beedham, 2005).

The vanillin metabolites may exhibit different pharmacokinetics and pharmacodynamics than the parent compound, which may have consequences for cancer cells in terms of cytostatic effects. For instance, vanillic acid and protocatechuic acid are inhibitors of phenolsulfotransferases (Yeh and Yen, 2003), which are responsible for sulfating xenobiotics during phase II metabolism (Brix et al., 1998; Coughtrie et al., 1998), including curcumin (section III.C.2.b), vanillin, and ferulic acid. Their formation may therefore benefit the disposition of curcumin and some of its metabolites and improve the cytostatic potency. With respect to pharmacodynamics, vanillic acid has been shown to exhibit a range of biological effects in melanocytes on transcription factors, receptors, and enzymes that were absent for vanillin (Chou et al., 2010).

b. Pharmacokinetics of ferulic acid. Ferulic acid is very effectively absorbed in both the stomach (Zhao et al., 2003a, 2004) and the intestines (Spencer et al., 1999), as a result of which ferulic acid exhibits high bioavailability after ingestion (Adam et al., 2002; Rondini et al., 2002; Zhao et al., 2003b). Studies in rats showed that, when stomachs containing intragastrically deposited ferulic acid were incubated ex vivo versus in situ for 25 minutes, respectively 80 ± 6% and 26 ± 11% of the ferulic acid was retrieved from the gastric content and 7 ± 1% and 4 ± 2% from the gastric mucosa (Zhao et al., 2004), indicating that more than one-half of the ferulic acid enters the circulation by means of gastric absorption. Similar experiments in rats revealed that 56 ± 2% of the ferulic acid perfused through the small intestine in situ is absorbed (Adam et al., 2002). The finding that no ferulic acid was detected in the ileum and cecum of rats that had been fed dietary ferulic acid for 9 days suggests that virtually all the ferulic acid is absorbed in the stomach and the duodenal and jejunal segments (Zhao et al., 2003a). Intestinal perfusion experiments with ferulic acid in bicarbonate buffer confirmed uptake of ferulic acid by the jejunum and minimal uptake by the ileum (~10% of the jejunal uptake), with the maximum absorption rate occurring between 60 and 70 minutes of perfusion (Spencer et al., 1999).

Biotransformation of ferulic acid does not appear to occur in the stomach, including the gastric mucosa (Zhao et al., 2004), or in the intestinal lumen (Adam et al., 2002). However, extensive sulfation, glucuronidation, and sulfoglucuronidation takes place after absorption of ferulic acid from the stomach and intestines, as these conjugates have been detected in plasma, urine, and bile after intragastric deposition (Zhao et al., 2004), transenteral perfusion (Adam et al., 2002), and per gavage or dietary intake (Rondini et al., 2002; Zhao et al., 2003b). Experiments with isolated rat jejunums perfused with ferulic acid demonstrated that approximately 80% of the perfused ferulic acid passes through the intestine in unconjugated form, whereas approximately 20% is glucuronidated (Spencer et al., 1999), indicating that first pass metabolism occurs in enterocytes. The plasma concentrations of ferulic acid and its metabolites peak at 15 minutes after a single, per gavage dose and the compounds are cleared from the blood within 2 (Zhao et al., 2003b) to 4 hours (Rondini et al., 2002). The metabolites are present in blood within 5 minutes postadministration (ferulic acid sulfoglucuronide > unconjugated form > > sulfate = glucuronide) (Zhao et al., 2003b), altogether reflecting rapid metabolism and systemic clearance.

In the in situ gastric deposition experiments (Zhao et al., 2004), ferulic acid was the predominant species in portal vein plasma (approximately 50% of total ferulic acid) but was found at very low concentrations in arterial plasma, bile, and urine. This indicates that ingested ferulic acid is highly susceptible to the second pass effect by the liver. Correspondingly, the largest fraction of total ferulic acid, including its metabolites, was found in bile 25 minutes after intragastric deposition (Zhao et al., 2004). Conjugation in the liver occurs in the order of sulfoglucuronidation > glucuronidation > sulfation = no conjugation (Zhao et al., 2004), although analysis of bile extracted from rats that had been subjected to in situ intestinal perfusion with ferulic acid showed that biliary ferulic acid, which comprised 5–7% of the perfused dose, was only present as glucuronidated and sulfated conjugates (Adam et al., 2002). The degree of sulfation may be influenced by the fact that, as vanillic acid and protocatechuic acid (section III.B.1.a), ferulic acid is an inhibitor of phenolsulfotransferases, albeit less potent (Yeh and Yen, 2003).

Ferulic acid and its conjugates were also retrieved in urine 25 minutes after intragastric deposition in the order of sulfoglucuronidated > glucoronidated ≈ sulfated ≈ unconjugated, although the total ferulic acid content was approximately one-half of that in the liver (Zhao et al., 2004). In rats that were given dietary ferulic acid, the maximum total ferulic acid content in the bladder was reached within 1 hour and the cumulative excretion of free ferulic acid and its metabolites (glucuronidate-, sulfate-, and sulfoglucuronodate adducts) plateaued at 1.5 hours after ingestion (Rondini et al., 2002). Because approximately 70% of per gavage administered total ferulic acid (single dose) was retrieved in urine 5 hours after dosing (Zhao et al., 2003b), it is probable that a fraction of the hepatic metabolites is basolaterally excreted or re-enters the systemic circulation after biliary excretion to ultimately undergo elimination via the renal system.

The pharmacokinetics of ferulic acid in humans differ in some respects from that in rats. First, the cumulative urinary excretion of ferulic acid and its conjugates (at least ferulic acid glucuronide) is

considerably slower and reaches a plateau after approximately 9 hours (Bourne and Rice-Evans, 1998). Second, the total fraction of free ferulic acid excreted over a 24-hour period comprises only 4–5% (0.9–3.0 mg in 1.2–3.0 liters of urine, i.e., 2–13 μM) of the ingested ferulic acid, whereas the fraction of total ferulic acid, including its metabolites, is less than 10% (Bourne and Rice-Evans, 1998). It should be noted that these data were collected in healthy volunteers who ingested tomatoes as a source of ferulic acid. In another study, where healthy volunteers drank coffee as a source of ferulic acid, the peak concentration of ferulic acid in urine was reached within 2 hours (Rechner et al., 2001). The bioavailability and pharmacokinetics of ferulic acid are impacted by the dietary source of the ferulic acid (Adam et al., 2002), which may explain the temporal differences in urinary peak concentration between tomato-derived and coffee-derived ferulic acid.

2. Pharmacodynamic Properties of Curcumin Catabolites (Generated In Vitro). Based on the pharmacokinetic properties it is evident that vanillin is more likely to exert an adjuvant chemopreventive and oncostatic effect in cancers of the gastrointestinal tract, given its poor uptake and systemic bioavailability. In contrast, ferulic acid is completely taken up from the gut, but undergoes biotransformation and systemic clearance via the urinary system within several hours after oral intake. If, however, ferulic acid is capable of accumulating in the tumor during the time its blood levels are still considerable, it may conspire with curcumin in killing cancer cells.

a. Pharmacodynamic properties of vanillin. Vanillin possesses potent antimutagenic properties, exemplified by its ability to reduce the extent of DNA lesions induced by several chemical mutagens (Ohta et al., 1986), UV light (Takahashi et al., 1990), as well as spontaneous mutations (Shaughnessy et al., 2006) in different *Esherichia coli* strains, mitomycin C-induced DNA lesions in (hybrid) Chinese hamster ovary cells (Sasaki et al., 1987; Gustafson et al., 2000), mouse bone marrow cells (Inouye et al., 1988), and somatic cells of *Drosophila melanogaster* (Santos et al., 1999), as well as X-ray-, UV light-, or H_2O_2-induced mutations in bone marrow-derived cells (Sasaki et al., 1990; Maurya et al., 2007) and hamster fibroblasts (V79 cells) (Imanishi et al., 1990; Tamai et al., 1992) and hybrid ovary cells (Gustafson et al., 2000). In human mismatch repair-deficient (*hMLH1*$^{-}$) HCT116 colon cancer cells, vanillin decreased the number of spontaneous mutations in a concentration-dependent manner (19–73% at concentrations of 0.5—2.5 mM) (King et al., 2007). Vanillin also improved the efficacy of DNA damage repair mechanisms under conditions of oxidative stress (Maurya et al., 2007) and in spontaneously mutating colon cancer (HCT116) cells (King et al., 2007). Ironically, vanillin (2.5 mM) itself appears to inflict a specific type of DNA damage, in consequence to which genes related to DNA damage, oxidative stress and stress responses, cell growth, and apoptosis are activated that in turn trigger DNA repair (by homologous recombination) of mutations not induced by vanillin without causing substantial cell death (King et al., 2007). Such a mechanism was also reported by others (Santos et al., 1999; Shaughnessy et al., 2006).

Microarray analysis of vanillin-treated human hepatocellular carcinoma (HepG2) cells provided insight into the comprehensive biochemical pathway-modulating properties of this catabolite in relation to its oncostatic and antiproliferative properties (Cheng et al., 2007a). At the LC_{50} (25 mM), 213 genes were downregulated and 347 genes were upregulated by vanillin. Transcriptional effects were also observed at sublethal concentrations; at 1 and 5 mM concentration, vanillin caused the downregulation of 26 and 119 genes and the upregulation of 28 and 84 genes, respectively. At a 5 mM concentration, 47 gene ontology classes were affected, mostly related to the cell cycle and apoptosis but also to tumor progression. These effects appeared to be centered around the Fos gene pathway, which codes for the protein c-Fos, a proto-oncogene that complexes with activator protein-1 (AP-1) to induce transformation and progression of cancer (Milde-Langosch, 2005). Proteomic follow up revealed that vanillin significantly suppressed the activity of AP-1 as a result of decreased levels of several nonphosphorylated mitogen-activated protein kinases (MAPKs), of which the phosphorylated extracellular signal-regulated protein kinases 1 and 2 (ERK-1/2) were also decreased, indicating that AP-1 activity in HepG2 cells was suppressed by vanillin through the ERK signaling pathway. Despite the fact that the vanillin concentrations used in this study were exceptionally high, high doses of vanillin were not toxic in healthy rats when administered per gavage or intraperitoneally up to 300 mg/kg (Ho et al., 2011). Vanillin is also capable of inducing apoptosis in human colorectal adenocarcinoma (HT-29) cells at a ~2.4-fold lower LC_{50} (2.6 mM) than in noncancerous fibroblasts (LC_{50} = 6.6 mM in NIH/3T3 cells) because of its concentration-dependent inhibition of cell cycle checkpoints (G_0/G_1 arrest at IC_{50} = 1.3 mM and G_2/M arrest at IC_{50} = 6.2 mM) (Ho et al., 2009).

Lastly, vanillin exhibits antimetastatic properties. Vanillin, but not vanillic acid, suppressed the metastasis of 4T1 mammary adenocarcinoma cells in mouse lungs and inhibited the invasion and migration of cancer cells as well as the enzymatic activity of cancer cell-secreted matrix metalloproteinase 9 (MMP-9) in vitro (Lirdprapamongkol et al., 2005). Inhibition of cell migration and angiogenesis by vanillin was also observed in hepatocyte growth factor-stimulated human lung adenocarcinoma (A549) cells (Lirdprapamongkol et al., 2009). These effects were attributed to vanillin-mediated inhibition of the α, β, δ (class IA), and γ (class IB) phosphoinositide 3-kinase (PI3K)

isoforms and Akt (Lirdprapamongkol et al., 2009), and thereby the PI3K/Akt signaling pathway, which in general is constitutively activated in cancers to facilitate proliferation and to reduce apoptosis (Hennessy et al., 2005; De Luca et al., 2012).

b. Pharmacodynamic properties of ferulic acid. As vanillin, ferulic acid possesses antimutagenic, anticarcinogenic, and antiproliferative properties. Ferulic acid was shown to inhibit mutations induced by several types of mutagens in *Salmonella typhymurium* (Wood et al., 1982; Yamada and Tomita, 1996) and *Eisenia gracilis* (Krizkova et al., 2000) as well as benzo[*a*] pyrene-induced mutagenesis in Chinese hamster fibroblasts (V79 cells) (Wood et al., 1982) at IC_{50} concentrations in the micromolar range. A ~55% reduction in benzo[*a*]pyrene-induced nuclear aberrations was also observed in intestinal cells of mice after dietary ferulic acid treatment (Wargovich et al., 1985).

In regard to carcinogenesis, the induction of tongue squamous cell papillomas and carcinomas by 4-nitroquinoline-1-oxide (Tanaka et al., 1993; Mori et al., 1999) and colonic neoplasms by azoxymethane (Mori et al., 1999, 2000; Kawabata et al., 2000) was inhibited by diet-fed ferulic acid in rats. The ferulic acid-mediated reduction in colon adenomas and adenocarcinomas concurred with increases in GST and quinine reductase in the liver and colon of colon tumor-bearing rats in a ferulic acid dose-dependent manner (Kawabata et al., 2000). Similarly, oral administration of ferulic acid resulted in the normalization of cytochrome P450 and b_5 (Alias et al., 2009) and of SOD, catalase, glutathione peroxidase, GST, and GSH to control levels in the liver and skin of skin tumor-bearing mice (Alias et al., 2009) and in the plasma and mammary tissue of mammary tumor-bearing rats (Baskaran et al., 2010). These data suggest that in vivo, both phase I and II detoxifying enzymes may aid in the chemopreventive action of ferulic acid.

Ferulic acid further exhibited anticarcinogenic effects in benzo[*a*]pyrene-induced pulmonary adenoma development in mice (Lesca, 1983) and ameliorated the 7,12-dimethylbenz[*a*]anthracene-induced development of skin cancers in mice (Kaul and Khanduja, 1998; Alias et al., 2009), mammary adenocarcinomas in rats (Baskaran et al., 2010), and carcinomas in the buccal pouch of Syrian golden hamsters (Balakrishnan et al., 2008). At the administered dosages, ferulic acid was not carcinogenic (Kaul and Khanduja, 1998; Balakrishnan et al., 2008; Alias et al., 2009; Baskaran et al., 2010). Moreover, in human breast cancer (T47D) cells, ferulic acid inhibited proliferation at an IC_{50} of 2.3 nM (Kampa et al., 2004), although inhibition of cell proliferation and apoptosis could not be reproduced in other human breast cancer cell lines at ferulic acid concentrations of up to 75 μM (Serafim et al., 2011). At concentrations of 150–1500 μM, however, ferulic acid was able to reduce cell viability in cultured rat hepatoma (HTC) cells (Maistro et al., 2011).

Several possible mechanisms have been elucidated that may in part explain the cytostatic effects of ferulic acid. Administration of ferulic acid to rats with 7,12-dimethylbenz[*a*]anthracene-induced mammary adenocarcinomas was associated with significantly downregulated expression of mutated p53, which is abundant in breast cancers (Chen et al., 2004; Tennis et al., 2006; Girardini et al., 2011) but also other cancers (Olivier et al., 2010; Freed-Pastor and Prives, 2012), and anti-apoptotic bcl-2 (Baskaran et al., 2010). In HepG2 cells, ferulic acid induced apoptosis in a dose-dependent manner by activating NADPH oxidase, which in turn produced exuberant amounts of ROS that ultimately led to cell death (Lee, 2005). Although ferulic acid failed to trigger apoptosis in estrogen-sensitive (MCF-7) and estrogen-insensitive (MDA-MB-231 and HS578T) human breast cancer cell lines, it was show to induce swelling and mitochondrial permeability transition in isolated rat mitochondria (Serafim et al., 2011), which is a precursor event for apoptosis (Petit et al., 1996; Hirsch et al., 1997).

c. Antioxidant and anti-inflammatory properties of curcumin catabolites (generated in vitro). The anticancer effects of curcumin catabolites are to an extent related to the intricate relationship between oxidative stress, inflammation, and mutagenesis/carcinogenesis as well as metastasis. Oxidative modification of DNA and lipids during oxidative stress is associated with replication defects, transcriptional deregulation, genomic instability, and aberrant modulation of signaling pathways, i.e., factors that directly induce carcinogenesis (Cerutti, 1985, 1989; Cerutti and Trump, 1991; Trush and Kensler, 1991; Cerda and Weitzman, 1997; Marnett, 1999; Bartsch and Nair, 2006; Federico et al., 2007). Carcinogenesis is also promoted indirectly through inflammation. Oxidative stress triggers inflammation (Rolo et al., 2012; van Golen et al., 2012a,b) by, for example, ROS-mediated modulation of redox-sensitive transcription factors and release of damage-associated molecular patterns, which activate nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and other signaling pathways that in turn lead to the release of various pro-inflammatory chemokines and cytokines (Karin and Greten, 2005; Reuter et al., 2010). When pervasive for longer periods of time, these inflammatory mediators can trigger carcinogenesis (Balkwill and Mantovani, 2001; Karin and Greten, 2005; Bartsch and Nair, 2006; Grivennikov and Karin, 2010; Grivennikov et al., 2010), which may be further exacerbated by the augmented levels of ROS and reactive nitrogen species produced during inflammation by leukocytes (Rolo et al., 2012; van Golen et al., 2012a,b). With respect to the link between oxidative stress and metastasis, elevated ROS production becomes instrumental once cells have adopted a cancer phenotype, because ROS intermediates are required

for the cytoskeletal rearrangements that underlie cell motility (Pani et al., 2010).

Several of the characterized curcumin catabolites are antioxidants, including vanillin (Sawa et al., 1999; Maurya et al., 2007; Shyamala et al., 2007; Chou et al., 2010; Makni et al., 2011; Tai et al., 2011; Galano et al., 2012), vanillic acid (Sawa et al., 1999; Shyamala et al., 2007; Chou et al., 2010; Kumar et al., 2011; Prince et al., 2011; Stanely Mainzen Prince et al., 2011; Galano et al., 2012), ferulic acid (Scott et al., 1993; Kaul and Khanduja, 1998; Kanski et al., 2002; Kikuzaki et al., 2002; Hirata et al., 2005; Srinivasan et al., 2007; Jung et al., 2009), and 4-vinylguaiacol (Tressl et al., 1976). These catabolites may therefore elicit anticancer effects in various phases of cancer biology. Ferulic acid has been shown to specifically deter lipid peroxidation in lipid-rich egg yolk homogenates (Islam et al., 2009), rat kidneys (Jung et al., 2009), plasma (Balakrishnan et al., 2008), and isolated liver microsomal membranes, especially in synergy with endogenous antioxidants such as α-tocopherol and β-carotene (Trombino et al., 2004). Peroxidized lipids and their reactive aldehyde derivatives such as 4-hydroxy-2-nonenal and malondialdehyde are known to be mutagenic and carcinogenic (Tudek et al., 2010) due to their DNA adduct-forming propensity (Voulgaridou et al., 2011). Ferulic acid may hence prevent mutagenesis/carcinogenesis by neutralizing the detrimental effects of peroxidized lipids and reactive aldehydes in addition to its general ROS scavenging activities. Accordingly, ferulic acid prevented DNA breakage induced by H_2O_2 in HT-29 cells (Ferguson et al., 2005). Feruloyl methane (Fig. 6B), (2*Z*,5*E*)-2-hydroxy-6-(4-hydroxy-3-methoxyphenyl)-4-oxohexa-2,5-dienal (Fig. 6B), the cyclized curcumin derivatives (Figs. 6, A and B, and 7B), and the dimerized catabolites (Fig. 7C) likely possess antioxidant properties as well given their phenylic hydroxyl group(s).

On top of the antioxidant properties, vanillin and ferulic acid can modulate the activity of numerous redox systems and help sustain normophysiological levels of endogenous antioxidants, which are generally compromised during carcinogenesis (Schwartz et al., 1993; Pappalardo et al., 1996; St Clair et al., 2005). In noncancerous hepatocytes, vanillin attenuated the CCl_4-induced reduction in the activity of the antioxidant enzymes catalase and SOD as well as GSH (Makni et al., 2011). In tumor-bearing animals, ferulic acid was shown to augment the activity/concentration of the molecular antioxidants α-tocopherol, ascorbic acid, glutathione, and the redox-sensitive enzymes GST, glutathione reductase, glutathione peroxidase, catalase, and SOD to near-normophysiological levels of control animals (Dean et al., 1995; Han et al., 2001; Balakrishnan et al., 2008; Alias et al., 2009; Baskaran et al., 2010). The vast majority of curcumin catabolites is therefore able to ameliorate oxidative stress directly and via endogenous molecular and enzymatic systems, such as the phase II detoxification enzymes, and thereby hamper the potential development and progression of cancer.

Additionally, some curcumin catabolites may indirectly bestow their anticarcinogenic effects by impacting the inflammation component in the oxidative stress/inflammation/carcinogenesis axis—an effect that has been proven for several classes of anti-inflammatory drugs (Wang et al., 2003; Greene et al., 2011; Thun et al., 2012). Vanillin has been shown to inhibit lipopolysaccharide-induced COX-2 gene expression and NF-κB activation in RAW 264.7 murine macrophages (Murakami et al., 2007) and to considerably reduce the expression of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), IL-1β, and IL-6 in rat livers after CCl_4 treatment (Makni et al., 2011). Vanillin also enhances pro-apoptotic signaling in cancer cells induced by TNF-related apoptosis-inducing ligand (TRAIL), a protein excreted by activated neutrophils (Simons et al., 2008), monocytes (Halaas et al., 2004), and macrophages (Halaas et al., 2000), via inhibition of NF-κB activation (Lirdprapamongkol et al., 2010). Similarly, vanillic acid inhibited TNF-α and IL-6 expression, suppressed the activation of NF-κB and caspase-1, and reduced the expression levels of COX-2 and prostaglandin (PG)E_2 in lipopolysaccharide-stimulated mouse peritoneal macrophages (Kim et al., 2011). Ferulic acid was able to decrease the extent of tetradecanoylphorbol acetate-induced inflammation (edema) in mouse ears (Fernandez et al., 1998) and inhibit prostanoid production in IL-1β-stimulated human colon fibroblasts (Russell et al., 2008) as well as COX-2 production in HT-29 cells (Ferguson et al., 2005).

C. Pharmacokinetics and Pharmacodynamic Implications of Curcumin Metabolites Generated in Biological Systems

The chemical modification of curcumin in vivo is significantly different from the in vitro degradation and modification mechanisms because the metabolic processes are mainly enzyme driven. Consequently, the metabolites also differ and mainly comprise end products of reductase-mediated methine bridge hydrogenation, sulfation, glucuronidation, and (Michael reaction-mediated) protein complexation, as illustrated in Fig. 12 (Pan et al., 1999; Fang et al., 2005; Anand et al., 2007; Jung et al., 2007; Marczylo et al., 2007; Dhillon et al., 2008; Marczylo et al., 2009). The disposition of curcumin and its biological metabolites is summarized in Table 1.

1. Pre-enterocytic Pharmacokinetics. The metabolism of orally ingested curcumin essentially starts in the epithelial cells of the intestinal mucosa, a highly regulated barrier composed of mucus-covered enterocytes that the curcumin molecules must pass through

to enter the systemic circulation. However, before intracellular metabolism occurs, several pharmacokinetic hurdles must be overcome. The mucus—a low pH-sustaining aqueous layer replete with glycoproteins and lipids (Legen and Kristl, 2003)—is known to avidly retain curcumin (Berginc et al., 2012), as a result of which the pre-epithelial curcumin concentration gradient is lowered and transmucosal passage of curcumin is impaired. Secondly, intestinal mucus contains μ- and π-class GSTs that catalyze the conjugation of GSH to electrophilic compounds (Samiec et al., 2000), including curcumin (Awasthi et al., 2000; Usta et al., 2007), resulting in pre-enterocytic biotransformation and reduced systemic bioavailability of the native compound. Lastly, the curcumin molecules that are taken up by enterocytes are further subject to apical efflux from the cells (Usta et al., 2007; Berginc et al., 2012) (section III.C.2.f), i.e., back into the intestinal lumen. This also applies to curcumin that has been reduced or conjugated in the enterocytes (sections III.C.2.a through III.C.2.d).

The pre-enterocytic pharmacokinetic hurdles are partly responsible for the very low concentration of curcumin and its metabolites at the basolateral end (i.e., the circulation), as evidenced by the vast difference between the orally administered curcumin dose and the peak curcumin levels retrieved in plasma (Supplemental Table 2) and the very high levels in the intestines and feces. Studies in rats showed that 35–40% (Ravindranath and Chandrasekhara, 1982), 75 ± 8% (Wahlstrom and Blennow, 1978), and 89 ± 3% (Holder et al., 1978) of the orally administered single dose of curcumin was present in feces 3 days after administration. It should be noted that a portion of the retrieved fecal curcumin had undergone basolateral uptake and subsequent hepatic clearance (the second pass effect) and biliary transport back into the intestines inasmuch as the fraction of fecal curcumin was higher 12 days after administration than 3 days after administration (Ravindranath and Chandrasekhara, 1982). Consequently, intestinal retention of curcumin as a result of the first pass effect and enteral re-entry of curcumin and its metabolites due to the second pass effect account for the high levels of curcumin and its derivatives in the gut and poor systemic bioavailability after oral intake.

2. Xenobiotic Metabolism in Enterocytes. The curcumin molecules that are taken up by enterocytes subsequently undergo phase I (modification) and phase II (conjugation) metabolism (Suzuki and Sugiyama, 2000) by several cytosolic and microsomal enzymes, most notably (oxido)reductases (section III.C.2.a), sulfotransferases (SULTs) (section III.C.2.b), glucuronosyltransferases (section III.C.2.c), and GSTs (section III.C.2.d), which further contributes to the low systemic curcumin levels. The poor bioavailability is also exacerbated by the fact that curcumin acts as a substrate for proteins that are present in enterocytes but not responsible for detoxification metabolism (section III.C.2.e). These proteins can bind and/or chemically modify curcumin. Aside from the specific examples addressed in section III.C.2.e, enzymes that may also catabolize curcumin include aldo-keto reductases (which can modify/cleave β-diketones) (Rosemond et al., 2004; Grogan, 2005), non-phase I metabolism (oxido)reductases that hydrogenate alkenes to alkanes (Pan et al., 1999), and alkene-cleaving enzymes (Mutti, 2012), although the involvement of these proteins in the modification of curcumin is presently unconfirmed.

a. Xenobiotic phase I metabolism in enterocytes: curcumin reduction. With respect to the phase I (oxido)reductases, several studies have demonstrated that reductive metabolism of curcumin (Fig. 12 and Table 1) occurs extensively in enterocytes (Ireson et al., 2002; Dempe et al., 2012). In fact, experiments performed with differentiated Caco-2 cells, which are enterocyte-resembling human colon cancer cells (Yee, 1997; Press and Di Grandi, 2008; Naruhashi et al., 2011) that exhibit phase I metabolism and express phase II enzymes such as sulfotransferases, uridine diphosphate-glucuronosyltransferases (UGTs), and GSTs (Meunier et al., 1995; Satoh et al., 2000), revealed that curcumin undergoes reduction to a much greater degree than conjugation by the sulfotransferases and glucuronosyltransferases (Dempe et al., 2012). The reduction reactions favor sulfated curcumin > native curcumin > glucuronidated curcumin (Dempe et al., 2012). When Caco-2 cells were incubated with 50 μM curcumin for 3 hours and curcumin and its metabolites were analyzed in the medium and cell lysate, curcumin sulfate was not detected in either medium or cell lysate, whereas curcumin glucuronide was only detected in medium. Contrastingly, reduced curcumin sulfate and, to a lesser extent, reduced curcumin glucuronide species were abundantly present in the medium, but not in cell lysates. In follow up apical→basolateral transport experiments with Caco-2 cells, no curcumin sulfate was found on the apical side and only very small quantities of curcumin sulfate (~4‰ of the added curcumin molecules) were observed on the basolateral side. On the other hand, reduced curcumin sulfate but not glucuronide was found at high levels on the basolateral side (Dempe et al., 2012), altogether suggesting that double (4 electrons) or triple reductions (6 electrons) of curcumin may drive intestinal phase II metabolism with respect to the SULTs.

It is not exactly known which enzymes reduce curcumin to its hydrogenated isoforms. The enzymatic source responsible for the reduction of curcumin is, however, strictly cytosolic, whereas the reduction of hexahydrocurcumin to octahydrocurcumin occurs in both the cytosol and microsomes (Ireson et al., 2002). In Caco-2 cell transport experiments, Wahlang et al. (2011) showed that itraconazole-mediated inhibition of cytochrome P450 CYP3A4, an isozyme that is replete

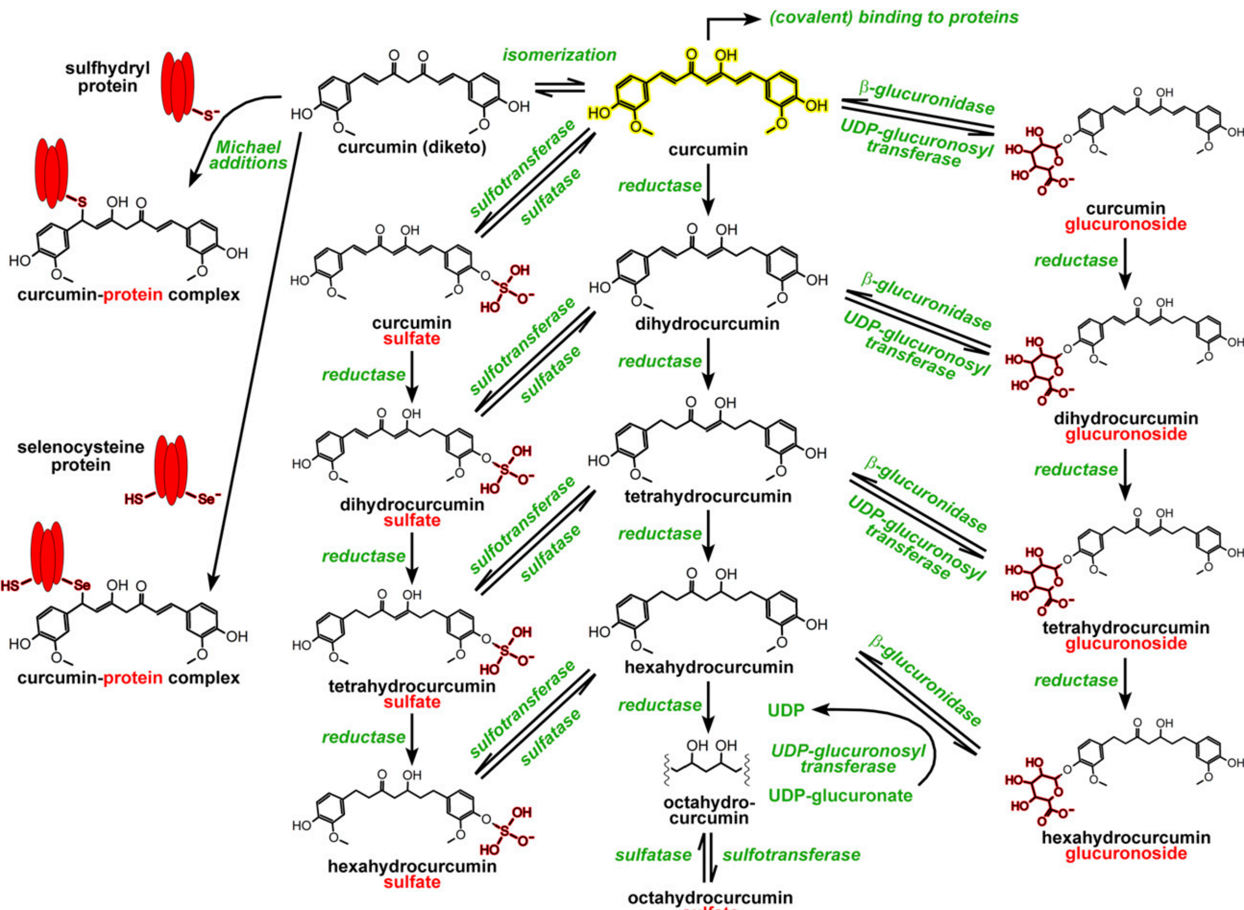


Fig. 12. Overview of curcumin metabolism in cells and living organisms. The starting compound (enolic curcumin) is indicated in yellow, the chemical processes and enzymatic reactions in green, and the curcumin adducts in red. Only one specific conjugation site is presented. However, glucuronidases and sulfotrasferases are capable of conjugating a glucuronic acid or sulfate, respectively, to any of the hydroxyl groups (Marczylo et al., 2009). The conjugation process typically involves the addition of a single moiety, although double glucuronidation has been reported to occur in isolated liver microsomes (Tamvakopoulos et al., 2007b) and diglutathionylated curcumin has been found in isolated reaction systems (Awasthi et al., 2000). The final reduction catabolite, octahydrocurcumin, is alternatively referred to as hexahydrocurcuminol. Data compiled on the basis of Pan et al. (1999), Ireson et al. (2002), Fang et al. (2005), Anand et al. (2007), Jung et al. (2007),Marczylo et al. (2007, 2009), Dhillon et al. (2008).

in the liver and intestines (Yokose et al., 1999) and capable of detoxifying certain drugs by reduction (Shahrokh et al., 2012), results in an increased curcumin permeability coefficient (Wahlang et al., 2011). This finding is suggestive of alterations in reductive biotransformation of curcumin by CYP3A4, which manifests itself in differential curcumin transport kinetics as a result of a shifted curcumin:reduced curcumin equilibrium and corollary effects on transporter activity. One of the alcohol dehydrogenase isozymes is also a potential mediator, as equine alcohol dehydrogenase was shown to reduce curcumin to hexahydrocurcumin in an isolated in vitro reaction system (Ireson et al., 2002). Another possible candidate but never-in-this-context-investigated oxidoreductase is duodenal cytochrome *b* (DcytB), an outer membrane-spanning ferric reductase that shuttles intracellularly transferred electrons (from ascorbate) across the enterocyte membrane to reduce extracellular Fe^{3+} (complexed to dehydroascorbate, the oxidized form of ascorbic acid) to its ferrous state, thus allowing enterocytes to take up iron (McKie et al., 2001). In addition to transition metals, DcytB is capable of reducing exogenous molecular electron acceptors, such as MTT, NBT (McKie et al., 2001), and dehydroascorbate (Vlachodimitropoulou et al., 2010). Moreover, it was recently shown that polyphenol-metal chelate complexes, such as intracellular quercetin-Fe^{2+}, transfer electrons to DcytB for extracellular reduction reactions (Vlachodimitropoulou et al., 2010). Given curcumin's ability to undergo reduction (Ireson et al., 2002; Dempe et al., 2012), donate electrons ("Chemical degradation and modification of curcumin," section II.C.1.b.i), and bind transitional metal cations (section II.A.4), it is not unlikely that curcumin plays a role in the DcytB redox axis at the electron donating and possibly the accepting end, of which the latter would account for the formation of the reduced curcumin isomers. The reduction of hexahydrocurcumin to octahydrocurcumin has been proposed to be mediated by cytochrome P450 reductase (Ireson et al., 2002).

b. Xenobiotic phase II metabolism in enterocytes: curcumin sulfation. In regard to the phase II enzymes,

TABLE 1

Overview of the in vivo disposition of curcumin and its metabolites

The x-marks indicate that the respective compound was found in the respective fluid or tissue, whereas the absence of an x-mark signifies that the compound was either not found or not assayed. Octahydrocurcumin is alternatively referred to as hexahydrocurcuminol. The database is chiefly comprised of studies in which orally administered curcumin or curcumin formulations were investigated, including liposomal curcumin (Maiti et al., 2007; Marczylo et al., 2007, 2009; Cuomo et al., 2011; Helson et al., 2012), curcumin + piperine (Shoba et al., 1998), curcumin + piperine + lecithin (Antony et al., 2008), and Biocurcumax (BCM-95 CG) (Antony et al., 2008). Exceptions to orally delivered curcumin include the dog study with intravenously administered curcumin in liposomal form (Helson et al., 2012) and studies where free curcumin was infused intraperitoneally or intravenously in addition to oral administration (Holder et al., 1978; Pan et al., 1999; Yang et al., 2007a).

Compound	Species	Blood	Urine	Bile	Intestine	Liver	Spleen	Kidney	Heart	Lungs	Brain	Muscle	Fat	References
Curcumin	human:	x	x		x									Shoba et al., 1998; Cheng et al., 2001; Garcea et al., 2004, 2005; Sharma et al., 2004; Lao et al., 2006; Antony et al., 2008; Dhillon et al., 2008; Carroll et al., 2011; Cuomo et al., 2011; Chen et al., 2012b; Irving et al., 2013
	rat:	x	x	x	x	x		x	x				x	Holder et al., 1978; Wahlstrom and Blennow, 1978; Ravindranath and Chandrasekhara, 1980, 1982; Shoba et al., 1998; Asai and Miyazawa, 2000; Ireson et al., 2001; Marczylo et al., 2007, 2009; Maiti et al., 2007; Yang et al., 2007b
	mouse:	x			x	x	x	x	x	x	x	x		Pan et al., 1999; Perkins et al., 2002; Zhongfa et al., 2012
	dog:	x												Helson et al., 2012
Curcumin glucuronoside	human:	x			x									Dhillon et al., 2008; Vareed et al., 2008; Carroll et al., 2011
	rat:	x	x											Ravindranath and Chandrasekhara, 1980; Asai and Miyazawa, 2000
	mouse:													
Curcumin monoglucuronoside	human:	x	x		x									Garcea et al., 2004, 2005; Sharma et al., 2004; Chen et al., 2012b; Irving et al., 2013
	rat:	x		x	x									Holder et al., 1978; Ireson et al., 2001; Marczylo et al., 2007, 2009
	mouse:	x												Pan et al., 1999; Zhongfa et al., 2012
Curcumin sulfate	human:	x			x									Dhillon et al., 2008; Vareed et al., 2008; Carroll et al., 2011
	rat:	x	x											Ravindranath and Chandrasekhara, 1980; Asai and Miyazawa, 2000
	mouse:				x									Perkins et al., 2002
Curcumin monosulfate	human:	x	x		x									Garcea et al., 2004, 2005; Sharma et al., 2004; Irving et al., 2013
	rat:	x			x	x								Ireson et al., 2001; Marczylo et al., 2007
	mouse:	x												Zhongfa et al., 2012
Curcumin glucuronoside sulfate	human:													
	rat:	x												Ireson et al., 2001
	mouse:													
Tetrahydrocurcumin	human:													
	rat:	x		x	x	x								Holder et al., 1978; Marczylo et al., 2007
	mouse:	x												Pan et al., 1999; Zhongfa et al., 2012
	dog:	x												Helson et al., 2012
Hexahydrocurcumin	human:					x								Garcea et al., 2004
	rat:	x		x	x	x		x						Holder et al., 1978; Ireson et al., 2001; Marczylo et al., 2007, 2009
	mouse:													
Octahydrocurcumin	human:					x								Garcea et al., 2004
	rat:	x												Ireson et al., 2001
	mouse:													
Dihydrocurcumin monoglucuronoside	human:													
	rat:													

(*continued*)

TABLE 1—*Continued*

Compound	Species	Blood	Urine	Bile	Intestine	Liver	Spleen	Kidney	Heart	Lungs	Brain	Muscle	Fat	References
	mouse:	x												Pan et al., 1999
Tetrahydrocurcumin monoglucuronoside	human:													
	rat:	x												Marczylo et al., 2007
	mouse:	x												Pan et al., 1999
Hexahydrocurcumin monoglucuronoside	human:													
	rat:	x												Ireson et al., 2001; Marczylo et al., 2007
	mouse:	x												Pan et al., 1999
Tetrahydrocurcumin monosulfate	human:													
	rat:				x	x								Marczylo et al., 2007, 2009
	mouse:													
Hexahydrocurcumin monosulfate	human:													
	rat:				x	x		x						Marczylo et al., 2007, 2009
	mouse:													
Ferulic acid	human:													
	rat:			x										Holder et al., 1978
	mouse:													
Dihydroferulic acid	human:													
	rat:			x										Holder et al., 1978
	mouse:													

intestinal epithelial cells contain a host of cytosolic SULTs, among which are members of the phenol SULT subfamily (SULT1) that are responsible for catalyzing the transfer of a sulfonyl group from 3′-phosphoadenosine 5′-phosphosulfate to a wide variety of phenolic xenobiotics (Eisenhofer et al., 1999; Nowell and Falany, 2006; Enokizono et al., 2007; Hempel et al., 2007; Teubner et al., 2007; Riches et al., 2009), including numerous dietary flavonoids (Huang et al., 2009). Two members of this subfamily, SULT1A1 and 1A3, are responsible for sulfating curcumin (Fig. 12) in human and rat intestines, whereby the 1A3 isozyme is more effective in catalyzing the sulfation of curcumin than the 1A1 isozyme (Ireson et al., 2002). As stated in the previous section, studies with differentiated Caco-2 cells have shown that sulfation of native curcumin occurs to a minimal extent in enterocytes and that reduced curcumin species, predominantly hexa- and octahydrocurcumin, constitute the preferred SULT substrates (Dempe et al., 2012).

c. Xenobiotic phase II metabolism in enterocytes: curcumin glucuronidation. In addition to UGTs, epithelial cells along numerous segments of the gastrointestinal tract contain several UGT isozymes, including UGT1A1 and UGT1A7 through UGT1A10 (Basu et al., 2004), all of which reside in the lumen of the endoplasmic reticulum (ER). Of these, UGT1A7, 1A8, and 1A10 are predominantly expressed in the gastrointestinal tract and not or minimally in the liver (Strassburg et al., 1999; Tukey and Strassburg, 2000), the organ most responsible for glucuronidation (Fisher et al., 2001). Glucuronidation of curcumin (Fig. 12) has been unequivocally demonstrated in differentiated Caco-2 cells (Berginc et al., 2012; Dempe et al., 2012) and in isolated rat and human intestinal microsomes (Ireson et al., 2002; Hoehle et al., 2007). Curcumin glucuronidation activity for different recombinant human UGTs occurs in the order of UGT1A1 > UGT1A8 = UGT1A10 > UGT1A3 > UGT2B7 > UGT1A7 > UGT1A9 > UGT1A6 in buffered solution (Hoehle et al., 2007) and transfected microsomes (Pfeiffer et al., 2007). Furthermore, hexahydrocurcumin (Hoehle et al., 2007; Dempe et al., 2012) and octahydrocurcumin (Dempe et al., 2012) undergo glucuronidation in enterocytes, which, as is the case with the SULTs, occurs more extensively with reduced curcumin than with nonreduced curcumin (Dempe et al., 2012). For hexahydrocurcumin, the glucuronidation activities are in the order of UGT1A8 = UGT1A9 > UGT2B7 > UGT1A1 = UGT1A10 > UGT1A7 > UGT1A3 (Hoehle et al., 2007), indicating that the affinity for the substrate binding site or catalytic activity are dependent on the reduction status of curcumin.

d. Xenobiotic phase II metabolism in enterocytes: curcumin glutathionylation. On top of their mucosal presence, GSTs (GSTM1a-1a and GSTP1-1) are contained in intestinal epithelial cells (Siegers et al., 1988; Ogasawara et al., 1989). GSTs have been shown to

glutathionylate curcumin in isolated reaction systems using recombinant or purified human enzymes, in human intestinal cytosol, and in Caco-2 cells (Awasthi et al., 2000; Usta et al., 2007). The reaction proceeds via a Michael addition (section II.A.7) of GSH to C_1/C_σ of curcumin (Fig. 1C), yielding mainly two diastereoisomeric monoglutathionyl curcumin conjugates (Awasthi et al., 2000; Usta et al., 2007), although diglutathionylation has also been observed (Awasthi et al., 2000). Both monoglutathionyl diastereoisomers are formed by α-class (GSTA1-1, GSTA2-2), μ-class (GSTM1a-1a), π-class (GSTP-1-1) but not θ-class (GSTT-1) GSTs in the order of GSTM1a-1a $>$ GSTA1-1 $>$ GSTP1-1 $\approx$ GSTA2-2 (Usta et al., 2007). The glutathionylated curcumin is relatively unstable and rapidly ($t_{1/2}$ = 4 hours) degrades into curcumin and several uncharacterized degradation products (Usta et al., 2007), which may comprise glutathionylated adducts of feruloyl methyl ketone, ferulic acid, and feruloyl aldehyde (Awasthi et al., 2000), i.e., curcumin catabolites that are also produced by (photo)chemical degradation (Fig. 6). However, it is not clear whether these catabolites form before or after conjugation.

e. Curcumin binding to and modification by nondetoxifying proteins. The last notable type of processes that contributes to the low systemic bioavailability of curcumin and/or the generation of curcumin metabolites entails the association of curcumin with intestinal nondetoxification proteins. In some instances the binding of curcumin to a protein relays an inhibitory effect, as has been shown for several ATP-binding cassette (ABC) transporters (Wortelboer et al., 2003, 2005; Shukla et al., 2009) and CYP isozymes (Volak et al., 2008), whereas in other instances the binding can be both antagonistic and biotransformative, as is the case for, e.g., SULTs (Eaton et al., 1996; Ireson et al., 2002; Volak et al., 2008; Fong et al., 2012), UGTs (Volak et al., 2008; Berginc et al., 2012; Dempe et al., 2012; Fong et al., 2012), and GSTs (Awasthi et al., 2000). With respect to nondetoxifying enzymes, the combined antagonistic and catalytic effects have been demonstrated for curcumin-COX-2 (Hong et al., 2004; Selvam et al., 2005; Griesser et al., 2011) and curcumin-lipoxygenase complexes (Skrzypczak-Jankun et al., 2000; Toth et al., 2000; Hong et al., 2004; Prasad et al., 2004), which result in the formation of 6-hydroxy-1-(4-hydroxy-3-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1,3,3*a*,6*a*-tetrahydro-4*H*-cyclopenta[*c*]furan-4-one (Fig. 7B). Similar effects have been reported for recombinant rat TrxR 1 (Dal Piaz et al., 2010) (section II.A.4), the activity of which was inhibited by curcumin due to covalent complexation with two curcumin molecules through a Michael addition (section II.A.7) at TrxR's selenocysteine and sulfhydryl moieties in the active site (Fang et al., 2005) (Fig. 12). Another illustrative example is the Michael addition-mediated covalent binding of curcumin at the tyrosine kinase domain of ErbB-2, which leads to intracellular depletion of the curcumin-ErbB-2 complexes via mainly nonproteasomal and nonlysosomal pathways (Jung et al., 2007). Inasmuch as COX-2, lipoxygenases, and ErbB receptors are expressed in intestinal mucosa (Kawajiri et al., 2002; Sagiv et al., 2007; McElroy et al., 2012) and TrxR1 is present in the colon (Lechner et al., 2003), the abovementioned processes negatively affect the systemic disposition of curcumin and expand the variety of curcumin metabolites in the intestinal mucosa.

f. Excretion of biological curcumin metabolites from enterocytes. The formation of conjugated or complexed curcumin derivatives not only abrogates the possibility of intermolecular interactions (section II.A.1 through II.A.7), and hence curcumin's cytostatic capacity (section III.E), but also changes the pharmacokinetics of curcumin in an unfavorable manner as a result of excretion. The reduction and subsequent conjugation of xenobiotics is mainly intended to render these compounds more hydrophilic so as to facilitate their renal or enteral excretion, whereby the latter may proceed directly from the intestinal mucosa back into the gut (for orally administered compounds) or indirectly via the circulatory-biliary route. Systemic clearance and enteral 'back-trafficking' prevents curcumin from reaching the tumor tissue, granted that the tumor resides outside the urinary and lower gastrointestinal system.

Because transmembrane passage of anionic substances requires facilitated transport, cells possess a host of transmembrane proteins that effectively traffic the biotransformed compounds across the hydrophobic barrier. In the context of conjugated curcumin, the most important (but not the only) transporters are those of the ABC superfamily that include P-glycoprotein [P-gp/multidrug resistance protein (MRP) 1 (MDR1)/ABC subfamily B member 1 (ABCB1)], present on the apical side of cells in the jejunum, colon, liver, and kidney (Thiebaut et al., 1987); breast cancer resistance protein (BCRP/ABCG2), abundantly expressed apically (Berginc et al., 2012) in intestinal and colonic epithelial cells (Campa et al., 2008); MRP1 (ABCC1), which most likely resides in the basolateral membrane of enterocytes (Nakamura et al., 2002); MRP2 (ABCC2), which is expressed in the apical membrane of cells in the liver, kidney, small intestine, colon, and gallbladder; MRP3 (ABCC3), which is present mainly basolaterally in the ileum, colon, liver, kidney, and gallbladder; and MRP7 (ABCC7), expressed in the kidney but topographically elusive (Deeley et al., 2006). These ABCB/C/G transporters have a high affinity for organic anions and mediate their transport in an ATP-dependent manner (Konig et al., 1999; Homolya et al., 2003). Accordingly, the transporters are responsible for the efflux of various sulfated (BRCP, MRP1, MRP2, MRP3), glucuronidated (P-gp, BRCA, MRP1, MRP2, MRP3, MRP7), and glutathionylated compounds (P-gp, BRCA, MRP1, MRP2, MRP7) out of the cell (Imai et al., 2003; Suzuki et al., 2003; Deeley et al.,

2006; Vaidya et al., 2009; An and Morris, 2011), some of which have been shown to transport curcumin conjugates.

With the use of transporter-specific inhibitors PSC883 (P-gp), MK571 (MRPs), and FumC (BRCP), it was shown that the efflux of intracellularly produced curcumin-glucuronide to the apical side of the Caco-2 cell monolayer was most likely mediated by P-gp and MRPs, whereas MRP1 and MRP3 were probably responsible for transport to the basolateral side (Berginc et al., 2012). These results were only partly corroborated in a similar experimental setup by Dempe et al. (2012), demonstrating that curcumin glucuronide efflux to the basolateral side indeed occurred—the extent of which was equal to that of curcumin and greater than that of curcumin sulfate—but that curcumin glucuronide transport to the apical side was absent. Additionally, hexahydrocurcumin and its sulfated and glucuronidated derivatives were shown to be basolaterally but not apically transported in the order of hexahydrocurcumin sulfate > > hexahydrocurcumin > hexahydrocurcumin glucuronide. Similarly, octahydrocurcumin and its sulfated and glucuronidated derivatives underwent basolateral but not apical efflux in the order of octahydrocurcumin sulfate > > octahydrocurcumin = octahydrocurcumin glucuronide. Reduced but nonconjugated curcumin exhibited extensive apical excretion. Because no transporter inhibitor assays were performed, the transporters responsible for the efflux of the reduced curcumin species and the reduced + conjugated species remains elusive and limited to several candidate transporters based on known localization.

3. Post-enterocyte Pharmacokinetics of Curcumin and Biological Curcumin Metabolites. The curcumin and its derivatives that have been retained in the intestinal lumen or apically transported after biotransformation in enterocytes, comprising mainly nonconjugated reduced curcumin (Dempe et al., 2012), are expected to be excreted in feces and not to contribute to pharmacodynamic effects in tumor tissue outside the alimentary tract. The potential antitumor effects of intestinally retained curcumin (metabolites) have been addressed in section III.A. Conversely, the curcumin and its metabolites that are transported basolaterally could potentially target to tumor tissue, but are also subject to the second pass effect and uptake by nonenterohepatic organs.

a. Tissue distribution of curcumin and implications on cancer pharmacodynamics. It is clear, from the extent of basolaterally transported curcumin derivatives compared with native curcumin (section III.C.2.f, Supplemental Table 2), that the majority of the fraction of curcumin that enters the circulation does so in biotransformed state. This, in combination with the high degree of intestinal retention and retroenteral efflux, translates to very low curcumin levels in tissue. In fact, 12 days after oral administration of radioactively ([^{3}H]) labeled curcumin (10, 80, or 400 mg) in rats (Ravindranath and Chandrasekhara, 1982), only 1–2% of the [^{3}H]curcumin was found in the urine (sampled during the entire 12-day period), 3% in the blood, 1% in the liver, and <1% in the kidneys. The remainder of the [^{3}H]curcumin was retrieved from fecal matter, which was also sampled during the entire 12-day period. In the 10-mg and 80-mg groups all the [^{3}H]curcumin was fully recovered from the abovementioned tissue/samples, whereas in the 400-mg group the recovery was 82%, which may reflect accumulation of [^{3}H]curcumin in the intestinal mucosa as alluded to in section III.C.1, given that the intestines were not assayed for radioactivity. In support of this, a preceding study in rats by the same group revealed that, 24 hour after per gavage administration of 400 mg of curcumin, approximately 40% of curcumin was present in the gastrointestinal tract and that curcumin levels in blood, liver, and kidneys were below the limit of detection (<5 μg/ml or g tissue, equivalent to 13 μM) (Ravindranath and Chandrasekhara, 1980). Furthermore, relatively high curcumin concentrations have been found in colorectal mucosa of patients after oral intake (Garcea et al., 2005; Carroll et al., 2011; Irving et al., 2013) and in feces as well as intestinal and colonic mucosa in rats after 1 week of dietary curcumin intake (Perkins et al., 2002). Given the very low curcumin levels in healthy tissue, it is likely that the accumulation of curcumin in nonenteral tumor tissue will follow a similar pattern.

The curcumin that is taken up by cancer cells is subsequently subjected to similar metabolic processing (phase I–II and excretion) as described for enterocytes (sections III.C.2.a through III.C.2.f) because cancer cells contain many of the enzymes responsible for phase I and II metabolism (Burchell et al., 1990; Tsuchida and Sato, 1992; Doherty and Michael, 2003; Rochat, 2009) and the transporters to facilitate bidirectional trafficking of xenobiotics and their conjugates (Rochat, 2009; Brozik et al., 2011; Tiwari et al., 2011). Accordingly, reductive metabolism of curcumin to hexahydrocurcumin and octahydrocurcumin as well as export of the metabolites into the culture medium was found in human endometrial adenocarcinoma (Ishikawa) cells and HepG2 cells (Dempe et al., 2008). Similarly, curcumin glucuronidation and export of the curcumin-glucuronide adducts were observed in HT-29 cells. Curcumin metabolism naturally has an impact on the cytostatic efficacy of curcumin, as addressed in the section on the pharmacodynamic implications of biological curcumin metabolites (section III.C.4).

b. Postenterocyte pharmacokinetics of curcumin and its metabolites: second pass effect. In addition to the abovementioned first pass effects, the curcumin levels in blood and ultimately tissue are reduced by the second pass effect, as all gut-derived compounds are initially subjected to the filtration and detoxification

machinery of the liver. Hepatic clearance and biliary excretion of systemic curcumin is very effective and rapid. Holder et al. (1978) showed that 74% and 85% of intravenously administered curcumin (as a single bolus) was present in bile 2 and 6 hours after administration, respectively, with peak bile concentrations occurring within 30 minutes after administration (Wahlstrom and Blennow, 1978). Moreover, biotransformation of curcumin in the liver is expected to be profound inasmuch as hepatocytes contain very high concentrations of most phase I and II enzymes compared with other organs, including all the enzymes known to reduce and conjugate curcumin (section III. C.2.a through III.C.2.e) (Sevior et al., 2012).

Accordingly, the reductive metabolism of curcumin has been demonstrated in hepatic microsomes of human (Pfeiffer et al., 2007; Tamvakopoulos et al., 2007b), rat (Pfeiffer et al., 2007), and mouse origin (Tamvakopoulos et al., 2007b) as well as human and rat hepatocytes (Ireson et al., 2001). On top of generating di-, tetra-, and hexahydrocurcumin (Ireson et al., 2001; Pfeiffer et al., 2007; Tamvakopoulos et al., 2007b), the formation of octahydrocurcumin from hexahydrocurcumin was observed in human and rat hepatocytes (Ireson et al., 2001). Furthermore, incubation of male rat liver slices with curcumin and subsequent enzymatic hydrolysis of the glucuronide and sulfate moieties yielded, in order of low to high abundance, tetrahydrocurcumin, two isoforms of dihydrocurcumin (ascribed to the reduction of the allylic or enolic double bond), two diastereomers of octahydrocurcumin, and hexahydrocurcumin (Hoehle et al., 2006).

With respect to conjugative metabolism, monoglucuronidation of curcumin was observed in human, rat, and mouse microsomes (Pfeiffer et al., 2007; Tamvakopoulos et al., 2007b) as well as rat liver slices (Hoehle et al., 2006). In liver slices, glucuronide conjugation proceeded in the order of hexahydrocurcumin > curcumin > octahydrocurcumin > tetrahydrocurcumin. When the liver slices of male rats were incubated with tetrahydrocurcumin, the glucuronidized metabolites comprised hexahydrocurcumin > > octahydrocurcumin > tetrahydrocurcumin, whereas the degree of glucoronidation of hexahydrocurcumin after the addition of hexahydrocurcumin was only slightly higher than that of octahydrocurcumin, again reflecting the dependency of enzymatic activity on curcumin's reduced state as alluded to in sections III. C.2.a and III.C.2.c. Sulfation was also observed in the liver slices, but was less extensive than glucuronidation and more selective. Only curcumin and hexahydrocurcumin were sulfated, whereby the SULTs exhibited a greater affinity for hexahydrocurcumin (Hoehle et al., 2006).

The liver contains numerous transporters to facilitate canalicular excretion of curcumin and its metabolites (Evans, 1996; Leslie et al., 2001; Deeley et al., 2006; Kusuhara and Sugiyama, 2009), as has been shown in bile analysis studies after intravenous administration of curcumin (Holder et al., 1978; Wahlstrom and Blennow, 1978). After 3-hour continuous infusion of curcumin (4 mg/h/rat), the collected bile contained 50–60% of the infused curcumin dose, of which ~95% comprised curcumin glucuronide and ~2% curcumin and ~2% curcumin sulfate (Holder et al., 1978). Additional metabolites that were detected in small quantities after liquid phase extraction and MS included tetrahydrocurcumin, hexahydrocurcumin, ferulic acid, and dihydroferulic acid (the reduced form of ferulic acid). Although the fraction of glucuronidated curcumin in bile has been reported to be lower, namely 20% of the total biliary excrement (Wahlstrom and Blennow, 1978), the data attest to the rapid and extensive metabolism and canalicular excretion of curcumin (derivatives) and confirm the greater propensity of hepatocytes to conjugate curcumin by glucuronidation versus sulfation. Curcumin sulfate has been retrieved from feces of patients who received curcumin orally (Sharma et al., 2001). Given the predominantly basolateral transport of sulfated curcumin species (section III.C.2.f), the fecal curcumin sulfate was most likely formed during second pass metabolism and biliary clearance. Intrahepatic glutathionylation and subsequent biliary excretion of curcumin-glutathione adducts has not been reported.

c. Postenterocyte pharmacokinetics of curcumin: nonenterohepatic organs. The curcumin that circumvents the first (intestines) and second pass (liver) effect becomes susceptible to uptake by other organs and tissue, as indicated in Table 1. Some of the organs that are known to take up curcumin are replete with phase I and II detoxification enzymes, including but not limited to the kidneys (contain all the enzyme systems that facilitate curcumin biotransformation) (Lock and Reed, 1998; Nishimura et al., 2009) and the lungs (Zhang et al., 2006; Nishimura et al., 2009). Many of the organs also contain (some of) the transporters that mediate trafficking/excretion of the curcumin metabolites, as has been described for the kidneys (Kusuhara and Sugiyama, 2009) as well as the lungs (Brechot et al., 1998; Leslie et al., 2001; Nishimura et al., 2009). Consequently, nonenterohepatic organs are capable of metabolizing curcumin and basolaterally transporting the metabolites for further systemic clearance by the kidneys and possibly the liver. It should be noted, however, that the contribution to the systemic depletion of curcumin levels has not been extensively investigated and is probably marginal given the already low blood levels of curcumin.

d. Postenterocyte pharmacokinetics of curcumin metabolites: systemic clearance. The curcumin metabolites that have been basolaterally exported from enterocytes, hepatocytes, or nonenterohepatic organ cells are most likely to undergo renal clearance, given that the kidneys are responsible for the elimination of

a myriad of conjugated compounds, including xenobiotic metabolites (Lohr et al., 1998). Although renal uptake and clearance of curcumin conjugates has never been directly demonstrated, it does constitute the most favorable route inasmuch as renal uptake increases with a compound's hydrophilicity, charge, and H-bonding capacity (Feng et al., 2010) and kidney cells are equipped with a plethora of basolateral importers and apical exporters (Kusuhara and Sugiyama, 2009; Morrissey et al., 2013) to eliminate the conjugated compounds via urine (Wang et al., 2011a).

The only indirect evidence for the renal clearance of conjugated curcumin was provided by a study in rats (Ravindranath and Chandrasekhara, 1980) and two phase I trials with colorectal cancer patients (Sharma et al., 2004; Irving et al., 2013). In the former study, rats were administered a single dose of curcumin (400 mg) per gavage and urinalysis was performed up to 44 days postadministration. The peak levels of curcumin glucuronide and curcumin sulfate in urine were observed on days 1 and 8, respectively, after administration. Glucuronidated curcumin was observed up to 13 days postadministration, whereas sulfated curcumin appeared in urine up to 44 days after dosing (Ravindranath and Chandrasekhara, 1980). In one of the two clinical studies, the urine of patients consuming 3600 mg of curcumin per day during the entire treatment period contained curcumin (0.1–1.3 μM), curcumin sulfate (19–45 nM), and curcumin glucuronide (210–510 nM) on day 29 of oral dosing (Sharma et al., 2004). In the other clinical study, in which patients were administered an oral curcumin dose of 1880 mg/day for 14 days and sampling was performed ~24 hours after the final administration, the urine levels of curcumin glucuronide were 8–176 nM, whereas the concentration of curcumin and curcumin sulfate was below the limit of detection (7 nM) (Irving et al., 2013). However, these studies only suggest and do not prove the renal uptake of conjugated curcumin, because the sulfation and glucuronidation may have occurred in the kidneys (section III.C.3.c).

The same may apply to the liver, as hepatocytes contain several organic anion transporters (Kusuhara and Sugiyama, 2009). Accordingly, the liver has been shown to take up glucuronidated compounds such as conjugated bilirubin (van de Steeg et al., 2010), sulfated compounds such as estrone-3-sulfate (Bossuyt et al., 1996; Craddock et al., 1998; Tschantz et al., 2008), and glutathionylated compounds such as *S*-2,4-dinitrophenylglutathione (Hinchman et al., 1993). Whether the liver is capable of taking up conjugated curcumin, however, has never been investigated and cannot be extrapolated on the basis of these substrates. Studies on the uptake of conjugated curcumin by organs/tissues other than the liver and kidneys, including tumor tissue, are also lacking.

Given the relatively high plasma levels of conjugated curcumin compared with native curcumin, detailed information on the pharmacokinetics of sulfated, glucuronidated, and glutathionylated curcumin and the uptake by cancer cells may bear therapeutic relevance. The conjugated curcumin is water soluble and could therefore be administered to patients intravenously at dosages that yield higher blood concentrations than when curcumin is given orally. If the conjugates are sufficiently taken up by cancer cells, as has been shown for various glucuronide adducts of anticancer agents such as doxorubicin (de Graaf et al., 2004; Grinda et al., 2011) and anthracycline prodrugs (Houba et al., 1996; Bakina et al., 1997), the conjugates could be cleaved by intracellular β-glucuronidases and sulfatases (Fig. 12) to yield curcumin and impart a pharmacological effect directly in the tumor cells. Such a mechanism was proposed by Hatcher et al. (2008) to be partly responsible for the anticancer effects of curcumin in vivo, i.e., by the enzymatic back-conversion of endogenously produced curcumin conjugates. Additionally, acute and chronic inflammatory cells in the tumor micro-environment release large quantities of lysosomal β-glucuronidase that cleaves the glucuronide moiety from conjugated compounds in the target tissue (Bosslet et al., 1998), which could further contribute to the pharmacological efficacy of curcumin in some tumors.

4. Pharmacodynamic Implications of Curcumin Metabolites Generated in Biological Systems. As is the case for the pharmacokinetics of biologically formed curcumin metabolites, relatively little is known about the oncostatic properties of these derivatives (Fig. 12 and Table 1), although some experimental evidence is available to support the theory that curcumin works in pharmacodynamic concert with its metabolites. In addition to ferulic acid (sections III.B.1.b and III.B.2.b), which is produced at very low quantities in vivo (Holder et al., 1978), the metabolites that have been investigated for their (potential) anticancer properties include tetrahydrocurcumin, hexahydrocurcumin, octahydrocurcumin, and curcumin glucuronide. With the summary of the biological curcumin metabolites provided in section III.C, it is evident that only a fraction of the known metabolites has been pharmacodynamically investigated. On top of that, the pharmacological importance of the most investigated species, namely nonconjugated reduced curcumin, may be questioned insofar as the reduced isomers are largely conjugated in cells, particularly during first pass (section III.C.2) and second pass metabolism (section III.C.3.b).

a. Pharmacodynamic implications of tetrahydrocurcumin. Of all the metabolites, tetrahydrocurcumin has been studied most extensively for its broad spectrum of biomodulatory properties that affect multiple hallmarks of cancer (section III.D). Tetrahydrocurcumin was shown to exert a deterrent effect on proliferation in four different colon cancer cell lines (Ryu et al., 2008) but

was not toxic to fibroblasts (NIH3T3 cells) at concentrations up to 100 μM (Yodkeeree et al., 2008), exhibited mild inhibition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in 7,12-dimethylbenz[*a*]anthracene-initiated skin cancers in mice (Huang et al., 1995), and considerably reduced the number of aberrant crypt foci in murine 1,2-dimethylhydrazine dihydrochloride-initiated (Kim et al., 1998) as well as azoxymethane-initiated colons (Lai et al., 2011). The latter effects were in part attributable to anti-inflammatory signaling via decreased levels of COX-2 and inducible nitric oxide synthase (iNOS) as a result of tetrahydrocurcumin-mediated ERK1/2 downregulation (Lai et al., 2011). A mild tetrahydrocurcumin-induced reduction in the mRNA expression and activity of iNOS, which is overexpressed in numerous tumor types (Thomsen et al., 1994, 1995; Cobbs et al., 1995) and sites of inflammation (Ohshima and Bartsch, 1994), was also observed in lipopolysaccharide-stimulated murine macrophages (RAW 264.7 cells). The effects were associated with a decreased activity of lipopolysaccharide-induced NF-κB and IKK1 (Pan et al., 2000), which regulate iNOS gene transcription (Griscavage et al., 1996). However, interferences in these signaling pathways appear to be cell type specific, as tetrahydrocurcumin did not modulate NF-κB or its downstream targets COX-2, cyclin D1, and vascular endothelial growth factor (VEGF) in human chronic myelogenous leukemia (KBM-5) cells (Sandur et al., 2007).

Anti-inflammatory effects were also manifested at the level of arachidonic acid metabolism (section III.E.3), where tetrahydrocurcumin inhibited the release of arachidonic acid from lipopolysaccharide-stimulated RAW 264.7 cells and A23187-stimulated HT-29 cells, PGE_2 and other arachidonic acid metabolites (RAW 264.7), and leukotriene B_4 (RAW 264.7), i.e., trademarks of inflammation and carcinogenesis (Greene et al., 2011), at a potency comparable to curcumin (Hong et al., 2004). Tetrahydrocurcumin also reduced expression levels of PGE_2 in phorbol-12-myristate-13-acetate-stimulated human colonic epithelial cells (Ireson et al., 2001). Another enzyme in this metabolic pathway, 5-lipoxygenase, that is involved in inflammation, cancer cell proliferation, resistance to apoptosis, and angiogenesis (Ghosh and Myers, 1998; Steele et al., 1999; Romano and Claria, 2003; Chen et al., 2006d) was also inhibited by tetrahydrocurcumin, albeit at fourfold higher IC_{50} values than curcumin (Hong et al., 2004). In addition to curtailing the cancer cell's resistance to apoptosis through 5-lipoxygenase, tetrahydrocurcumin is capable of stimulating autophagic cell death in human leukemia cells (HL-60) by downregulating PI3K/3-phosphoinositide-dependent protein kinase 1 (PDK1)/Akt and ERK1/2- and c-Jun N-terminal kinase (JNK)1/2 MAPK signaling and by inhibiting the phosphorylation of mammalian target of rapamycin (mTOR), its downstream effector p70 ribosomal protein S6 kinase, and GSK-3β (Wu et al., 2011). Tetrahydrocurcumin also inhibited cell proliferation in these cells (Wu et al., 2011) as well as in HepG2 cells (Yoysungnoen et al., 2008) and reduced the degree of angiogenesis in xenografted HepG2 tumors (Yoysungnoen et al., 2008) and human melanoma (B16F-10) tumors in mice (Leyon and Kuttan, 2003). The latter concurred with a decrease in serum •NO and TNF-α concentrations (Leyon and Kuttan, 2003).

Furthermore, tetrahydrocurcumin decreased Wnt-1 and β-catenin protein levels in chemically initiated mouse colons (Lai et al., 2011) as well as in human embryonic kidney cells (Ryu et al., 2008) and reduced the extent of GSK-3β phosphorylation in colonic tissue (Lai et al., 2011). These proteins play an important role in cell proliferation, differentiation, and oncogenesis (Wodarz and Nusse, 1998). Concurrently with inhibiting Wnt-1/β-catenin signaling, tetrahydrocurcumin significantly reduced protein levels of connexin-43 (Lai et al., 2011), a gap junctional intercellular communication protein that is in part regulated by the GSK-3β/β-catenin signaling pathway (Xia et al., 2010) and that has been implicated in breast cancer lymph node metastases (Kanczuga-Koda et al., 2006), non-small cell lung cancer (Jinn and Inase, 2010), endometrioid adenocarcinomas (Wincewicz et al., 2010), and skin carcinogenesis (Kamibayashi et al., 1995).

With respect to metastasis, tetrahydrocurcumin was shown to inhibit the invasion and migration of human fibrosarcoma (HT1080) cells through Matrigel matrices in a concentration-dependent manner, owing to a reduced secretion of extracellular matrix degradation proteins MMP-2, MMP-9, and urokinase-type plasminogen activator (uPA) (Yodkeeree et al., 2008) (section III.E.1.e). The decreased release of MMP-2 was attributable to tetrahydrocurcumin's downregulation of protein levels of tissue inhibitor of metalloproteinases-2 and membrane type 1 MMP, which are responsible for extracellularly activating MMP-2 from its zymogen state (Sato et al., 1994; Butler et al., 1998; Visse and Nagase, 2003).

Several studies have demonstrated that curcumin is capable of inhibiting various ABC transporters (Wortelboer et al., 2003, 2005; Shukla et al., 2009), which is likely to affect the transport kinetics of curcumin and its metabolites in, e.g., enterocytes and hepatocytes, because these cells are replete with multiple ABC transporters (section III.C.2.f). Cancer cells also overexpress multiple ABC transporters (Brozik et al., 2011) that confer multidrug resistance (Tiwari et al., 2011). Some of these transporters in cancer cells, including P-gp, BCRP, and MRP1 (Brozik et al., 2011), are also capable of transporting curcumin and curcumin metabolites (section III.C.2.f). Tetrahydrocurcumin significantly inhibits the transport functionality of P-gp, BCRP, and MRP1 by directly binding to the proteins, but without being transported itself, and reverses the MDR phenotype

in human cervical carcinoma cells (KB-V-1), BCRP-transfected breast cancer cells (MCF-7), and MRP1-transfected human embryonic kidney cells (HEK 293) (Limtrakul et al., 2007). Consequently, tetrahydrocurcumin may act as a chemosensitizer that enhances the intracellular retention of curcumin and its derivatives, thereby augmenting their pharmacological efficacy.

Lastly, in section III.B.2.c the relationship between oxidative stress, inflammation, mutagenesis/carcinogenesis, and metastasis was addressed in the context of the anticancer properties of the curcumin metabolites generated in vitro, some of which retain their capacity to scavenge radicals and thus interfere in cancer development and spreading. The antioxidant capacity of curcumin is not affected by reduction, as tetrahydrocurcumin is a potent antioxidant. In streptozotocin-nicotinamide-induced diabetic rats, per gavage dosing of tetrahydrocurcumin (80 mg/kg/day) resulted in a significant decrease in hepatic and renal oxidative stress, as evidenced by a decrease in the amount of formed hydroperoxides and thiobarbituric acid reactive substances (Murugan and Pari, 2006a,b). The main oxidants that are neutralized by tetrahydrocurcumin are those responsible for lipid peroxidation, such as alkoxyl and peroxyl radicals, as was revealed by *tert*-butyl hydroperoxide-induced lipid peroxidation assays in erythrocyte ghost membranes (Sugiyama et al., 1996). Specific antioxidant inhibitors (SOD and catalase, mannitol and DMSO as •OH scavengers, DABCO as $^1O_2$ scavenger, and DTPA as transition metal chelator) did not prevent lipid peroxidation in erythrocyte membrane ghosts. Similar results were obtained in linoleic acid oxidation experiments and in rat liver liver microsomes (Osawa et al., 1995). Moreover, all studies on the antioxidant capacity of tetrahydrocurcumin revealed that tetrahydrocurcumin is a stronger antioxidant than curcumin (Osawa et al., 1995; Sugiyama et al., 1996; Murugan and Pari, 2006a,b) and that, just as vanillin and ferulic acid (section III.B.2.c), tetrahydrocurcumin enhances the antioxidant milieu by increasing (or deterring a decrease in) the activity of numerous endogenous antioxidant systems, including SOD, catalase, glutathione peroxidase, GST, GSH, ascorbic acid, and α-tocopherol (Murugan and Pari, 2006a). Interestingly, methylation of both phenylic hydroxyl groups of tetrahydrocurcumin produced the same oxidation products as tetrahydrocurcumin, suggesting that the enolic hydroxyl group is responsible for the antioxidant properties of tetrahydrocurcumin (Sugiyama et al., 1996). Taken altogether, these data suggest that tetrahydrocurcumin may act as a chemopreventive agent through its antioxidant capacity.

b. Pharmacodynamic implications of hexahydrocurcumin. Hexahydrocurcumin exhibits a similar pharmacodynamic profile as tetrahydrocurcumin, which is not surprising given the fact that the reduction of the last alkene in the methine bridge (at the C_α) has no bearing on intermolecular interactions (section II.A). As tetrahydrocurcumin, hexahydrocurcumin was shown to reduce the number of dimethylhydrazine-induced aberrant crypt foci in rat intestines more effectively than curcumin (Srimuangwong et al., 2012a). The neoplasm-reducing effects were accompanied by a reduction in COX-2 expression, but not COX-1 expression, as well as induction of apoptosis in the epithelial cells in the focal crypts, which are usually resistant to apoptosis. In vitro studies in HT-29 cells corroborated that hexahydrocurcumin reduces cell viability and causes a decrease in COX-2 mRNA and protein levels (Srimuangwong et al., 2012b). A reduction in mRNA expression and activity of iNOS was also observed in lipopolysaccharide-stimulated RAW 264.7 macrophages incubated with hexahydrocurcumin, which was associated with a decreased activity of NF-κB and IKK1 (Pan et al., 2000). Hexahydrocurcumin also interferes with arachidonic acid metabolism, as it reduces protein levels of PGE_2 in phorbol-12-myristate-13-acetate-stimulated human colonic epithelial cells at equal magnitude as tetrahydrocurcumin (Ireson et al., 2001). Moreover, incubation of human colorectal cancer (SW480) cells with hexahydrocurcumin led to extensive G_1/G_0 cell-cycle arrest (Chen et al., 2011) (section III.E.1.c), although these effects could not be reproduced in other cancer cell types (Ishikawa cells) (Dempe et al., 2008).

c. Pharmacodynamic implications of other biological curcumin metabolites. In addition to tetrahydrocurcumin and hexahydrocurcumin, only two other curcumin metabolites have been pharmacologically investigated in a very limited number of studies, i.e., octahydrocurcumin and curcumin glucuronide. For octahydrocurcumin a different bonding behavior may be expected because the H-bond accepting β-ketone is transformed to an H-bond donating enol after reduction (section II.A.1). However, the pharmacodynamic properties of octahydrocurcumin mirror those of tetra- and hexahydrocurcumin in that octahydrocurcumin was shown to inhibit the extent of lipopolysaccharide-induced iNOS activation and protein levels in RAW 264.7 cells, which coincided with a reduction in NF-κB and IKK1 activity (Pan et al., 2000).

Curcumin has further been shown to form reactive glucuronide adducts during phase II metabolism that inhibited microtubule assembly in a cell-free polymerization system (Pfeiffer et al., 2007). The inhibition of microtubule assembly was only facilitated by curcumin glucuronide but not hexahydrocurcumin glucuronide (Pfeiffer et al., 2007), suggesting that the association between curcumin glucuronide and microtubule proteins occurs via a Michael addition (section II.A.7). Michael addition-mediated bonding is not in conflict with the reactivity criteria for curcumin glucuronide-protein interactions, namely that the phenylic hydroxyl group (the preferred site for glucuronidation) must be in the para-position relative to the methine bridge and

that the methine bridge must comprise a conjugated system (Pfeiffer et al., 2007), given that a Michael addition shares the second criterion for this reaction to proceed at the sterically unhindered β-enol. It is well-established that the chemical reactivity of glucuronidated nonsteroidal anti-inflammatory drugs (Bailey et al., 1998; Pfeiffer et al., 2007) and other drugs such as valproic acid (Cannell et al., 2002) toward microtubular proteins causes the proteins to lose their ability to form microtubules. Covalent modification of other biomolecules, including albumin, dipeptidyl peptidase IV, and UGTs by glucuronidated metabolites has also been reported (Bailey and Dickinson, 2003). Consequently, glucuronidated curcumin may affect the structure and functionality of other proteins via this mechanism, although this needs to be confirmed experimentally.

5. Pharmacodynamic Implications of Curcumin Metabolites: Final Considerations. In light of the extensive formation of curcumin degradation products (in vitro) and metabolites (in vivo), some of which exhibit anticancer activity, there are several important considerations that should be accounted for in curcumin studies. First, given the rapid alkaline hydrolysis of curcumin under culture conditions, it is advisable that cytotoxicity testing and/or determination of LC_{50} or IC_{50} values are also performed for the degradation products (Fig. 6B), at least the most important ones. The results can then be corrected for the effects ascribable to the curcumin metabolites to ascertain the efficacy of curcumin alone. This is important when long incubation times are used (Supplemental Table 1), particularly in light of the fact that a weak but significant negative correlation exists between incubation time and LC_{50} values (Supplemental Fig. 6). In analogy, many different healthy and cancerous cells have the capacity to metabolize xenobiotics, as a result of which reduced and conjugated curcumin (derivatives) should ideally also be included in the experiments as proper controls. Second, experimental results may vary between different cancer cell lines derived from one type of cancer, between different cancer cell lines regardless of anatomic origin (as illustrated in Fig. 10), and between cells extracted from different species. These differences could be very subtle. For example, the enzymatic conversion of hexahydrocurcumin to octahydrocurcumin occurs more extensively in rat hepatocytes than in human hepatocytes (Ireson et al., 2001).

Third, considerable differences in experimental results obtained in animals versus humans may apply, so caution should be exercised when data from animal studies are extrapolated to the clinical setting. The best illustration of these differences is provided by Ireson et al. (2002): "The extent of sulfation of curcumin in the cytosol of human intestinal tissue was four times that in rat intestine, whereas in human liver cytosol, it was only a fifth of that observed in rat liver cytosol. Curcumin sulfation was 3-fold higher in cytosol from human intestine than in that from human liver, whereas in rats, intestinal sulfation was only a seventh of that in the liver. Microsomal metabolism of curcumin generated as much as 16 times more curcumin glucuronide in the intestine of humans than in the equivalent tissue in rats, but human liver microsomes generated only a third of the amount of curcumin glucuronide found in rat liver microsomes. Microsomal glucuronidation of curcumin in human intestine exceeded that seen in the liver by a factor of 2.5, whereas in rats, the amount of curcumin glucuronide formed in the intestine was only a 60th of that measured in the liver." Moreover, sex differences may exist in regard to curcumin pharmacokinetics, because considerable differences in the reduction of curcumin have been found between male and female rats (Hoehle et al., 2006). Also, significant interspecies differences exist with respect to xenobiotic transport kinetics. For instance, differences in P-gp transport activity have been described for humans versus animals, which are also substrate-dependent (Lin and Yamazaki, 2003).

Finally, differences have been found between curcumin levels in cancerous tissue versus the respective healthy tissue. In colorectal cancer patients, the concentration of curcumin in malignant colorectal tissue of patients who received daily oral doses of curcumin for 1 week was 34–61% lower than in normal colorectal tissue (Garcea et al., 2005), indicating that the pharmacokinetics of curcumin in healthy tissue cannot directly be extrapolated to the targeted malignancies.

In the final analysis, numerous factors should be taken into account when designing experiments with curcumin so as to maximize the accuracy, representability, and extrapolatability of the data.

Although curcumin has received some "bad press" regarding its chemical stability and pharmacokinetic attributes in the preceding sections, curcumin's anticancer properties, particularly in vitro, make up for its chemical and pharmacokinetic issues *as long as* the compound reaches the target tissue at a sufficiently high concentration and in its native chemical, pharmaceutically active state (section II.C.2.a.i). In the following sections, the molecular basis for the tumoricidal mechanisms of curcumin are addressed in the context of the hallmarks of cancer, whereby it was assumed that curcumin is either delivered to or taken up by cancer cells with its molecular structure intact. The many biomolecules affected by curcumin notwithstanding (Johnson and Mukhtar, 2007; Anand et al., 2008; Gupta et al., 2011), the focus of this review is only on the direct curcumin-target molecule interactions that have been empirically proven beyond reasonable doubt.

D. Hallmarks of Cancer

The complex nature of cancer cells has been thoroughly studied over the last few decades,

encompassing an intricate network of (bio)molecules and signaling cascades. In an effort to consolidate the wealth of experimental and clinical data into a coherent conceptual framework, Hanahan and Weinberg (2000, 2001) categorized the phenotypical manifestations of cancer cells according to eight hallmarks that symbolize the divergence between benign and malignant cellular behavior. A slightly modified version of the hallmarks, indicated in Roman numerals with a prefix "H," is provided below.

The chief characteristic of a cancer cell is the ability to sustain a state of continuous proliferation (H-I) because of an increase in growth factor availability, overexpression of growth factor receptors, and/or activating mutations in either the growth factor receptor tyrosine kinase domain or the mitotic signaling cascades that operate downstream to growth factor receptor ligation (Samuels et al., 2004; Schubbert et al., 2007; Witsch et al., 2010). These lead to the (trans)activation of numerous signaling pathways [e.g., B-Raf (Dibb et al., 2004), Akt (Manning and Cantley, 2007)] that converge at the nucleus to upregulate the transcription of promitotic genes and, ultimately, direct cell cycle progression and cell division (Lemmon and Schlessinger, 2010).

Healthy tissue counteracts excessive cell proliferation by producing growth inhibitors such as transforming growth factor (TGF) β (Hannon and Beach, 1994) and cell cycle inhibitors (e.g., p16, p19, p19Arf, p21, and p27; section III.E.1.c). Malignant cells, however, are typically insensitive to anti-growth signals (H-II), predominantly because of loss-of-function mutations affecting the *TP53* (Levine and Oren, 2009) and/or retinoblastoma (*RB1*) gene(s) (Knudsen and Knudsen, 2008), which serve as hubs that integrate antiproliferative messages to steer cell cycle arrest and/or apoptosis. Moreover, in regular cells unbridled cell division would expedite cell death due to the accelerated loss of telomeres, which are the protective nucleotide repeats that cap the ends of DNA strands and shorten with each replicative cycle (Blasco, 2005). To circumvent accelerated cell death, malignant cells overexpress telomerase, an enzyme that maintains telomeres at their original length, thus bestowing cancer cells with unlimited replicative potential (H-III) (Artandi and DePinho, 2000, 2010).

Cancer development is additionally accommodated by the immune system, which fails to eradicate (emerging) tumors (H-IV). It has been postulated that cancer development is subject to a form of natural selection, whereby highly immunogenic cells are cleared by the immune system, leaving behind only the cells with low immunogenic potential that can grow uncontested (Dunn et al., 2002). In addition, tumor cells modulate the local cytokine balance to favor a tolerogenic cellular milieu, which is reflected by the low intratumor levels of cytotoxic T lymphocytes and natural killer cells (Grivennikov et al., 2010). The few immune cells that do attempt to induce cancer cell apoptosis are hindered by defective death receptor signaling networks, which fail to translate outside-in signaling by surface receptors such as TRAIL or FAS to actual cell death (Johnstone et al., 2008). This is paralleled by the fact that malignant cells exhibit an imbalance in pro- and anti-apoptotic mediators in favor of the Bcl-2 family of survival proteins (Cory and Adams, 2002, 2005; Chipuk et al., 2010), which means that neoplastic tissue is resistant to both the intrinsic as well as extrinsic pathway of apoptosis (H-V) (Ashkenazi, 2002; Igney and Krammer, 2002; Cotter, 2009).

To fuel the rampant expansion of the incipient tumor, cancer cells reprogram their energy metabolism (H-VI) (Gatenby and Gillies, 2004; Tennant et al., 2010), displaying disproportionally high rates of glucose uptake and (aerobic) glycolysis (Warburg, 1956; Gatenby and Gillies, 2004; Vander Heiden et al., 2009). When the boosted energy supply is insufficient to meet the metabolic demands, the tumor micro-environment establishes novel supply routes in the form of blood vessels, i.e., angiogenesis (H-VII). The so-called "angiogenic switch" (Bergers and Benjamin, 2003) is predominantly driven by myeloid lineage cells that are chemoattracted to the tumor micro-environment (Murdoch et al., 2008). Upon accumulation, these leukocytes release a variety of proteases and chemokines that not only liberate potent angiogenic triggers such as VEGF (Ferrara, 2002), but additionally repress the action of antiangiogenic messengers like IL-12 (Murdoch et al., 2008). Once the proper cellular climate for survival and progression of the primary lesion has been established, invaded myeloid cells assist malignant cells in their efforts to dissociate from the restraints of cell-cell adhesions and extracellular matrix networks (Joyce and Pollard, 2009). In doing so, cancer cells ultimately disseminate throughout the body to invade and colonize remote organs, i.e., metastasize (H-VIII) (Bamboat et al., 2009; Nurnberg et al., 2011).

The eight aforementioned deviations from normal cellular behavior not only provide a phenotypical cross-section of the cancer cell, but also identify a set of molecular targets that can be exploited in the development and evaluation of novel chemotherapeutic compounds.

E. Direct Molecular Targets of Curcumin

1. ErbB Family of Receptors. The ErbB family of receptors consists of four members: ErbB-1 [epidermal growth factor receptor (EGFR), HER1], ErbB-2 (HER2/neu), ErbB-3 (HER3), and ErbB-4 (HER4). Structurally, all ErbB proteins are transmembrane peptides containing extracellular cysteine-rich ligand binding domains and an intracellular domain that contains a tyrosine kinase. Upon EGF binding, the ErbB proteins form homo- or heterodimers, which leads to

autophosphorylation at the tyrosine residues in the kinase domain and induction of intracellular signaling (Yarden and Sliwkowski, 2001; Avraham and Yarden, 2011). Although structurally similar, the ErbB receptors are not functionally redundant. ErbB-1 and ErbB-4 are autonomous and bind mainly mutually exclusive ligands (Jones et al., 1999), whereas ErbB-3 binds the same ligands as ErbB-4 but lacks kinase activity (Guy et al., 1994). Conversely, ErbB-2 has no ligand binding properties but amplifies the kinase activity through the formation of heterodimers with activated ErbB family members (Klapper et al., 1999).

ErbB proteins are known to activate Ras, which in turn activates Raf (stimulation of cell division), PI3K (survival, cell division), and PKC (resistance to apoptosis, cell motility, angiogenesis) (Downward, 2003) (section III.E.2). Attributable to the stimulatory effect of ErbB-1 signaling on several cancer hallmarks, cancer cells typically evolve means to exacerbate the impact of the ErbB-1 signaling. This includes the overexpression, evolutionary acquisition of constitutively activating mutations within ErbB proteins (Gorgoulis et al., 1992; Wong et al., 1992; Irish and Bernstein, 1993; Moscatello et al., 1995; Uribe and Gonzalez, 2011), and overproduction of ErbB-binding growth factors other than EGF, i.e., TGFα and neuregulin 1 (Yamanaka et al., 1993; Salomon et al., 1995; Scher et al., 1995; Krane and Leder, 1996). Accordingly, monoclonal antibodies against ErbB-2 (trastuzumab) and ErbB-1 (cetuximab) as well as ErbB-1 tyrosine kinase inhibitors (gefitinib and erlotinib) have been developed and approved for cancer treatment (Hynes and MacDonald, 2009).

a. Interaction between curcumin and ErbB. With the use of [^{3}H] labeling, Jung et al. (2007) established that [^{3}H]-curcumin binds covalently to ErbB-2. Two potential Michael reaction acceptor sites in curcumin's methine bridge are responsible for the curcumin-ErbB-2 interaction inasmuch as the substitution of [^{3}H]curcumin by [^{3}H]tetrahydrocurcumin, which lacks the conjugated alkenes between the methoxyphenyl and the β-diketo moiety required for a Michael reaction (section II.A.7), abrogated the binding. Through the deletion of various cytosolic ErbB-2 domains, it was found that curcumin binds to the tyrosine kinase domain of ErbB-2, which causes ubiquitination by C terminus of HSC70-interacting protein, also known as CHIP, and subsequent receptor degradation (Jung et al., 2007). Proteasomal inhibitors were unable to prevent ubiquitinated ErbB-2 degradation, indicating that curcumin-induced degradation proceeds in a proteasome-independent manner (Tikhomirov and Carpenter, 2001; Jung et al., 2007). Corroboratively, Hong et al. (1999) also found that curcumin-induced ErbB-2 degradation is accompanied by reduced kinase activity, decreased association between ErbB-2 and its chaperone GRP94, and an increased cytoplasmic localization of ErbB-2 versus its plasma membrane-bound state. These events concurred with considerably retarded cell growth in ErbB-2 overexpressing human breast cancer cells after curcumin treatment (Hong et al., 1999).

Numerous studies on the effects of curcumin on ErbB signaling found reduced protein levels of ErbB-1 (Chen and Xu, 2005; Chen et al., 2006a; Patel et al., 2008; Somers-Edgar et al., 2008; Thangapazham et al., 2008b; Majumdar et al., 2009; Giommarelli et al., 2010; Lee et al., 2011a; Nautiyal et al., 2011a), ErbB-2 (Hong et al., 1999; Ohori et al., 2006; Thangapazham et al., 2008b; Majumdar et al., 2009; Nautiyal et al., 2011a), and ErbB-3 (Majumdar et al., 2009; Nautiyal et al., 2011a), as well as reduced phosphorylation of ErbB proteins (Chen and Xu, 2005; Reddy et al., 2006; Somers-Edgar et al., 2008; Khafif et al., 2009; Majumdar et al., 2009) after incubation of cells with curcumin. It should be noted that the effects of curcumin on ErbB levels and function varied per cancer cell type (Squires et al., 2003; Kim et al., 2006; Reddy et al., 2006; Lev-Ari et al., 2007; Khafif et al., 2009; Lin et al., 2009). Interestingly, protein levels of insulin-like growth factor 1 receptor, which contains a similar tyrosine kinase domain as the ErbB receptors, were also found to be reduced by curcumin (Reddy et al., 2006; Majumdar et al., 2009; Nautiyal et al., 2011a), as were protein levels of VEGF receptor (VEGFR)1 (Somers-Edgar et al., 2008).

Because the tyrosine kinase domain is essential for downstream signaling of ErbB-1, ErbB-2, and ErbB-4 (but not ErbB-3) and the domain is evolutionary conserved with high sequence homology (Mirza et al., 2010), the covalent binding of curcumin to the kinase domain of ErbB-2 may be applicable to all subtypes of the ErbB family, although direct experimental evidence beyond ErbB-2 is currently not available. Nevertheless, a large body of evidence exists regarding the perturbed downstream signaling pathways of ErbB-1 and ErbB-2 by curcumin, as is discussed below.

b. Sustained proliferation (H-I).

i. Curcumin and the Ras-Raf-MAPK Pathway. After autophosphorylation of the ErbB kinase domain, phosphotyrosine-binding protein GRB2 is recruited to ErbB to act as a scaffold for further signaling by mobilizing polypeptides such as the Son of Sevenless proteins. Son of Sevenless proteins act as a guanine exchange factor for Ras, causing Ras to become activated, after which activated Ras phosphorylates Raf. Raf activates the MAPK/ERK kinases 1 and 2 (MEK1 and MEK2) that in turn activate ERK1 and ERK2 (Kyriakis et al., 1992). ERK1/2 phosphorylates transcription factors of the Ets family as well as the transcription factor Egr-1, culminating in the synthesis of cyclin D (Pruitt and Der, 2001; Downward, 2003). Additionally, ERK1/2 activates Myc and AP-1 (Kolch and Pitt, 2010), causing transcriptional upregulation of *CCND1* that encodes cyclin D1 (Peeper and Bernards,

1997) (Fig. 13). The D-type cyclins are essential for cell division; upon binding to Cdk4/6, the cyclin D-Cdk4/6 complex phosphorylates members of the RB protein (pRB) family that subsequently dissociate from E2F transcription factors, allowing these to initiate transcription of genes required for G_1/S transition and of S phase-promoting genes (Meyerson and Harlow, 1994; Weinberg, 1995; Malumbres and Barbacid, 2001), ultimately resulting in cell proliferation (section III.E.1.c).

Parallel to but less extensive than the ERK1/2 pathway, Raf-activated JNK1 (MAPK8) mobilizes the immediate-early transcription factors Jun and Fos, resulting in increased expression of A- and E-type cyclins and S-phase progression. Jun and Fos drive the transcription of various growth factors (e.g., heparin-binding EGF-like growth factor (HB-EGF), keratinocyte growth factor, VEGF-D) and growth factor receptors (ErbB-1), further sensitizing cells to proliferative signaling (reviewed in Eferl and Wagner, 2003) (Fig. 13).

Curcumin has been implicated in the ErbB-induced Ras-Raf-MAPK pathway. In line with degradation of ErbB-2, protein levels of k-Ras and Raf (Giommarelli et al., 2010) were compromised in human colon cancer and skin cancer cells after incubation with curcumin. Moreover, curcumin was found to inhibit the phosphorylation of MEK and ERK1/2 without affecting total protein levels (Lev-Ari et al., 2007; Zhou et al., 2007; Ji et al., 2008; Khafif et al., 2009). Interestingly, curcumin also reduced the levels of proliferative transcription factors c-Myc (Ohori et al., 2006; Kanwar et al., 2011; Tung et al., 2011), c-Met (Lee et al., 2011a), Egr-1 (Chen et al., 2006a), and Fos and impaired nuclear translocation of Jun (Squires et al., 2003). A possible explanation, albeit not confirmed experimentally, may be the constant degradation of these transcription factors due to curcumin-induced abrogation of upstream signal transduction. Not surprisingly, expression levels of D-, A-, E-, and B-type cyclins were found to be reduced when tumor cell lines were treated with curcumin (Chen and Xu, 2005; Ohori et al., 2006; Cai et al., 2009; Huang et al., 2011; Lee et al., 2011a; Tung et al., 2011). Similar to cyclin D, protein levels of Cdk4 and Cdk1 (which bind to cyclin B to promote M-phase entry, section III.E.1.c) were also depleted after curcumin treatment (Cheng et al., 2010; Giommarelli et al., 2010), providing a rationale for the reduced proliferation rates induced by curcumin in various human cancer cell lines. The absence of cyclin-Cdk complexes by impaired ErbB-1 signaling causes the members of the pRB family to remain dephosphorylated and bound to E2F transcription factors, which need to be released from pRB to become active. Inhibited E2F-induced gene transcription results in cell cycle arrest in G_1 and hence inhibition of cell proliferation.

The curcumin-induced direct inhibition of proliferative signaling through the Ras-Raf-MAPK and JNK1 pathways is further amplified by processes that indirectly inhibit cell proliferation. These processes include curcumin-mediated amelioration of VEGF signaling by means of impaired VEGF production in the prostaglandin synthesis pathway (section III.E.3.c) and curcumin-induced degradation of ErbB-1 produced as a result of Jun/Fos-activated ErbB-1 transcription in the JNK1 pathway (Fig. 13).

ii. Curcumin and the PI3K-Akt Pathway. PI3K is the main trigger for the Akt (protein kinase B) pathway that regulates cell survival, proliferation, and growth (Fig. 14). The p85 subunit of PI3K contains an SH2 domain, so it can be directly activated as a result of ErbB-1 autophosphorylation. Alternatively, PI3K can be activated by Ras (section III.E.1.b.i) via its Ras-binding domain. Upon activation, PI3K converts phosphatidylinositol 3,4-phosphate into phosphatidylinositol 3,4,5-phosphate that recruits and activates Akt (reviewed in Vivanco and Sawyers, 2002). With respect to proliferation, Akt is well known for its stimulatory action on the mTOR complex by phosphorylation-mediated inactivation of its inhibitor, tuberous sclerosis complex 2 (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002). Activated mTOR stimulates ribosomal synthesis through the activation of S6K1 and inhibition of 4E-BP1, thereby increasing the capacity of protein synthesis required for proliferation (Vivanco and Sawyers, 2002; Wullschleger et al., 2006). Synchronously with the Ras-Raf-MAPK signaling pathway (section III.E.1.b.i), Akt further promotes cyclin D activation by inhibiting the expression of GSK-3β, which normally phosphorylates (i.e., inactivates) D-type cyclins to restrict cell-cycle progression (Diehl et al., 1998) (Fig. 14).

The downstream effects of curcumin-ErbB-1 interactions on the PI3K-Akt pathway include diminished Akt protein levels (Patel et al., 2008, 2010; Somers-Edgar et al., 2008; Cheng et al., 2010; Lee et al., 2011a; Nautiyal et al., 2011a) and reduced Akt phosphorylation (Zhou et al., 2007; Ji et al., 2008; Somers-Edgar et al., 2008), although several studies have reported contradictory findings, albeit in different cell lines (Reddy et al., 2006; Lin et al., 2009). Furthermore, protein levels of mTOR, 4E-BP1, S6K1 (Beevers et al., 2006), and GSK-3β (Wu et al., 2011) were reduced after curcumin treatment. Although a causal relationship between curcumin-induced ErbB-1 inhibition and impaired Akt signaling was not directly investigated in some of the cited studies, the synergetic inhibition of the PI3K-Akt and Ras-Raf-MAPK pathways by curcumin-ErbB-1 binding appears to ubiquitously impair the proliferative capacity of cancer cells.

c. Insensitivity to anti-growth signals (H-II). Tumor cells sustain constant and unrestricted proliferation by combining continuous proliferative signaling with resilience against anti-growth signals. Given that cell division is the driving force behind proliferation and

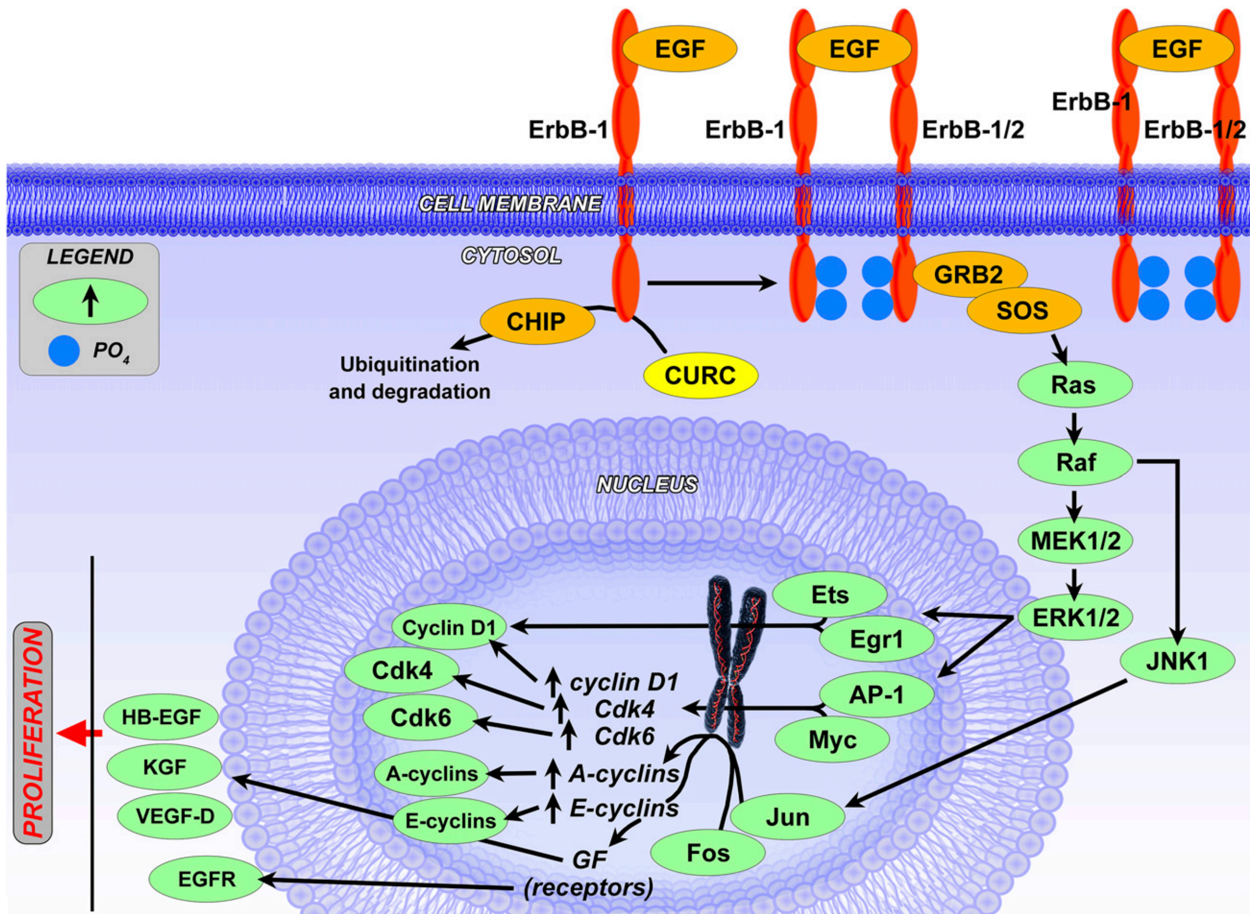


Fig. 13. Ras-Raf-MAPK and JNK1 pathways initiated by the binding of EGF to its cognate receptor ErbB-1 (and possibly the ErbB-2 heterodimer) in relation to cancer cell proliferation (H-I) affected directly through cell cycle regulators and indirectly through growth factors (HB-EGF, keratinocyte growth factor, VEGF-D) and growth factor receptors (ErbB). The molecules in circles represent proteins, whereas the molecules written in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein and blue spheres indicate phosphorylation (legend) under native conditions, i.e., unaffected by curcumin. The molecular pathway relating A/D/E cyclins, Cdk4, and Cdk6 to proliferation is further detailed in section III.E.1.c. Curcumin (CURC) inhibits this pathway by covalent interaction with the tyrosine kinase domain of ErbB-1, leading to the ubiquitination and degradation of ErbB-1 and ultimately reduced cell proliferation. CHIP, C terminus of Hsc70-interacting protein; Egr1, early growth response protein 1; Ets, E-26 transformation-specific or E-26; GRB2, growth factor receptor-bound protein 2; KGF, keratinocyte growth factor; SOS, Son of Sevenless.

the cell cycle dictates the advancement of cell division, most anti-growth signals culminate in the activation of inhibitors of cyclin/Cdk complexes inasmuch as dimerization of cyclins and their Cdk counterparts are rudimentary to the progression of the cell cycle. Cyclin D-Cdk4/6 complexes stimulate the G_1/S transition by phosphorylating members of the RB family that consequently facilitate E2F-mediated expression of S-phase promoting genes (Meyerson and Harlow, 1994; Weinberg, 1995; Malumbres and Barbacid, 2001). Cyclins E and A are produced in the late G_1- and S-phase, respectively, as a result of E2F activation and bind/activate Cdk2 to promote S-phase progression, DNA replication, and hyperphosphorylation of pRB (Malumbres and Barbacid, 2001; Blagosklonny and Pardee, 2002). In the G_2 phase, cyclin B is produced to bind and activate Cdk1, which mediates M-phase initiation (Vermeulen et al., 2003; Lindqvist et al., 2009) (Fig. 15).

In case of oncogenic stress (i.e., activation of oncogenes such as c-Myc), DNA damage (activation of p53), contact inhibition, and other types of cellular stress, healthy cells activate cyclin kinase inhibitors (CKIs) to inhibit cyclin-Cdk complexes and arrest the cell cycle (Sherr and Roberts, 1999; Malumbres and Barbacid, 2001). Cyclin D-Cdk4/6 complexes are inhibited by CKIs $p15^{INK4B}$, $p16^{INK4A}$, $p18^{INK4C}$, and $p19^{ARF}$ by competitive binding to Cdks 4 and 6, thereby blocking the formation of the cyclin D-Cdk4/6 complex. CKIs $p21^{CIP1}$, $p27^{KIP1}$, and $p57^{KIP2}$ stimulate the formation of cyclin D-Cdk4/6 complexes, but associate with cyclin E/A-Cdk2 and cyclin B-Cdk1 complexes to inhibit their activity (Agami and Bernards, 2002; Chu et al., 2008) and thus deter cell cycle progression to the S- and M-phases, respectively (Fig. 15). Activation of CKIs is context-dependent and relies on intricate signaling networks that regulate cell cycle progression.

There is ample evidence that curcumin induces cell cycle inhibitors pRB, p53, $p16^{INK4A}$, $p21^{CIP1}$, and $p27^{KIP1}$ (Aggarwal et al., 2007; Shankar and Srivastava, 2007; Srivastava et al., 2007; Su et al., 2010;

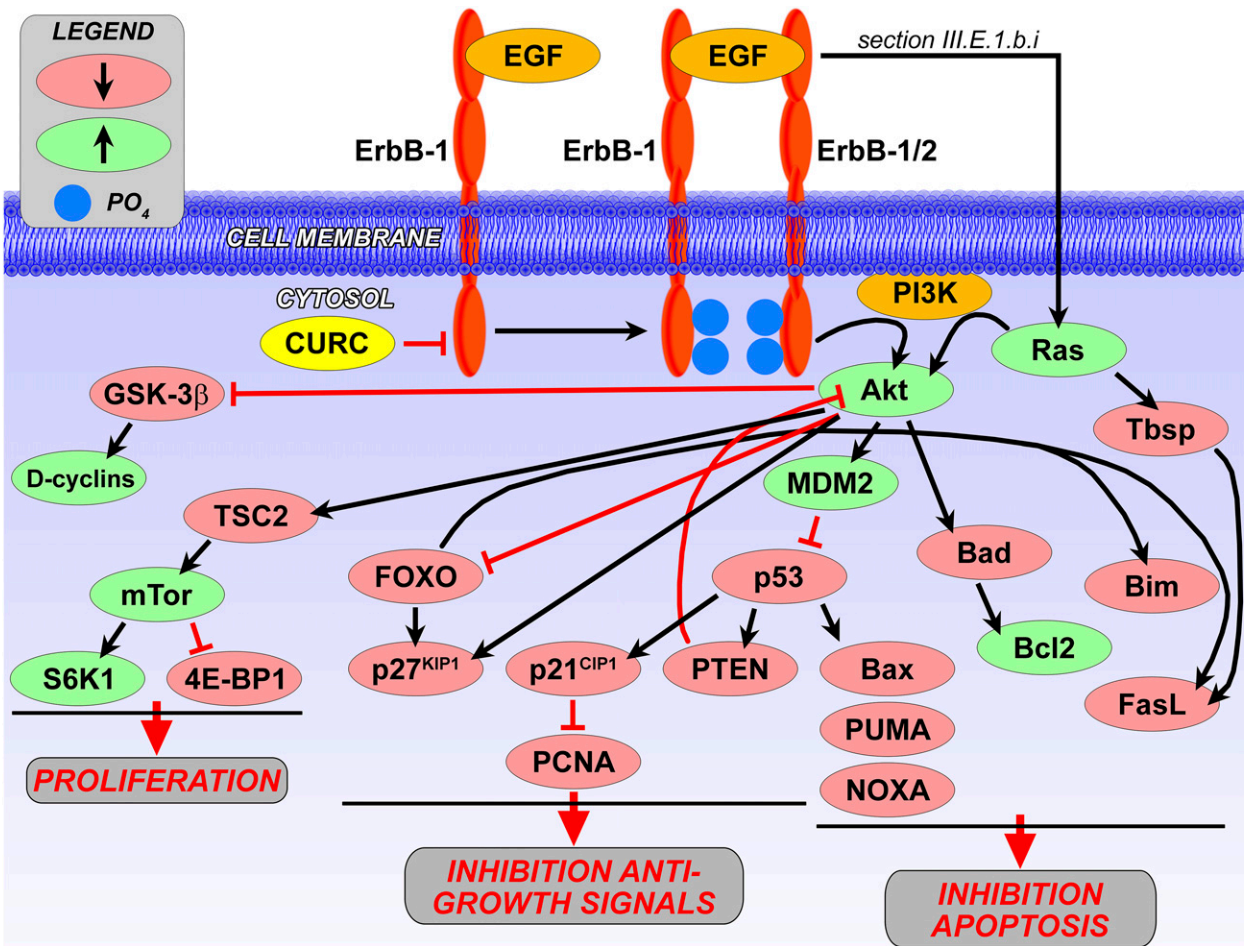


Fig. 14. PI3K-Akt pathway initiated by the binding of EGF to its cognate receptor ErbB-1 (and possibly the ErbB-2 heterodimer) in relation to cancer cell proliferation (H-I), inhibition of antigrowth signals (H-II), and resistance to apoptosis (H-V). The molecules in circles represent proteins. Green circles indicate activation of or translational/expressional increase in the respective protein, red circles indicate deactivation of or translational/expressional decrease in the respective protein, and blue spheres indicate phosphorylation (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits this pathway by covalent interaction with the tyrosine kinase domain of ErbB-1, leading to the ubiquitination and degradation of ErbB-1 and ultimately reduced cell proliferation, sensitization of cancer cells to antigrowth signals, and apoptosis induction. PCNA, proliferating cell nuclear antigen; TSC2, tuberous sclerosis 2.

Veeraraghavan et al., 2010; Gogada et al., 2011). These events can be linked to the ErbB-1-Akt signaling axis. Increased protein levels of pRB may be ascribed to reduced pRB phosphorylation by cyclin-Cdk complexes, resulting in sustained pRB-E2F complexation, sustained inhibition of E2F transcription factors, and inhibition of the S-phase (Fig. 15). With respect to p53 activation, Akt typically phosphorylates murine double minute 2 (MDM2) to degrade p53 so as to ensure cell cycle progression (Mayo and Donner, 2001) (Fig. 14). Curcumin may interfere with this process by restraining ErbB-1-mediated activation of Akt, leading to p53 stabilization and activation in case of genomic or oncogenic stress. Target genes of p53 include phosphatase and tensin homolog (PTEN), the major inhibitor of Akt, and $p21^{CIP1}$ (Levine, 1997), which is able to induce cell cycle arrest in the S- or G_2-phase in the presence of DNA damage by binding and inhibiting proliferating cell nuclear antigen (Fig. 14), thereby stalling DNA replication forks (Abbas and Dutta, 2009).

The production of $p27^{KIP1}$ is induced by various extracellular signals, including contact inhibition, differentiation, and growth inhibitory factors (e.g., TGFβ) (Hengst and Reed, 1996; Liang and Slingerland, 2003; Besson et al., 2004; Chu et al., 2008). Akt is able to inactivate FOXO transcription factors and thereby reduce $p27^{KIP1}$ expression as well as to phosphorylate and inactivate $p27^{KIP1}$ directly to inhibit cell cycle arrest (Brunet et al., 1999; Dijkers et al., 2000) (Fig. 14). Curcumin-mediated inhibition of the ErbB-1-Akt pathway may therefore restore $p27^{KIP1}$ levels in tumor cells treated with curcumin and induce cell cycle arrest.

$p16^{INK4A}$, p53, and $p21^{CIP1}$ are essential for mediating oncogenic stress-induced cell cycle arrest (Serrano et al., 1997; Jacobs and de Lange, 2005) as a result of, e.g., DNA damage or telomere shortening (Bartkova et al., 2006). Interestingly, curcumin not only engages these proteins to induce cell cycle arrest, but it also invokes the conditions (i.e., stress) necessary for the activation of some of these cell cycle inhibitors in

certain cancers. One prime example is the induction of p53 and p16^{INK4A} by curcumin-mediated telomere shortening. Tumor cells typically maintain telomere length by activation of the telomerase subunit hTERT. It was demonstrated that both the expression and nuclear translocation of hTERT is inhibited by curcumin in H1299 human non-small cell lung carcinoma cells (Lee and Chung, 2010), which could lead to telomere shortening and activation of p53 and p16^{INK4A}.

Amplified protein levels of p21^{CIP1} may be a direct result of p53 activation, but there are additional p53-independent mechanisms that are responsible for p21^{CIP1} induction. The promoter region of *CDKN1A* (codes for p21^{CIP1}) contains binding sites not only for p53, but also for breast cancer type 1 susceptibility protein (BRCA1), Ras (section III.E.1.b.i), E2F1 and E2F3 (Fig. 15), vitamin D receptor (VDR) (section III. E.4), STAT transcription factors, and various growth and transcription factors (Abbas and Dutta, 2009). Repression of *CDKN1A* may also be achieved by binding of various Myc-containing complexes to the promoter region of *CDKN1A* (Gartel and Shchors, 2003). The exact mechanism by which p21^{CIP1} is produced as a result of curcumin treatment in cancer cells is largely elusive, because many of the above-mentioned factors are influenced by curcumin. It is clear, however, that curcumin treatment abrogates the cancer cell's insensitivity to anti-growth signals through the absence of stimulators of proliferation (i.e., inhibition of the Ras and Akt pathways), DNA damage, oncogenic stress, and contact inhibition by relieving the inhibitory effect of Akt on multiple tumor suppressor proteins as described in this section.

d. Resistance to apoptosis (H-V). The resistance to apoptosis imparted by ErbB-1 signaling is mostly regulated through the PI3K-Akt pathway (Manning and Cantley, 2007) (section III.E.1.b.ii). Akt phosphorylates pro-apoptotic Bad, causing it to dissociate from anti-apoptotic Bcl2 proteins that are subsequently activated (Datta et al., 1997). The anti-apoptotic effects of Akt-MDM2-mediated inhibition of p53 are actualized by silencing of the p53 target genes Bax, PUMA, and NOXA, which are pro-apoptotic members of the Bcl2 family (Miyashita and Reed, 1995; Oda et al., 2000; Nakano and Vousden, 2001) (Fig. 14). Similarly, the inhibition of FOXO transcription factors by Akt diminishes the expression of Bim (Bcl2l11), another pro-apoptotic member of the Bcl2 family, and of the pro-apoptotic transmembrane protein Fas ligand (FasL) (Brunet et al., 1999; Dijkers et al., 2002) (Fig. 14). In the FasL and Fas receptor pathway, thrombospondin (Tbsp) is inactivated by Ras signaling, which further contributes to the insensitivity to the extrinsic apoptotic pathway as a result of reduced Tbsp levels, resulting in reduced transcription of FasL (Armstrong and Bornstein, 2003) (Fig. 14).

Inhibition of the ErbB-1-PI3K-Akt pathway by curcumin should therefore trigger the production of

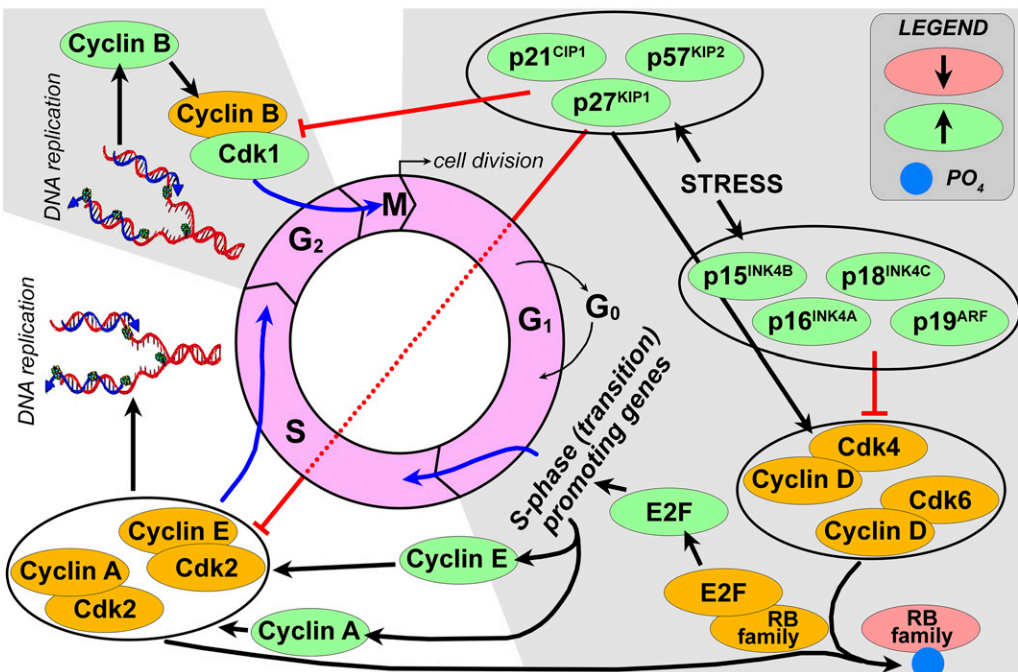


Fig. 15. Cell cycle progression and its regulation by pertinent signaling proteins (ovals, as in legend). Green circles indicate activation of or translational/expressional increase in the respective protein, red circles indicate deactivation of or translational/expressional decrease in the respective protein, and blue spheres indicate phosphorylation (legend) under native conditions, i.e., unaffected by curcumin. With the exception of the stress-induced transcription factors, the molecular events are categorized according to the phase in which they occur (G_0, G_1, and G_2 phases correspond to gray background, S and M phases correspond to white background). Note that the RB family of proteins comprises pRB, p107, and p130. Inhibition is designated as ⊣.

the aforementioned pro-apoptotic proteins and stimulate tumor cells to undergo apoptosis. Accordingly, increased apoptosis of cultured tumor cells is almost always observed in studies with curcumin. Increased protein levels of pro-apoptotic Bak (Shankar and Srivastava, 2007; Jin et al., 2009), Bax (Chendil et al., 2004; Shankar and Srivastava, 2007; Jin et al., 2009; Veeraraghavan et al., 2010; Wu et al., 2010), Bad (Wu et al., 2010), and a concomitant reduction in levels of anti-apoptotic Bcl2 (Chendil et al., 2004; Shankar and Srivastava, 2007; Jin et al., 2009; Wu et al., 2010), Bcl-xl (Veeraraghavan et al., 2010;Wu et al., 2010), Mcl1 (Veeraraghavan et al., 2010), and X-linked inhibitor of apoptosis (Wu et al., 2010) were found in various cancer cell lines. Less extensively studied, but also upregulated were mRNA levels of *Puma* and *Noxa* in curcumin-treated cells, in which p53 was found to be co-upregulated (Shankar and Srivastava, 2007). Differences in specific Bcl2 protein family member levels after incubation with curcumin are most likely attributable to cell type differences and phenotype-related differences (e.g., some cancer cell types amplify anti-apoptotic Bcl2 family proteins, whereas other cancer cell types delete or downregulate pro-apoptotic Bad, Bax, etc), although curcumin treatment almost ubiquitously culminates in apoptosis induction by the production of pro-apoptotic proteins and downregulation of anti-apoptotic proteins, thus sensitizing cancer cells to programmed cell death.

e. Angiogenesis (H-VII) and metastasis (H-VIII). The curcumin-mediated degradation of ErbB-1 and the consequential reduction in Ras signaling inhibits tumor cell development and progression by the pathways described above but also by inhibiting angiogenesis and reducing the tumor's metastatic potential. The ErbB-1-Akt-Ras pathway stimulates angiogenesis by its proliferative effect in endothelial cells (ECs) (Downward, 2003), but also by the induction of NF-κB (Fig. 16). Activated NF-κB induces COX-2 and PG synthesis (sections III.E.3.a and III.E.3.c), the production of proangiogenic growth factors VEGF, platelet-derived growth factor, and basic fibroblast growth factor (bFGF) (Rak et al., 1995), as well as the production and release of chemokines and cytokines such as several chemokine (C-X-C motif) ligand (CXCL) isotypes, IL-6, and IL-8, which trigger angiogenesis and extracellular matrix (ECM) remodeling (Sparmann and Bar-Sagi, 2004; Ancrile et al., 2008).

To facilitate vessel sprouting and migration of tumor cells (metastasis), modification of the ECM is a necessity. Ras signaling facilitates ECM remodeling by inducing MMP-9 (section III.E.2.b), MMP-2, and uPA (Pepper, 2001; Kranenburg et al., 2004) (Fig. 16). Metastasis is further potentiated by the abrogation of cell-cell adhesions through ErbB-1-Ras-mediated negative regulation of E-cadherin (Schmidt et al., 2005; Horiguchi et al., 2009). In addition, ErbB-1 can induce Rho to impart actin cytoskeleton rearrangements required for cell motility and migration (Ridley, 2001; Sahai and Marshall, 2002) (Fig. 16).

With respect to ErbB-1-induced angiogenesis, curcumin was found to reduce mRNA and protein levels of VEGF (Shao et al., 2002; Kunnumakkara et al., 2009; Sun et al., 2011; Tung et al., 2011) and bFGF (Shao et al., 2002), although these growth factors may not have been regulated exclusively through the ErbB-1-Ras-MAPK pathway but by other curcumin-affected pathways, including the VEGFR-PG pathway (section III.E.3.c) and the interrelated EP2/4-VEGF pathway (section III.E.3.c). In addition, curcumin inhibited NF-κB-induced production of CXCL1 and CXCL2 in breast cancer cells (Bobrovnikova-Marjon et al., 2004), which also may have been caused by its degrading effect on ErbB-1 and corollary suspension of downstream ErbB-1 signaling (section III.E.1.a). Ras signaling downregulates Tbsp (Fig. 14), leading to reduced angiogenesis, although the exact mechanism remains unclear [possible mechanisms are reviewed in Armstrong and Bornstein (2003)]. Curcumin treatment was found to increase levels of Tbsp1 (Zabrenetzky et al., 1994; Rak et al., 2000), which was most likely mediated by reduced Ras signaling due to inhibition of ErbB-1 tyrosine kinase phosphorylation by curcumin (Fig. 13).

In regard to metastasis, proteolytic degradation of the tumor ECM was reported to be inhibited by curcumin, which is likely a downstream consequence of reduced MMP-9 and MMP-2 levels due to curcumin-induced ErbB-1 degradation. This has been observed in multiple studies, although often with varying results depending on the cancer cell line (Shao et al., 2002; Kunnumakkara et al., 2009; Lin et al., 2010; Weissenberger et al., 2010; Sun et al., 2011). uPA levels were also decreased in curcumin-treated cells, in which inhibition of NF-κB activity was identified as the chief culprit (Zong et al., 2012). Similarly, inhibition of tumor metastasis by curcumin has been frequently observed and attributed to the reduction in MMP-2, MMP-9, uPA, and aquaporin 3 protein levels (Ji et al., 2008; Weissenberger et al., 2010; Wong et al., 2010; Soung and Chung, 2011). Although the mechanism is relatively elusive, aquaporins play a role in tumor growth and metastasis (Nico and Ribatti, 2010), during which MMPs are upregulated via the PI3K-Akt pathway (Xu et al., 2011) to facilitate these processes. Corroboratively, curcumin reduced the levels of aquaporin 3 by inhibiting ErbB-1 signaling in human ovarian carcinoma cells, as a result of which the cells were less prone to migrate in vitro (Ji et al., 2008). Moreover, curcumin treatment is associated with increased levels of E-cadherin (Wong et al., 2010), possibly as a result of downregulating its transcriptional repressor SNAIL. SNAIL is induced by the ErbB-1-Ras and Akt pathways (De Craene et al., 2005; Weissenberger et al., 2010), which are inhibited by curcumin (section III.E.1.b.i and III.E.1.b.ii, respectively).

Lastly, tumor cells that were less prone to migrate and metastasize in vitro as a result of curcumin treatment exhibit reduced Rho A activity, often in combination with diminished levels of its downstream targets MMP-2 and MMP-9 (Lin et al., 2010; Calaf et al., 2012).

In summary, the antiangiogenic effects and inhibitory impact on ECM modification by curcumin appear to emanate from its effect on NF-κB, as evidenced by decreased NF-κB protein levels and reduced association with DNA. These originate at the level of ErbB-1 inhibition by curcumin and ultimately translate to a reduced production of proangiogenic proteins and increased synthesis of angiogenesis-inhibiting proteins such as Tbsp. Curcumin further exerts an inhibitory effect on metastasis through ErbB-1 degradation-mediated reduction in Ras and Rho signaling, leading to decreased ECM proteolytic activity and sustained cell-cell adhesion.

2. Protein Kinase C. The PKC family encompasses more than 10 serine/threonine kinases that are classified as conventional (cPKCs: α, βI, βII, and γ), novel (nPKCs: δ, ε, η, and θ), or atypical [aPKCs: ζ and τ (human) or λ (mice)] according to the mechanism of activation and substrate specificity (Mellor and Parker, 1998). Each PKC isoform exhibits a tissue- and cell type-specific expression pattern (Erdbrugger et al., 1997). PKC activity can be driven by G-protein coupled receptors (GPCRs) and VEGFR2 (Mackay and Twelves, 2007), which use Ca^{2+} and/or diacylglycerol as second messengers to activate the PKCs and, as such, induce critical cellular processes including proliferation and survival (Yang and Kazanietz, 2003). Insofar as the GPCR and VEGFR2 signaling pathways play key roles in malignancies (Ferrara, 2002; Dorsam and Gutkind, 2007; Youssoufian et al., 2007), excessive PKC signaling has been linked to the development, progression, and metastasis of malignant tumors (Griner and Kazanietz, 2007). Accordingly, cancer has been traditionally considered a disease state associated with increased PKC activity (O'Brian et al., 1985; Takenaga and Takahashi, 1986). Over the last years, however, it has become apparent that each isoform is entangled in a number of different, often opposing signaling pathways (Griner and Kazanietz, 2007). The oncogenic

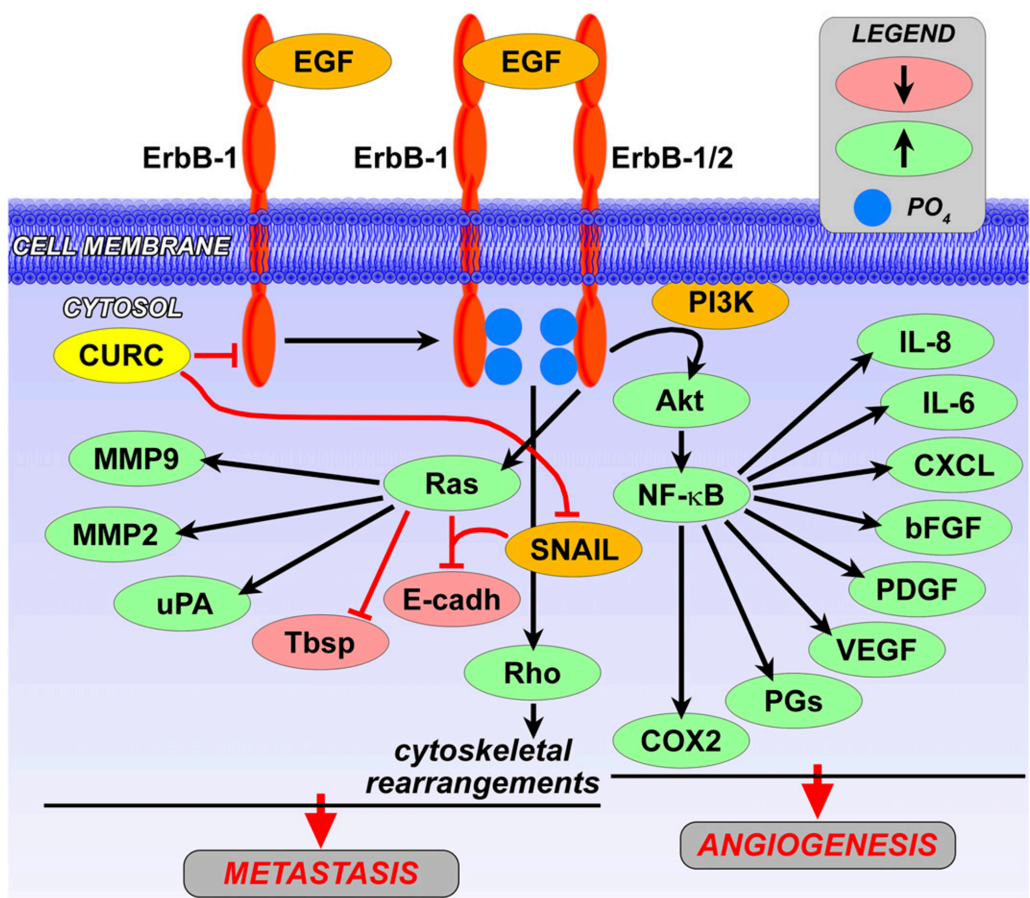


Fig. 16. PI3K-Akt pathways initiated by the binding of EGF to its cognate receptor ErbB-1 in relation to cancer metastasis (H-VII) and angiogenesis (H-VIII). The molecules in circles represent proteins. Green circles indicate activation of or translational/expressional increase in the respective protein, red circles indicate deactivation of or translational/expressional decrease in the respective protein, and blue spheres indicate phosphorylation (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits this pathway by covalent interaction with the tyrosine kinase domain of ErbB-1, leading to the ubiquitination and degradation of ErbB-1 and ultimately reduced cancer metastasis and angiogenesis.

effects of each individual PKC isoform may therefore emanate from either an increase or decrease in activity, which in turn varies per cancer type (Mackay and Twelves, 2007).

Because of these pleiotropic attributes, the efficacy of PKC-targeted therapies is ideally evaluated in accordance with isoform specificity, and in that respect, the first structural insights into (n)PKC-curcumin interaction have recently surfaced. By using isolated PKCδ agonist-binding (C1B) domains, it was shown that curcumin quenched the intrinsic PKC (tryptophan) fluorescence at an IC_{50} of 10.6 μM compared with an IC_{50} of 5.1 μM of the prototypical PKC agonist TPA (Majhi et al., 2010). Molecular docking studies indicated that curcumin forms two H-bonds with the PKCδ C1B domain: one between the phenylic hydroxyl group of curcumin and the Leu251 carbonyl moiety and the second between the phenylic oxygen of curcumin and the Gln257 amide group (Majhi et al., 2010). Molecular docking studies further revealed that curcumin also binds to PKCθ through H-bonding between the phenylic hydroxyl group and Gly254 on the PKCθ C1B domain (Das et al., 2011). Although all nPKCs and cPKCs contain a structurally related C1B domain (Kazanietz et al., 1995; Colon-Gonzalez and Kazanietz, 2006), the slight variations in curcumin binding to nPKC subclass members renders the extrapolation of the results to other PKC isoforms indefinite. Nevertheless, curcumin has been shown to promote apoptosis and to inhibit angiogenesis in cancer cells by modulating PKC signaling.

a. Resistance to apoptosis (H-V).

i. PKCα. The dichotomous nature of PKC signaling becomes evident in the context of cancer cell survival. In hepatoma Hep3B cells (Kao et al., 2011) and leukemia K562 cells (Semsri et al., 2011), curcumin (20 μM and 15 μM, respectively) was found to inhibit PKCα, resulting in the downregulation of Wilms' tumor-associated gene (*WT1*) transcription (Semsri et al., 2011). *WT1* was originally identified as a tumor suppressor gene (Yang et al., 2007b), but WT1 protein (*WT1* product) overactivity has subsequently been implicated in the inception of various cancers (Glienke et al., 2007; Han et al., 2007; Perugorria et al., 2009), predominantly by bolstering cellular resistance to apoptosis (Algar et al., 1996; Tatsumi et al., 2008).

In cancer cells, WT1 drives the transcription of the anti-apoptotic *Bcl2* (Mayo et al., 1999) and Bcl-2-associated athanogene 3 (*BAG3*) (Cesaro et al., 2010) genes by binding to the respective promoter regions (Fig. 17). BAG3 operates in concert with the molecular chaperone heat-shock protein 70 (Hartl et al., 2011), together positively affecting the survival of cancer cells by binding to various pro- and anti-apoptotic mediators (Fig. 17) (Gamerdinger et al., 2009; Rosati et al., 2011). First, cytosolic BAG3 binds the pro-apoptotic protein BAX, thereby preventing the mitochondrial translocation of BAX required for apoptosis induction (Festa et al., 2011). Second, BAG3 stabilizes the anti-apoptotic proteins Bcl-xl, Mcl-1, and Bcl-2, rescuing them from proteasomal degradation and retaining them at the mitochondrial outer membrane to preserve mitochondrial integrity (Cory and Adams, 2002; Jacobs and Marnett, 2009). Lastly, BAG3 prevents proteolysis of IKKγ, which is an activator of NF-κB that predominantly conveys prosurvival messages (Ammirante et al., 2010; Ben-Neriah and Karin, 2011). Concomitantly with the BAG3 pathway (Fig. 17), curcumin-mediated inhibition of PKCα signaling could disrupt cell survival signaling by abrogating the transcriptional repression of c-Myc by WT1 (Hartkamp et al., 2010). The transcription factor c-Myc is known to promote apoptosis by activating BAX (Eischen et al., 2001b; Soucie et al., 2001), by suppressing the formation of the anti-apoptotic proteins Bcl-2 and Bcl-xl (Eischen et al., 2001a,c), and/or by upregulating transcription of the functionally linked (Zhang et al., 1998; Sherr, 2006) tumor suppressors $p14^{ARF}$ and p53 (Fig. 17) (Hermeking and Eick, 1994; Zindy et al., 1998). The inhibitory effect of curcumin on PKCα thus shifts the balance between pro- and antisurvival mediators toward the latter, thereby sensitizing malignant cells to programmed cell death.

ii. PKCδ. In contrast to PKCα, PKCδ activity was shown to be increased by curcumin (10 μM), resulting in apoptosis of HCT116 human colon cancer cells (Scott and Loo, 2004, 2007). The observed effects probably stem from curcumin's ability to perturb the intracellular redox equilibrium (Domenicotti et al., 2003), leading to PKCδ mobilization by either direct oxidation of the enzyme's active site (Gopalakrishna and Anderson, 1989) or via sequential activation of the redox-sensitive PI3K (Niwa et al., 2003) and PKD1 (Fig. 17) (Le Good et al., 1998; Ziegler et al., 1999). Curcumin increases intracellular oxidative stress by intensifying the production of ROS (Ahsan and Hadi, 1998; Bhaumik et al., 1999), in part by alkylating the antioxidant TrxR to yield an enzyme with NADPH oxidase activity (Fang et al., 2005). Concomitantly, curcumin diminishes the cellular antioxidant reservoir [i.e., protein thiols (Sies, 1999; Requejo et al., 2010)] by covalently scavenging GSH (Wortelboer et al., 2003; Scott and Loo, 2007), thereby buttressing a pro-oxidative milieu.

In HCT-116 cells treated with curcumin (10 μM), PKCδ directs apoptosis via the leucine zipper transcription factor growth arrest and DNA damage-inducible gene 153 (GADD153, also known as C/EBP homologous protein) (Scott and Loo, 2004). GADD153 induces apoptosis by transcriptionally downregulating Bcl-2 (McCullough et al., 2001), which is paralleled by an increase in generation of the pro-apoptotic BH3-only proteins Bim (Puthalakath et al., 2007; Selimovic et al., 2011) and PUMA (Li et al., 2006; Um et al., 2011). It has been reported that GADD153 concurrently

coordinates cell death by activating ER oxidase 1α (Marciniak et al., 2004), which provokes a pro-oxidative environment in the ER membrane as well as the cytoplasm (Malhotra et al., 2008; Li et al., 2010), both culminating in cell death (Tabas and Ron, 2011). Curcumin's ability to induce a pro-apoptotic phenotype via elevated PKCδ signaling is supported by the fact that PKCδ is typically downregulated in malignancies, including brain cancer (Mandil et al., 2001), bladder cancer (Varga et al., 2004), and squamous cell carcinoma (Yadav et al., 2010b).

b. Angiogenesis (H-VII). The proliferation and advancement of various gastrointestinal cancers, including colon carcinoma and pancreatic duct adenocarcinoma, is promoted by the hormone neurotensin (Reubi et al., 1998; Maoret et al., 1999), which is released by the gut epithelium in response to the intake of fat (Ferris et al., 1985). Since these cancers overexpress high affinity neutrotensin receptors (Reubi et al., 1998; Maoret et al., 1999), the downstream signaling networks are typically hyperactivated. Ligation of the neurotensin receptor first confers a rise in cytosolic Ca^{2+}, leading to activation of the Ca^{2+}-sensitive cPKCs (Warhurst et al., 1994; Guha et al., 2003). Activated cPKCs in turn phosphorylate c-Raf-1 at multiple serine residues (Kolch et al., 1993; Carroll and May, 1994) to consecutively activate MEK1/2 and ERK1/2 (Guha et al., 2003; Kisfalvi et al., 2005). Activated ERK1/2 in turn phosphorylates the carboxy-terminal transactivation domain of the AP-1 component c-Fos (Murphy et al., 2002; Monje et al., 2003, 2005), resulting in the formation of functional AP-1 dimers (Frost et al., 1994; Watts et al., 1998; Ryder et al., 2001). The transcription factor AP-1 is known to encode a plethora of cancer-pertinent genes that relay mitogenic (section III.E.1.b), anti-apoptotic (section III. E.1.d), and proangiogenic signals (section III.E.1.e) (Eferl and Wagner, 2003; Roux and Blenis, 2004; Lopez-Bergami et al., 2010) (Fig. 18).

In human HCT-116 colon cancer cells, the tumor-propagating effects of neurotensin could be deterred by curcumin treatment (10–25 μM), which conceivably hindered cPKC activation by inhibiting the release of Ca^{2+} from the ER (Dyer et al., 2002; Wang et al., 2006a). Corroboratively, curcumin (20 μM) impaired PKC activity in human astroglioma cells stimulated with the PKC agonist TPA (Woo et al., 2005). In both cases, abrogated PKC signaling decelerated the MEK1/2-ERK1/2 pathway and downscaled the transcription of the proangiogenic AP-1 target genes *IL-8* in HCT-116 cells (Wolf et al., 2001; Wang et al., 2006a) and *MMP-9* in astroglioma cells (Arnott et al., 2002; Woo et al., 2005).

IL-8 and MMP-9 promote neovascularization via an interconnected pathway. The chemokine IL-8 advances angiogenesis by attracting immature myeloid lineage cell types to the tumor micro-environment (Pruijt et al., 1999; Sparmann and Bar-Sagi, 2004). Upon accumulation, these cells release MMP-9 from their storage granules (Yang et al., 2004b; Ahn and Brown, 2008; Du et al., 2008; Yan et al., 2010). MMP-9 regulates angiogenesis via proteolytic remodeling of the ECM (Liotta et al., 1980),

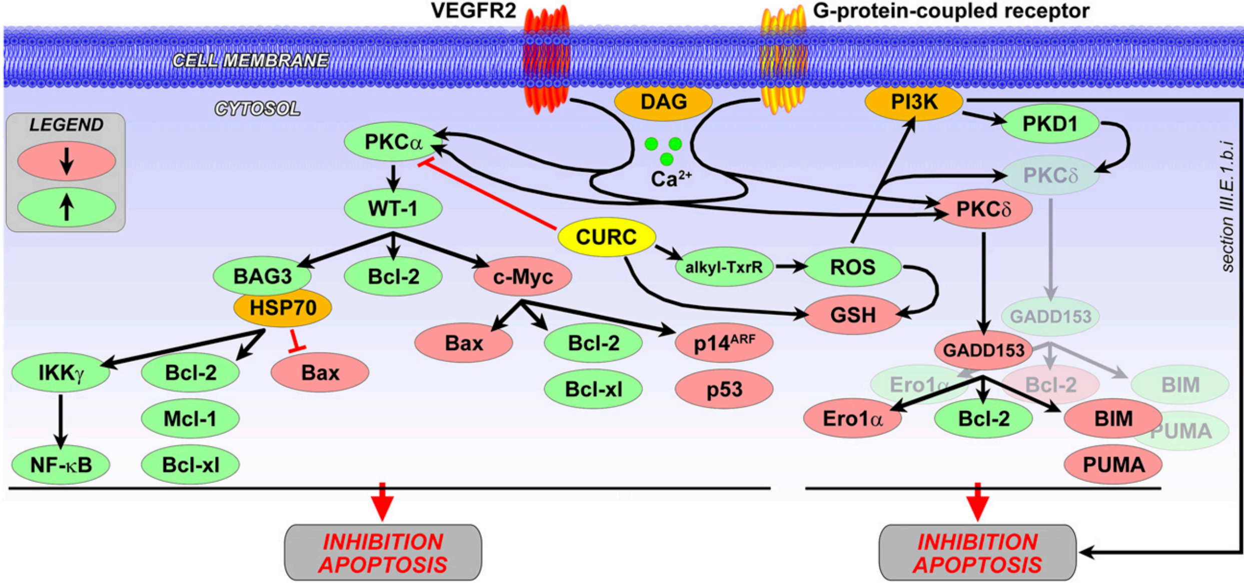


Fig. 17. PKCα and δ signaling pathways with respect to cancer cell apoptosis and the effects of curcumin. The molecules in circles represent proteins. Green circles indicate activation of or translational/expressional increase in the respective protein, and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits (⊣) the PKCα-WT1 pathway by its direct binding to and inhibition of PKCα. Curcumin's effect on the PKCδ pathway proceeds indirectly through the induction of oxidative stress. PKCδ is either directly oxidized (activated) by ROS or via ROS-mediated PI3K-PKD1 signaling, illustrated in the semi-opaque pathway in the background. An alternative PI3K-mediated pathway that leads to the inhibition of apoptosis in cancer cells, which may be affected by curcumin-induced ROS generation, is described in section III.E.1.b.ii. HSP, heat shock protein; IKK, IκB kinase.

thereby not only creating space for the formation of blood vessels, but, more importantly, liberating the potent angiogenic agents bFGF (Ardi et al., 2009) and VEGF (section III.E.3.c) (Bergers et al., 2000) from their ECM-bound state. In a similar fashion, MMP-9 cleaves kit ligand (stem cell factor) from the surface of stromal and bone marrow-derived cells to recruit endothelial progenitor cells and hematopoietic stem cells to the tumor micro-environment (Heissig et al., 2002), which are required for shaping the novel vessel (Luttun et al., 2002). Inhibition of PKC activity by curcumin could therefore retard neovascularization in tumors by disrupting the proangiogenic signaling through the ERK-AP-1-MMP-9 pathway.

3. Enzymes Involved in Prostaglandin Synthesis. PGs comprise a group of lipid messengers produced in an enzymatic cascade generally initiated in response to pro-inflammatory mediators (e.g., IL-1β, TNF-α), GPCR-agonists (e.g., bradykinin), or growth factors (e.g., EGF, TGF-β) (Clark et al., 1995; Gately and Li, 2004). As a first step in this cascade, cytosolic phospholipase A_2 ($cPLA_2$) hydrolyzes the ester bond of plasma membrane phospholipids at the *sn*-2 position to release arachidonic acid (AA) (Clark et al., 1995). The inducible cytosolic enzyme COX-2 subsequently drives a series of redox reactions that catalyze the conversion of AA to PGH_2 (Smith et al., 2011). Being an unstable intermediate, PGH_2 is converted to the biologically active end products prostacyclin (PGI_2), thromboxane, PGF_2, PGD_2, and PGE_2 (Funk, 2001) (Fig. 19). Of these, PGE_2, which is synthesized via microsomal prostaglandin E_2 synthase-1 (mPGES-1) and mPGES-2 as well as cytosolic PGES-3, is most relevant in cancer biology (Greenhough et al., 2009).

Normally, PG production is transiently intensified as part of an inflammatory response so as to readjust vascular tone, incite fever, and make the host aware of pain (Funk, 2001; Bazan and Flower, 2002). When constitutively generated, however, PG signaling stretches beyond the borders of inflammation, additionally steering cellular proliferation (e.g., via APC-β-catenin) and survival (e.g., via PI3K-AKT) (Greenhough et al., 2009). Consequently, perpetual PG production is considered both a cause and consequence of malignant tissue transformation (Grivennikov et al., 2010; Ben-Neriah and Karin, 2011).

On a cellular level, the tumor micro-environment (Grivennikov et al., 2010; Roussos et al., 2011) incessantly propels PG anabolism through generalized oxidative stress (Feng et al., 1995; Sun et al., 2009), the availability of pro-inflammatory mediators (Feng et al., 1995; Fukata et al., 2007), and the self-sufficiency in mitogenic signaling (Subbaramaiah et al., 1998; Di Popolo et al., 2000; Wang et al., 2012). Consequently, COX-2 and its downstream enzymes are typically overexpressed in most human premalignant and malignant lesions (Dannenberg et al., 2001; Radmark and Samuelsson, 2010), accounting for the supraphysiological PG concentrations seen in a variety of neoplasms (Eberhart et al., 1994; Pugh and Thomas, 1994; Wang and DuBois, 2004).

The central role of PGs in oncogenesis has led to the evaluation of COX-2 inhibitors as chemopreventive or oncostatic agents, primarily to delay the onset and/or spread of colorectal cancer (Fenwick et al., 2003; Arber et al., 2006). Although promising results were achieved, the cardiovascular toxicity associated with the chronic use of potent and selective COX-2 inhibitors (Bresalier et al., 2005; Kerr et al., 2007) has fueled an ongoing search for alternatives. In that respect, curcumin meets the demands of a suitable candidate, acting in

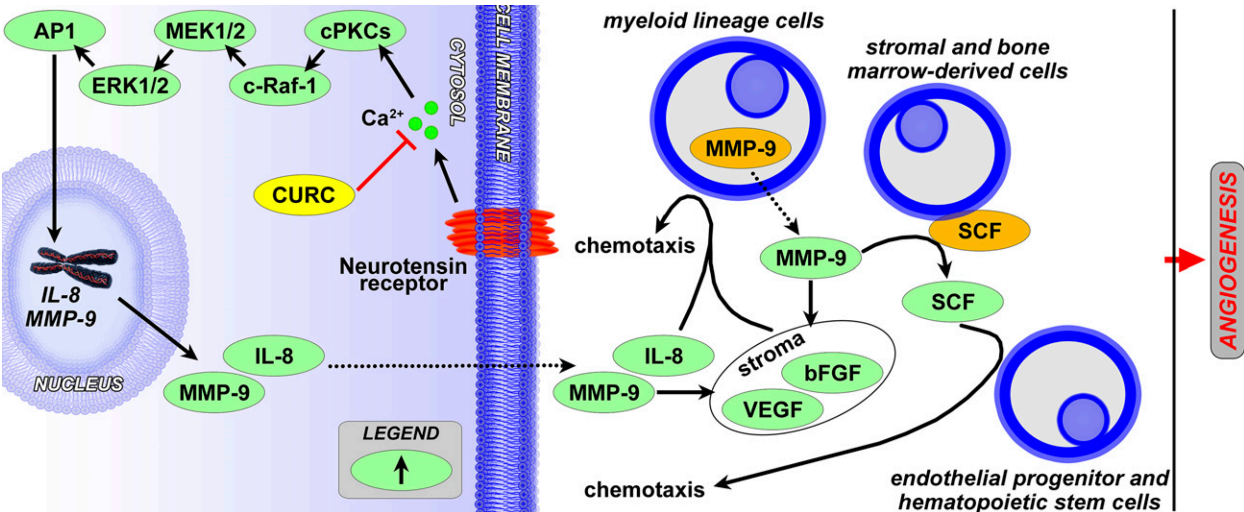


Fig. 18. Neurotensin-mediated signaling pathway with respect to cPKCs and angiogenesis. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits (⊣) this pathway by deterring the release of calcium ions from the endoplasmic reticulum, which normally occurs as a result of neurotensin receptor ligation. SCF, stem cell factor.

a multifarious fashion to normalize rather than abolish PG production rates.

a. Curcumin interferes with prostaglandin synthesis. Initially, the inhibitory effects of curcumin on PG synthesis were solely ascribed to the inhibition of ERK and/or NF-κB (Plummer et al., 1999; Kojima et al., 2000; Chun et al., 2003), resulting in the down-regulation of *PTGS2* transcription, which codes for COX-2, and reduced COX-2 protein levels (Fig. 19). However, molecular docking studies have evinced that curcumin can directly bind to the active site of COX-2 by forming an H-bond between the carbonyl oxygen of curcumin and Arg120 (Selvam et al., 2005) of the COX-2 catalytic site, thereby inhibiting enzyme activity (Fig. 19). This finding was substantiated by experiments with isolated ovine COX-2, which have shown that curcumin represses COX-2 activity by ≤20%, depending on the curcumin concentration (10–50 μM) (Hong et al., 2004).

In addition to the modest direct effect on COX-2, curcumin disrupts the PG synthesis cascade at other levels. For instance, curcumin (20 μM) blocks the phosphorylation of $cPLA_2$ in human colon cancer and esophageal squamous cell carcinoma cells, thereby limiting COX-2 substrate (AA) availability (Fig. 19) (Rao et al., 1995; Hong et al., 2004). The inhibition of PLA_2 phosphorylation by curcumin conceivably occurs via inhibition of MEK in the MAPK pathway (Hong et al., 2004) and/or by preventing the accumulation of cytosolic calcium required for PLA_2 mobilization (Clark et al., 1991; Hong et al., 2004). In human A549 lung carcinoma cells, curcumin directly debilitates the catalytic activity of mPGES-1 (Koeberle et al., 2009), thereby also acting downstream to COX-2 (Murakami et al., 2000, 2003). In doing so, curcumin deters the conversion of COX-2-derived PGH_2 to PGE_2 (Fig. 19) (Jakobsson et al., 1999; Samuelsson et al., 2007), a reaction that recently was deemed critical in the onset and progression of colorectal cancer (Sasaki et al., 2012). Because of these PG-inhibitory effects, curcumin has been shown to promote cancer cell apoptosis, decelerate angiogenesis, and facilitate the clearance of malignant lesions by the immune system.

b. Resistance to apoptosis (H-V). PGs positively influence the survival of cancer cells by impelling NF-κB to produce anti-apoptotic microRNA-21 (miR-21) (Shin et al., 2011). The role of miR-21 in cancer cell survival is underpinned by the finding that miR-21 expression is increased in various cancers (Chan et al., 2005; Meng et al., 2007; Schetter et al., 2008) and inversely correlated to survival of patients with tongue squamous cell carcinoma (Li et al., 2009a).

The miR-21 signaling cascade is initiated by the ligation of EP2 or EP4 receptor by PGE_2, resulting in a rise in intracellular cAMP (Sugimoto and Narumiya, 2007) and subsequent activation of PKA (Chen et al., 2006b; Wang et al., 2010). PKA phosphorylates the p65

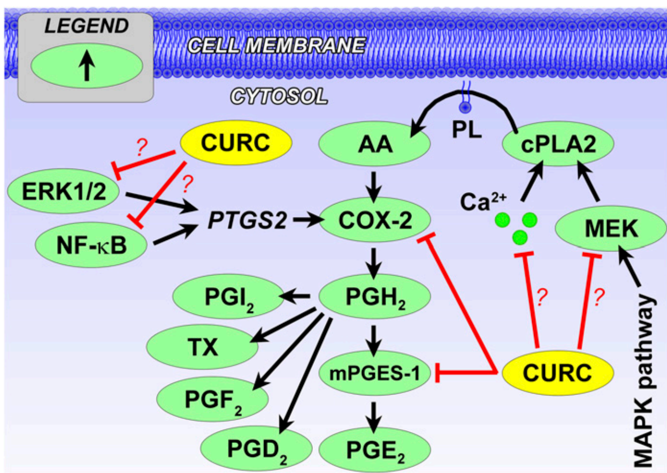


Fig. 19. Prostaglandin synthesis pathway. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits (⊣) this pathway by its direct inhibition of COX-2 and mPGES-1, and possibly by inhibiting the release of calcium ions from the endoplasmic reticulum and/or inhibition of MEK in the MAPK pathway and *PTGS2* gene transcription. The question marks indicate unconfirmed pathways (release of calcium ions, MEK) or elusive pathways (ERK1/2, NF-κB). PL, phospholipid.

(RelA) subunit of NF-κB at Ser276, which in response translocates to the nucleus dimerized with the p50 NF-κB subunit to activate NF-κB target genes (Zhong et al., 1998). Via this route, PGE_2 has been found to target p65 to the promoter region of *MIR21* in human gastric adenocarcinoma cells (Fig. 20) (Shin et al., 2011). The translational product miR-21 conveys its anti-apoptotic messages by silencing the tumor suppressor grainyhead-like 3 and thereby its direct transcriptional target PTEN (Fig. 20) (Darido et al., 2011). PTEN dephosphorylates phosphatidylinositol-3,4,5-trisphosphate to phosphatidylinositol 4,5-bisphosphate. Inhibition of PTEN therefore leads to reduced phosphatidylinositol-3,4,5-trisphosphate dephosphorylation that in turn results in the transactivation of the PI3K-AKT survival pathway (Hollander et al., 2011)—a process that prevails in numerous cancers (Manning and Cantley, 2007). The anti-apoptotic effects of the PI3K-AKT pathway are described in section III.E.1.d and include inactivation of the apoptosis inducers (Cory and Adams, 2002; Green, 2005) Bad (Datta et al., 1997) and caspase 9 (Cardone et al., 1998).

In MIAPaCA-E human pancreatic carcinoma cells, curcumin (4–10 μM) curtailed COX-2 activity and downstream PGE_2 production, resulting in decreased miR-21 levels (Ali et al., 2010). Inhibition of PGE_2-miR-21 signaling restored PTEN levels to baseline and decreased AKT activation (Ali et al., 2010), which was mirrored by a drop in cell viability due to the augmented induction of apoptosis (Ali et al., 2010). These findings have been validated by other reports that causally related curcumin-induced apoptosis in

cancer cells to suppressed PGE_2 synthesis (Lev-Ari et al., 2005; Lee et al., 2009; Sreekanth et al., 2011) and/or decreased AKT phosphorylation (Ghosh et al., 2009; Elamin et al., 2010; Watson et al., 2010a; Prakobwong et al., 2011; Wong et al., 2011).

c. Angiogenesis (H-VII). The angiogenic switch (Bergers and Benjamin, 2003) is primarily triggered by VEGF (Keck et al., 1989; Leung et al., 1989), the expression of which is upregulated in most human cancers (Ferrara and Davis-Smyth, 1997). The overexpression of VEGF is partially controlled by PG signaling (Hernandez et al., 2001).

Being produced by both malignant as well as stromal cell types (Fukumura et al., 1998; Ferrara and Kerbel, 2005), VEGF mainly promotes neovascularization via VEGFR2 (Ferrara, 2002; Hicklin and Ellis, 2005; Potente et al., 2011), which steers intracellular signaling pathways via specific phosphorylation sites on its cytoplasmic domain (Koch et al., 2011). Upon ligation of VEGFR2 by VEGF, Tyr1175 phosphorylation leads to activation of phospholipase C γ, which triggers PKC signaling by increasing cytosolic Ca^{2+} and diacylglycerol levels (Fig. 21, section III.E.2.b) (Bunney and Katan, 2010; Koch et al., 2011). The PKCs subsequently mobilize the Raf-MEK-ERK axis to activate $cPLA_2$ and induce PG synthesis (Fig. 21) (Meadows et al., 2001; Koch et al., 2011). Alternatively, it has been reported that phosphorylation of VEGFR2 Tyr1214 leads to recruitment of the adapter protein Nck, which drives COX-2-mediated $PG(E_2)$ production via proto-oncogene tyrosine-protein kinase Fyn and p38 MAPK in the MAPK pathway (Fig. 21) (Lamalice et al., 2006; Binion et al., 2008). PGE_2 in turn binds to EP2 and EP4 receptors (Spinella et al., 2004; Su et al., 2004) and conceivably upregulates VEGF transcription via the sequential activation of ERK and hypoxia inducible factor-1α (Fig. 21) (Pai et al., 2001; Fukuda et al., 2003). These pathways comprise a self-amplifying feedback loop inasmuch as the produced VEGF leads to additional PG and VEGF synthesis after binding to VEGFR2. Via this route, PGs have been shown to promote VEGF-mediated angiogenesis in both in vitro and in vivo models of breast cancer (Chang et al., 2004).

VEGF mainly triggers neovascularization via VEGFR2 on the surface of ECs (Ferrara and Kerbel, 2005; Weis and Cheresh, 2005). Phosphorylation of Tyr1175 leads to the consecutive activation of phospholipase C γ, PKC, and eNOS, which produces NO to increase vascular permeability (Fig. 22) (Wu et al., 1999). The concomitant phosphorylation of Tyr951 reinforces this effect by inducing the detachment of EC-cell adhesion proteins (e.g., VE-cadherin through inhibition of β-catenin and occludin by inhibition of tight junction protein ZO-1) via the recruitment of c-Src tyrosine kinase (Fig. 22) (Eliceiri et al., 1999; Avizienyte et al., 2002, 2004; Basuroy et al., 2003). The leaky vasculature allows the extravasation of plasma proteins, which serve as a docking matrix for ECs and their cellular precursors that are chemoattracted to shape the novel vessel (Bergers and Benjamin, 2003; Matsumoto et al., 2005). Additionally, VEGFR2 Tyr1175 phosphorylation upholds the proangiogenic environment by directing both the survival and proliferation of ECs (Olsson et al., 2006) by activating endothelial PI3K/AKT (Fujio and Walsh, 1999; Dayanir et al., 2001) and Ras-Raf-MAPK pathways (Takahashi et al., 1999; Meadows et al., 2001; Shu et al., 2002), respectively (Fig. 22).

Considering the bilateral relationship between PGs and VEGF, curcumin (1–20 μM) has been found to restrict the proangiogenic effects of VEGF through inhibition of COX-2 and $cPLA_2$ in the PG synthesis pathways (Figs. 21 and 22). The interference with PG synthesis by curcumin-mediated inhibition of $cPLA_2$ has been described in section III.E.3.a and in Fig. 19, whereby the downstream effects on VEGF signaling are similar to the COX-2 pathway. With respect to the COX-2 pathway, curcumin treatment induced a decline in COX-2 expression and PG production in primary human intestinal microvascular ECs (HIMECs) incubated with VEGF, resulting in impaired HIMEC proliferation, migration, and capillary-like tube formation (Binion et al., 2008). Both the proliferative and migratory capacity of HIMECs could be restored by reconstituting curcumin-pretreated cells with the PGI_2

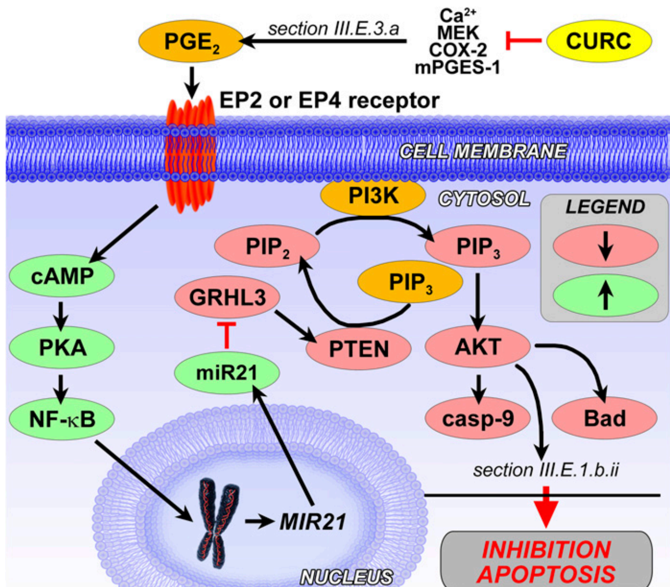


Fig. 20. PGE_2-induced signaling pathway following binding to the EP2 or EP4 receptor in relation to inhibition of apoptosis. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits (⊣) this pathway by inhibiting constituents in the PGE_2 synthesis pathway (section III.E.3.a) and thereby reducing PGE_2 levels and downstream signaling. The Akt-mediated pathway that leads to inhibition of apoptosis in cancer cells is detailed in section III.E.1.b.ii. GRHL3, grainyhead-like 3; PIP_n, phosphatidylinositol n-phosphate.

analogue carbacyclin (Binion et al., 2008), which suggests that PGI_2 is the chief PG mediator of angiogenesis. All aforementioned effects of curcumin could be replicated by treating HIMECs with the selective COX-2 inhibitor NS398 (Binion et al., 2008), which is in line with auxiliary reports that causally link PG signaling to angiogenesis in colon cancer cells (Tsujii et al., 1998), breast cancer cells (Chang et al., 2005), and sarcoma 180 cells (Amano et al., 2003). The therapeutic potential of inhibiting the PG-VEGF axis with curcumin is underscored by the fact that VEGF-targeted therapy has successfully made the transition to clinical application over the last years (Hurwitz et al., 2004; Heidemann et al., 2006; Batchelor et al., 2007; Llovet et al., 2008).

d. Evasion of immune destruction (H-IV). In an immunocompetent host, malignant cellular attributes are swiftly sensed by the immune system, which mounts an immune response to eliminate the transformed cells. The efficacy of any antitumor immune response is dictated by the local cytokine homeostasis. The cytokine balance in turn is controlled by PGE_2, which is used by cancer cells to skew immune cells toward a tolerant phenotype so as to circumvent their clearance. By using a murine Lewis non-small cell lung carcinoma model, it was shown that tumor-derived PGE_2 is prerequisite for uncontested tumor development, which was attributed to the stimulatory effects of PGE_2 on the local production of tolerogenic IL-10 by lymphocytes. The immunotolerance signaling occurs at the expense of pro-inflammatory IL-12 release by intratumoral macrophages (Halak et al., 1999; Stolina et al., 2000).

PGE_2 signals through the EP2 and EP4 receptors on T-cells to induce the expression of the forkhead box P3 (*FOXP3*) gene (Fig. 23) (Sharma et al., 2005). As such, PGE_2 steers the regulatory T-cell lineage (Tregs) to a tolerogenic phenotype ($CD4^+$ $CD25^+$ $FOXP3^+$) (Baratelli et al., 2005) that does not participate in the destruction of cancer cells (Sharma et al., 2005). $FOXP3^+$ Tregs hamper the destruction of cancer cells by excreting IL-10, which suppresses the release of type 1 cytokines (e.g., IL-12, TNF-α, interferon γ) by effector T-cells and macrophages (Fiorentino et al., 1989; Rohrer and Coggin, 1995; Nataraj et al., 2001; Mahic et al., 2006; Li and Flavell, 2008). In addition, it has been shown that $FOXP3^+$ Tregs themselves release PGE_2 in a COX-2-dependent fashion (Mahic et al., 2006), not only reinforcing the cascade of events as detailed hitherto, but additionally inhibiting the release of pro-inflammatory CCL3 (MIP-1α) and CCL4 (MIP-1β) by dendritic cells (Jing et al., 2003, 2004) and CCL5 (RANTES) production by macrophages (Fig. 23) (Takayama et al., 2002). Corroboratively, in patients with breast cancer (Pockaj et al., 2004) and gastric cancer (Yuan et al., 2010), the observed high PGE_2 levels correlated to low intratumoral T-cell activity, which, in the case of gastric cancer, was paralleled by high FOXP3 expression.

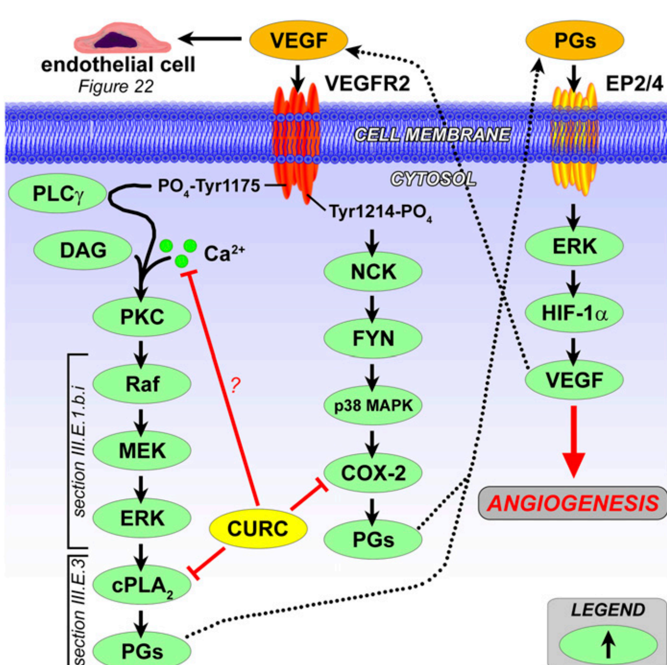


Fig. 21. VEGF-mediated signaling via the PG synthesis pathway in relation to angiogenesis. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin (CURC) inhibits (⊣) this pathway by its direct interaction with COX-2 and $cPLA_2$ and consequent PG synthesis. Curcumin may also inhibit the PKC pathway by deterring the cytosolic release of calcium ions. The effect of VEGF on angiogenesis through endothelial cells is depicted in Fig. 22. FYN, member of the proto-oncogene tyrosine-protein kinases; HIF, hypoxia inducible factor; NCK, non-catalytic region of tyrosine kinase adaptor protein 1.

These results collectively attest to the immunosuppressive properties of PGs, which signal via the EP2 and EP4 receptors to silence immune cells and allow the uncontested expansion of tumors. Consequently, curcumin-mediated blockage of $PG(E_2)$ production and PGE_2 binding conceivably restores the cytotoxic capabilities of the immune system, enabling sentinel cell types (e.g., macrophages, dendritic cells) to release the appropriate range of cytokines required to mobilize an adaptive immune response (Fig. 23). By reinstating the crosstalk between innate and adaptive immunity, curcumin treatment could instate an immune destruction of cancer cells.

4. Vitamin D Receptor.

a. Vitamin D metabolism and cancer. The steroid hormone $1\alpha,25(OH)_2D_3$ (calcitriol) is the active metabolite of vitamin D_3, the synthesis of which is initiated in the skin where, under the influence of sunlight, the precursor 7-dehydrocholesterol is converted to pre-D_3 (Haussler et al., 1998). The latter subsequently circulates to the liver and kidneys, which catalyze the hydroxylation of pre-D_3 to $25(OH)D_3$ and of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$, respectively (Haussler et al., 1998)

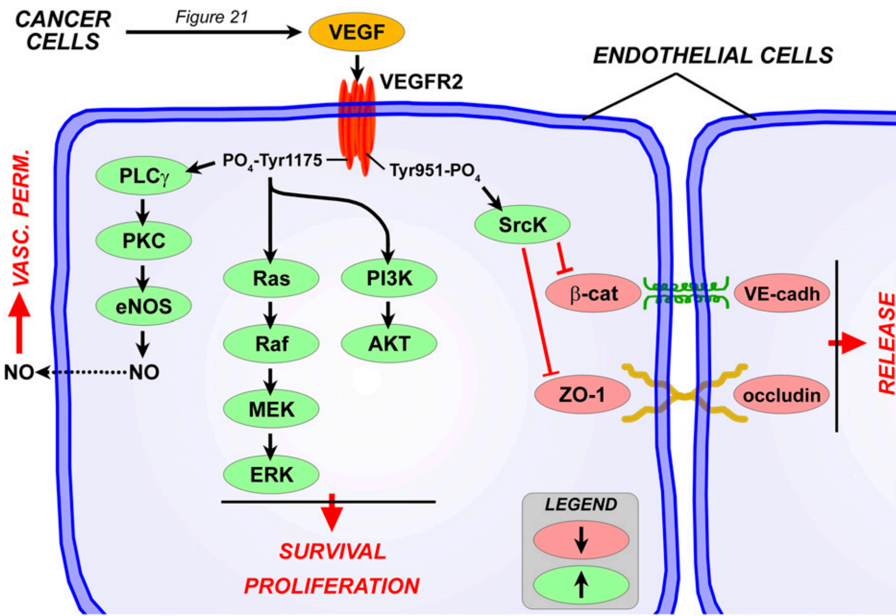


Fig. 22. VEGF-mediated signaling in endothelial cells in relation to cancer angiogenesis. The molecules in circles represent proteins. Green circles indicate activation of or translational/expressional increase in the respective protein and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. In case of VE-cadherin and occludin, the red circles indicate redistribution away from the tight junctions.

(Fig. 24). Although long considered to primarily maintain calcium homeostasis (Holick, 1996), 1α,25$(OH)_2D_3$ is now also recognized as a an anticarcinogen that controls cell survival and proliferation (Deeb et al., 2007). To confer the biochemical cues, 1α,25$(OH)_2D_3$ binds to VDR (Clemens et al., 1988; Crofts et al., 1998; Jurutka et al., 2007) located in the nuclear membrane, which, after forming a heterodimer with the retinoid X receptor, regulates transcriptional events by binding to genes containing a vitamin D response element (VDRE) (Bouillon et al., 2008) (Fig. 24).

The oncostatic potential of 1α,25$(OH)_2D_3$ predominantly emanates from its cell cycle inhibitory action (Deeb et al., 2007), which is achieved by upregulating VDRE-containing genes such as *CDKN1A* (encodes $p21^{CIP1}$) (Liu et al., 1996; Saramaki et al., 2006) and *GADD45A* (encodes GADD45α) (Hildesheim et al., 2002; Jiang et al., 2003). The downstream effects of $p21^{CIP1}$ activation in cancer cells (Fig. 24) are further addressed in section III.E.4.c, whereas GADD45α signaling blocks the G_2/M transition by deterring Cdk1-cyclin B1 complex formation (Wang et al., 1999; Jin et al., 2002) and promotes apoptosis via MAP kinase kinase 4-activated p38/JNK pathways (Takekawa and Saito, 1998; Harkin et al., 1999; Miyake et al., 2007) (Fig. 24), although it should be noted that the GADD45α-mediated induction of apoptosis via p38/JNK has been contested (Sheikh et al., 2000). As indirect effects, 1α,25$(OH)_2D_3$ has been shown to slow down the proliferation of various cancer cell types by inhibiting ErbB-1 signaling (Koga et al., 1988; Tong et al., 1999) (section III.E.1) while concomitantly activating the TGF-β pathway (Chen et al., 2002; Li et al., 2005a), which inhibits cell proliferation (Hannon and Beach, 1994). Moreover, it has been reported that 1α,25$(OH)_2D_3$ evokes acute nongenomic responses (e.g., cellular Ca^{2+} uptake) (Losel and Wehling, 2003; Losel et al., 2003) through the VDR (Nguyen et al., 2004; Zanello and Norman, 2004), although the significance of these effects on cancer biology and biochemistry remains ill-defined (Deeb et al., 2007).

By virtue of these functions, both experimental (Kallay et al., 2001; Zinser et al., 2005; Banwell et al., 2006) and epidemiologic data have confirmed the antitumor properties of 1α,25$(OH)_2D_3$, demonstrating that a vitamin D deficiency increases the risk of malignancies such as breast (Bertone-Johnson, 2009) and colorectal cancer (Garland and Garland, 1980; Garland et al., 1989). Even without pre-existing deficiencies, cancer cells are typically insensitive to 1α,25$(OH)_2D_3$, which is attributable to an acquired enzymatic imbalance in favor of 1α,25$(OH)_2D_3$ catabolism (Albertson et al., 2000; Bises et al., 2004; Matusiak et al., 2005; Parise et al., 2006). Because of the proven treatment efficacy of 1α,25$(OH)_2D_3$ (analogues) in experimental tumor models (Zhang et al., 2005; Banach-Petrosky et al., 2006), clinical trials aiming to evaluate the antitumor potential of 1α,25$(OH)_2D_3$ have been in the limelight over the last years (Deeb et al., 2007).

b. Curcumin is a vitamin D receptor agonist. Curcumin has recently been identified as a direct VDR agonist (Bartik et al., 2010; Menegaz et al., 2011), which, in view of the above mentioned, implies that curcumin could be used to compensate for the lack of 1α,25$(OH)_2D_3$ signaling in certain cancer cells. In that

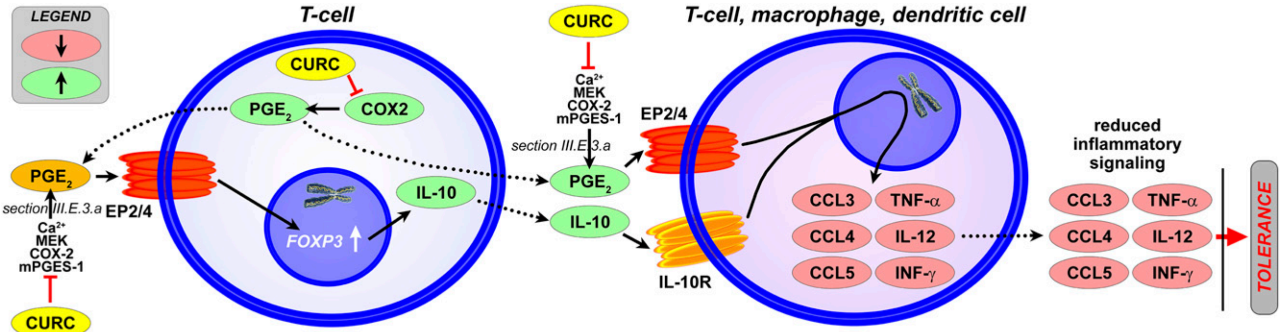


Fig. 23. Mechanisms of immunotolerance induction by cancer cells via cells of the innate and adaptive immune system. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein, and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin abrogates the tolerogenic signaling by T-cells by inhibiting constituents of the PGE_2 synthesis pathway (section III. E.3.a).

respect, the range of effects that the VDR exerts depends on the structural conformation of the ligand, which defines whether the agonist binds to the genomic domain (Rochel et al., 2000) or the partially overlapping alternative binding pocket (Mizwicki et al., 2004). Whereas the genomic domain prefers a "bowl-shaped" ligand to trigger transcriptional responses, the alternative binding pocket exhibits a higher affinity for "planar-shaped" compounds and mainly accounts for the acute effects associated with VDR signaling (Rochel et al., 2000; Norman et al., 2001; Mizwicki et al., 2004).

Similar to the structurally flexible ligand $1\alpha,25(OH)_2D_3$ (Okamura et al., 1995), curcumin can associate with both VDR domains (Menegaz et al., 2011). By using radiolabeling, it was demonstrated that curcumin competes with 0.4 nM $1\alpha,25(OH)_2D_3$ for VDR binding with a K_i of 2.9 μM (Bartik et al., 2010) when assuming a K_d of 10^{-10} M for the $1\alpha,25(OH)_2D_3$-VDR complex (Bouillon et al., 2008). Molecular docking studies revealed that the keto-enol isoform of curcumin, which adopts a planar geometry, shows a strong binding affinity for the VDR alternative binding pocket (Menegaz et al., 2011). In contrast, a 'bowl-shaped' β-diketo conformation of curcumin prefers the genomic domain and thus provokes the associated genomic responses (Menegaz et al., 2011). When the physiological bias for the keto-enol isoform is taken into consideration, curcumin has an overall inclination to bind the VDR alternative binding pocket (Menegaz et al., 2011), although numerous docking studies with the keto-enol isomer have revealed the adoption of nonplanar configurations of curcumin in bound state (section II.A.6). Consequently, higher intracellular curcumin concentrations may be required to elicit the VDR genomic domain-dependent transcriptional response, which confers the most profound cytostatic effects.

c. Sustained proliferation (H-I). Interestingly, recent in vitro data have demonstrated that curcumin is able to trigger VDR-dependent genomic responses in cancer cells (Bartik et al., 2010). First, it was shown that curcumin (10 μM) was able to induce luciferase that was under the control of a VDRE-containing promoter in transfected Caco-2 human colon cancer cells (Bartik et al., 2010). The transcriptional upregulation resulted from enhanced VDR-retinoid X receptor dimerization and amplified recruitment of the VDR transcriptional coactivator nuclear receptor coactivator 1 (SRC-1) (Bartik et al., 2010). To rule out an unspecific effect on nuclear receptors, Caco-2 cells were transfected with a vector containing the response element of an archetypical nuclear receptor [the glucocorticoid receptor (Gustafsson et al., 1987)], which proved unsusceptible to curcumin-induced transcriptional activation (Bartik et al., 2010). It was subsequently shown that exposure of Caco-2 cells to 50 μM curcumin elicited a genomic response in these cells (Bartik et al., 2010). Curcumin treatment increased the expression of various VDRE-containing genes, most notably the tumor suppressor *CDKN1A* ($p21^{CIP1}$) (Bartik et al., 2010). Upregulation of $p21^{CIP1}$ by curcumin was further corroborated in VDR-overexpressing Caco-2 cells, demonstrating that curcumin increases $p21^{CIP1}$ expression in a VDR-dependent fashion (Bartik et al., 2010).

$P21^{CIP1}$ is an integral sensor of cellular stress (Abbas and Dutta, 2009) and, in response, inhibits the kinase activity of various cyclin/Cdk complexes (e.g., cyclin E-Cdk2, cyclin A-Cdk1/2, cyclin B-Cdk1) (Gu et al., 1993; Harper et al., 1993; Chen et al., 1995, 1996; Luo et al., 1995; Shiyanov et al., 1996; Dulic et al., 1998; Gillis et al., 2009). In doing so, $p21^{CIP1}$ is able to arrest the cell cycle at both the G_1/S and G_2/M-transition (section III.E.1.c, Fig. 24) (Abbas and Dutta, 2009). Additionally, $p21^{CIP1}$ inhibits the activity of promitotic transcription factors implicated in cancer development, such as STAT3 (Paulson et al., 1999; Coqueret and Gascan, 2000; Yu et al., 2009) and Wnt4 (Devgan et al., 2005; Ranganathan et al., 2011), by hampering recruitment of the transcriptional coactivator p300-CBP (Iyer et al., 2004). Moreover, $p21^{CIP1}$ amplifies the transcriptional activity of p300-CBP (Snowden et al.,

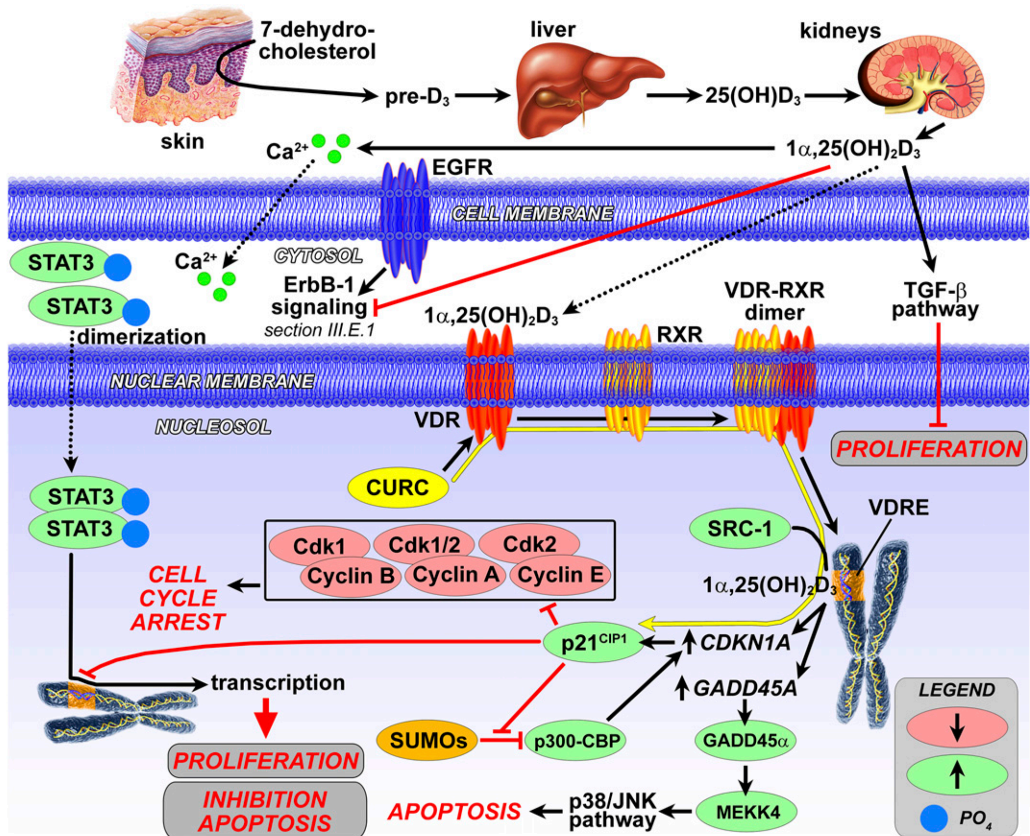


Fig. 24. Production of and signaling by $1\alpha,25(OH)_2D_3$ (calcitriol), the active metabolite of vitamin D_3, in relation to its oncostatic properties. The molecules in circles represent proteins. Green circles indicate activation of or translational/expressional increase in the respective protein and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under conditions of vitamin D binding or curcumin agonism of VDR. The biological effects that are delineated in gray boxes represent those in cancer cells under normophysiological conditions (i.e., in the absence of $1\alpha,25(OH)_2D_3$ or curcumin binding), whereas the biological effects that are indicated in red lettering only represent those that are induced by $1\alpha,25(OH)_2D_3$ or curcumin. Curcumin shares two common pathways with $1\alpha,25(OH)_2D_3$ in inhibiting cancer cell proliferation, inducing and abrogating the inhibition of apoptosis, and arresting cell cycle progression. First, both compounds are antagonists of ErbB-1 signaling as described in section III.E.1. The second common pathway is initiated with the activation of VDR, which culminates in the upregulation of $p21^{CIP1}$ expression (yellow arrow). MEKK, MAP kinase kinase kinase; RXR, retinoid X receptor.

2000) by abrogating post-transcriptional modification [SUMOylation (Geiss-Friedlander and Melchior, 2007)] of the p300-CBP cell cycle regulatory domain 1 by small ubiquitin-related modifiers (SUMOs) (Girdwood et al., 2003). Considering that p300-CBP activity is frequently suppressed in tumors (Iyer et al., 2004) and that the *CDKN1A* gene (encoding $p21^{CIP1}$) is a direct target of p300-CBP, $p21^{CIP1}$ could reciprocally stimulate its own production (Gartel and Tyner, 1999; Abbas and Dutta, 2009) to further enhance its inhibitory effects on cell cycle progression (Fig. 24). Via these routes, the curcumin-VDR axis could provide a novel chemopreventive or therapeutic avenue.

5. DNA.

a. Curcumin binds to DNA. During cell division, it is imperative that DNA is undamaged so as to ensure accurate relay of genetic information from the mother cell to the daughter cells. Damaged DNA triggers a DNA damage response that leads to either repair of the lesion(s) or induction of apoptosis if the damage is too catastrophic. Inasmuch as the rate of cell division is greatly augmented in tumors, chemotherapy has traditionally been directed toward DNA because the replicative processes are more susceptible to genetic anomalies in cancer cells than in healthy cells. Compounds that bind DNA may induce DNA damage, interfere with protein binding, or inhibit the function of DNA through epigenetic modifications. Examples include alkylating agents such as platinum compounds (DNA crosslinking) (Kelland, 2007), base-substituting agents such as 5-fluorouracil (recognized as base damage) (Longley et al., 2003), and topoisomerase inhibitors such as camptothecin and topotecan (inhibition of DNA replication) (Pommier, 2006).

Curcumin has been characterized as a DNA binding agent (Reuter et al., 2011). A study on curcumin-DNA interactions using circular dichroism spectroscopy and molecular docking revealed that curcumin binds to the minor groove of DNA in a pH- and sodium concentration-dependent manner (Zsila et al., 2004). By using circular dichroism, UV-vis spectroscopy, and FT-IR spectroscopy, Nafisi et al. (2009) established that curcumin binds DNA at T-bases in the minor groove, G and A bases in the major groove, and the

phosphate backbone. They also found that curcumin binds RNA, albeit with a lower affinity than DNA.

b. DNA damage signaling. Cycling cells control DNA damage by a DNA damage response that recognizes DNA lesions and facilitates repair or halts cell cycle progression in the G_1-, G_2-, or S-phase, which may culminate in apoptosis. Several types of DNA damage exist, which are typically recognized through different sensor protein complexes (Fig. 25). DNA double strand breaks are recognized by a complex of MRE11-NBS1-RAD50 that binds and activates ataxia-telangiectasia mutated (ATM) (Rogakou et al., 1998; Harper and Elledge, 2007). ATM in turn phosphorylates histone H2AX at Ser139 (Rogakou et al., 1998), which attracts DNA damage repair proteins such as mediator of DNA damage checkpoint protein 1, tumor suppressor p53-binding protein 1 (53BP1), and BRCA1 (Bartek and Lukas, 2007). Additionally, 53BP1 acts as a central transducer of the DNA damage signal to p53 when 53BP1 accumulation at discrete nuclear foci becomes too excessive (Wang et al., 2002), culminating in cell cycle arrest. Single strand breaks are recognized by ATR interacting protein and replication protein A that bind and activate ATM and RAD3-related (ATR) to consequently phosphorylate H2AX and recruit the DNA repair machinery (Zou and Elledge, 2003; Bartek and Lukas, 2007). Other DNA damaging events include base-mismatches (mismatch repair), base damage (base-excision repair), and UV-induced damage (nucleotide excision repair), all of which recruit ATM or ATR to the site of the lesion at some point (Lazzaro et al., 2009).

ATM and ATR are responsible for inducing cell cycle arrest and apoptosis through the p53 signaling pathway in response to DNA damage. Both ATM- and ATR-containing DNA-repair complexes inhibit MDM2, an inhibitor of p53 (Vassilev et al., 2004) as well as transcriptional activation of *p53* (Ofir-Rosenfeld et al., 2008), and thus activate transcription of p53 target genes. Furthermore, ATM and ATR activate Chk2 and Chk1, respectively, which also activate p53 independently of the ATM/ATR-mediated activation (Harper and Elledge, 2007; Kruse and Gu, 2009). As discussed in section III.E.1.c, p53 triggers cell cycle arrest by inducing the expression of $p21^{CIP1}$ that in turn inhibits cyclin E/A-Cdk2 and cyclin B-Cdk1 activity (Fig. 15). In addition, and independently of p53, Chk1 and Chk2 both phosphorylate (deactivate) cell division cycle 25, which is required for cyclin B-Cdk1 activity and M-phase entry (Lobrich and Jeggo, 2007) (Fig. 15).

c. Curcumin-induced DNA damage. Numerous studies have observed curcumin-induced DNA damage in cancer cells, leading to cell cycle arrest and apoptosis (Cao et al., 2007; Lin et al., 2008; Sahu et al., 2009; Jiang et al., 2010; Mendonca et al., 2010; Lu et al., 2011). Two putative mechanisms are responsible for these effects, namely direct curcumin-induced DNA damage and curcumin-mediated impairment of DNA repair mechanisms. With respect to the former, curcumin induces oxidation of guanine residues to the typical oxidation product 8-oxo-guanine (Jiang et al., 2010), which activates the mismatch repair machinery by transcriptional upregulation of various MSH, MLH, and PMS isoforms (Ni et al., 1999) (Fig. 25). In addition, curcumin treatment of tumor cells increased ATM-mediated H2AX phosphorylation (Sahu et al., 2009; Jiang et al., 2010), indicating that curcumin induces double strand breaks in DNA (Rogakou et al., 1998; Harper and Elledge, 2007). ATM and Chk1 were found to be activated after curcumin treatment in mismatch repair-deficient human colon carcinoma (HCT116) cells and human pancreatic cancer (BxPC-3) cells (Sahu et al., 2009; Jiang et al., 2010), suggesting that curcumin induces both double and single strand DNA breaks. Chk1 inactivates the p53 inhibitor MDM2 and activates p53 (Harper and Elledge, 2007; Kruse and Gu, 2009), which leads to cell cycle arrest via the induction of *CDKN1A* transcription and consequent $p21^{CIP1}$ upregulation, and stimulates apoptosis by the induction of Bax, PUMA, and NOXA (Levine, 1997;

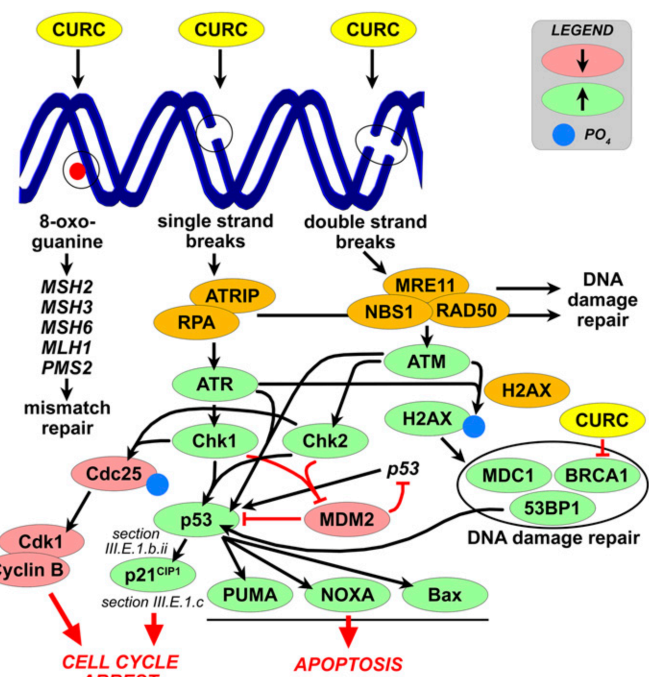


Fig. 25. Modes of DNA damage and downstream signaling in relation to DNA repair mechanisms as well as cancer cell apoptosis and cell cycle arrest. The molecules in circles represent proteins, the molecules in italics represent genes. Green circles indicate activation of or translational/expressional increase in the respective protein and red circles indicate deactivation of or translational/expressional decrease in the respective protein (legend) under native conditions, i.e., unaffected by curcumin. Curcumin promotes cell cycle arrest and apoptosis in cancer cells by oxidizing guanine residues and by inducing single and double strand breaks (encircled). Concomitantly, curcumin interferes with the repair of single and double strand breaks by inhibiting BRCA1. ATRIP, ATM and RAD3-related interacting protein; Cdc, cell division cycle; RPA, replication protein A.

Villunger et al., 2003) (Fig. 25). So far, the activation of p53 and the corollary increase in p21^{CIP1}, Bax, PUMA, and NOXA have been discussed in the context of telomere shortening (section III.E.1.c) (recognized as single strand DNA breaks) caused by the inhibition of ErbB-1 signaling (section III.E.1.b.ii). In addition to the upregulated expression of these proteins through the ErbB-1 signaling axis, however, base oxidation as well as single and double strand breaks induced directly by curcumin may be an alternative mechanism for the activation of these proteins.

Another mechanism revolves around curcumin's ability to inhibit DNA damage repair proteins, although the exact dynamics remain elusive. In human breast cancer cells, exposure to 10 μM curcumin induced DNA damage and concomitantly inhibited DNA repair by preventing nuclear translocation of BRCA1 (Rowe et al., 2009). Contrastingly, treatment of human lung adenoma A459 cells with 30 μM curcumin reduced protein levels of Chk1 and cytosolic (dephosphorylated) cell division cycle 25, which is indicative of reduced DNA damage and repair signaling. However, levels of p53 and p21^{CIP1} were increased, cyclin B1 levels were diminished, and the cells were arrested at the G_2/M transition (Lin et al., 2008). Levels of ATM, ATR, and Chk2 were not investigated, so it remains unclear as to which type of DNA damage was induced and which signaling mechanisms were responsible for cell cycle arrest and apoptosis in A459 cells. Additional studies on this subject are required to verify the exact mechanism (s) by which curcumin induces DNA damage.

d. DNA damage as a result of ErbB-1 inhibition by curcumin. There is evidence that ErbB-1 signaling modulates DNA repair genes, as reviewed in Meyn et al. (2009). First, ErbB-1 was found to directly interact with DNA-dependent protein kinase, catalytic subunit (Bandyopadhyay et al., 1998; Huang and Harari, 2000; Dittmann et al., 2005a,b; Das et al., 2006, 2007), which is essential in the repair of DNA double strand breaks by nonhomologous end-joining (Khanna and Jackson, 2001). Second, ErbB-1 induces the expression of DNA repair genes such as *XRCC1* (base-excision repair) through the Ras/Raf/MAPK pathway (Yacoub et al., 2001; Brem and Hall, 2005; Toulany et al., 2008) and *Rad51* (involved in homologous recombination repair of double strand breaks) (Chinnaiyan et al., 2005; Ko et al., 2008). Third, ErbB-1 signaling modulates the activation of several DNA repair proteins, which includes DNA-dependent protein kinase, catalytic subunit (Toulany et al., 2006), and ATM (Golding et al., 2007). Given that curcumin inhibits ErbB-1 (section III.E.1.a), it is not unlikely that DNA binding by curcumin in combination with inhibition of ErbB-1 signaling leads to DNA damage and a concomitantly impaired DNA repair machinery.

In summary, molecular docking and experimental studies have established that curcumin is capable of binding to DNA and inducing DNA damage in cancer cells. Curcumin-induced DNA damage always concurred with apoptosis, which likely resulted from the simultaneous inhibition of DNA repair proteins (possibly co-mediated by inhibition of ErbB-1) and proapoptotic signaling. The association between curcumin and DNA in continuously cycling cells may not only interfere with replication forks through oxidative damage to nucleotides and consequent DNA mismatches, but curcumin also inhibits DNA repair of single and/or double strand breaks. With a prevalent state of DNA damage and insufficient repair, cells will ultimately perish as a result of apoptosis. Nonmalignant cells may be considerably less prone to this type of damage because of the fact that genomic instability and hyperproliferation are mostly absent in healthy cells.

IV. Concluding Remarks

In the final analysis, curcumin can best be described as pharmacodynamically fierce but pharmacokinetically feeble. Its pharmacodynamic fierceness is derived from the fact that curcumin interferes with a plethora of vital pathways in cancer cells attributable to its promiscuous binding behavior, as a result of which curcumin is capable of inhibiting all hallmarks of cancer. In doing so, curcumin exhibits antimutagenic (i.e., chemopreventive), cytostatic, cytotoxic (i.e., curative), and antimetastatic properties. The association between curcumin and target molecules is facilitated through curcumin's unique chemical attributes, namely, a log P value that ensures transmembrane passage without excessive retention in lipophilic compartments, allowing curcumin to reach its molecular targets; the capacity to undergo H-bonding, chelate (non)metal cations, as well as undergo hydrophobic interactions; conformational adaptability as a result of rotamerization around multiple bonds, which facilitates optimal docking into, e.g., the substrate binding site of enzymes; and the potential to act as a Michael acceptor, leading to covalent associations with target molecules. In cancer cells, curcumin's pleiotropic binding results in the deterrence of numerous critical metabolic processes and the activation of cell death pathways through different molecular initiators, which collectively amplify its tumoricidal efficacy. Moreover, curcumin is cytoprotective to healthy cells, owing to its strong antioxidant properties, and is reported to work in concert with many types of chemotherapeutic agents, often producing an adjuvant effect.

On the other hand, the pharmacokinetically feeble character is attributable to curcumin's chemical instability, poor systemic uptake, and extensive biotransformation, at least in case of orally administered formulations. Curcumin is susceptible to degradation upon UV and visible light exposure and very prone to degradation under physiological conditions, namely in

an aqueous environment at pH = 7.4 and 37°C, which a priori complicates in vitro and in vivo studies. In that respect, oral administration of curcumin is ideal inasmuch as the acidic and dark gastrointestinal tract is protective. However, such protection is of limited value when it is not accompanied by efficient systemic uptake. Curcumin exhibits very poor bioavailability, which is partly due to its adherence to the enteral mucosa, strongly limiting its uptake by enterocytes. The curcumin molecules that are taken up by enterocytes are subsequently excreted apically or predominantly biotransformed before undergoing apical and basolateral transport. Moreover, curcumin is avidly cleared from the enterohepatic circulation by the liver, where it is also metabolized and excreted into the biliary system. The biotransformation of curcumin entails phase I and II xenobiotic metabolism and export in both enterocytes and hepatocytes, altogether accounting for very low plasma concentrations of the parent compound. In fact, the peak plasma levels of curcumin are 2 to 3 orders of magnitude lower than its in vitro IC_{50} values, even when very large quantities (12 g) of curcumin are ingested.

It is therefore not surprising that, in clinical trials for tumors outside of the gastrointestinal tract, curcumin was shown to be of suboptimal therapeutic value, despite the fact that some of the main metabolites (e.g., the reduced curcumin species and ferulic acid) retain anticancer properties. Even when uptake-enhancing agents such as piperine, turmerone, or liposomal formulations (e.g., Meriva, Indena SpA, Milan, Italy) are (co-)administered orally, the systemic curcumin concentrations are elevated but still remain below therapeutic levels. Such formulations are therefore not expected to significantly enhance therapeutic outcomes in patients with these types of cancer. For cancers of the gastrointestinal system, however, and colorectal malignancies in particular, oral curcumin formulations have yielded clinical results that are more encouraging. The poor prospectus of oral curcumin therapy for nongastrointestinal cancers notwithstanding, it is our opinion that long-term use of oral curcumin formulations may have a chemopreventive effect. The curcumin plasma levels peak within 2 hours after administration, and complete clearance usually occurs within a few hours thereafter. If dosed properly, e.g., intake of lower dose curcumin formulations containing an uptake-enhancing agent three times a day at equal intervals, sufficient curcumin plasma levels could be sustained for a chemopreventive effect. However, currently there are no long-term clinical studies to corroborate this claim.

To exploit the pharmacodynamic fierceness of curcumin for nongastrointestinal cancers, either chemically more stable and equally or more toxic curcumin analogues must be used or curcumin needs to be administered intravenously so as to circumvent the pharmacokinetic hurdles. In case of the latter, a drug carrier system is required for purposes of solubility in plasma. Despite the demonstrated toxicity of some curcumin analogues in various human cancer cell lines, there are several important disadvantages associated with the use of these compounds. Most of the compounds have a log P that is comparable with that of curcumin's, as a result of which these compounds must be taken orally or administered intravenously using a drug delivery system. Furthermore, insofar as the pharmacokinetics, in vivo pharmacodynamics, and toxicity profiles have not been established for the majority of curcumin analogues, there are no clear advantages of these compounds over the use of curcumin, at least not at this stage. The FDA classifies curcumin as generally recognized as safe—a status that is annulled upon the slightest modification of curcumin's chemical structure. Any modification makes the curcumin analogue subject to the entire preclinical and clinical development trajectory, which takes a lot of years and money. Consequently, unless there are pronounced and unequivocal advantages of a curcumin analogue over the parent compound (i.e., more stable, more toxic to cancer cells, more favorable pharmacokinetics, and a generally recognized as safe status), investing considerable time and resources in research and development is impractical and risk-laden.

Encapsulation of curcumin into a drug delivery system such as liposomes is therefore the most suitable option for the intravenous administration of curcumin and delivery to a tumor. In addition to the nontoxicity of phospholipids, the stabilizing and solubilizing effect of liposomal encapsulation in aqueous solutions, and several FDA-approved precedents (e.g., liposomal doxorubicin and vincristine), liposomes constitute ideal drug carriers for selective targeting to the tumor because of their preparatory versatility. Liposomes can be formulated such that they accommodate a specific physiological context and drug delivery requirements. For instance, long-circulating liposomes can be prepared that passively accumulate in the tumor as a result of the enhanced permeability and retention effect caused by the leakiness of intratumoral vasculature. Similarly, specific epitope recognition ligands can be grafted onto the liposome surface to facilitate targeting to abundantly expressed surface proteins on tumor cells. The most common issue with liposomes is that there is always some uptake by cells of the reticuloendothelial system, which may impair final intratumoral drug concentrations. However, liposomes can handle very high payloads to compensate for unspecific clearance, and the uptake by noncancerous cells is not expected to impart dire consequences given the cytoprotective properties of curcumin in non-to-slowly dividing cells. It is therefore anticipated that liposomal curcumin, or curcumin encapsulated in a different biocompatible drug delivery system, constitutes the best approach for

the treatment of nongastrointestinal cancers. Fortunately, this type of nanotechnology is very common nowadays and accessible to virtually every standard laboratory.

Authorship Contributions

Performed data analysis: Heger, van Golen.

Wrote or contributed to the writing of the manuscript: Heger, van Golen, Broekgaarden, Michel.

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