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Curcumin analog cytotoxicity against breast cancer cells: exploitation of a redox-dependent mechanism

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ABSTRACT

A series of novel curcumin analogs, symmetrical dienones, were previously shown to possess cytotoxic, anti-angiogenic and anti-tumor activities. Analogs **1** (EF24) and **2** (EF31) share the dienone scaffold and serve as Michael acceptors. We propose that the anti-cancer effects of **1** and **2** are mediated in part by redox-mediated induction of apoptosis. In order to support this concept, **1** and **2** were treated with L-glutathione (GSH) and cysteine-containing dipeptides under mild conditions to form colorless water-soluble adducts, which were identified by LC/MS. Comparison of the cytotoxic action of **1, 2** and the corresponding conjugates, **1**-$(GSH)_2$ and **2**-$(GSH)_2$, illustrated that the two classes of compounds exhibit essentially identical cell killing capabilities. Compared with the yellow, somewhat light sensitive and nearly water insoluble compounds **1** and **2**, the glutathione conjugates represent a promising new series of stable and soluble anti-tumor pro-drugs.

Curcumin (diferuloylmethane) is a α,β-diketone constituent of turmeric with antioxidant properties. It has been used in traditional medicine for liver disease, indigestion, urinary tract infections, rheumatoid arthritis, and insect bites.[1] This phytochemical has also demonstrated both anti-cancer and anti-angiogenic properties.[2,3] Among other things, curcumin blocks several important cellular targets such as nuclear factor κ-B (NF-κB).[4–6] This interaction, in turn, induces apoptosis and retards the function of protein kinase C, epidermal growth factor receptor tyrosine kinase and human epidermal growth factor receptor-2 (HER-2).[7,8] Recent therapeutic efficacy against pancreatic cancer in a phase II clinical trial further supports the use of curcumin as a lead for the development of a new class of anti-cancer agents.[9] Unfortunately, due to the low potency and poor absorption characteristics of curcumin, its clinical potential may prove to be limited.[10] Nonetheless, the compound remains an ideal lead compound for design of derivatives with improved water solubility.[11]

About 100 curcumin analogs have been synthesized in our laboratory and tested for anti-cancer and anti-angiogenesis properties.[12] A subset of 10 was further evaluated in the 60 panel NCI cancer cell lines and in several in vitro anti-angiogenesis screens. Analog **1** with *ortho*-fluoro groups and its 2-pyridine analog **2** exhibit superior cytotoxic activities compared with other members of the series (Fig. 1). Analog **1** has been shown to inhibit the growth of cancer cells at a ca. 10-fold lower dose than curcumin,[12] induce apoptosis[13] and block the growth of human breast tumors in a mouse xenograft model with relatively low toxicity.[14] Our most recent study identified I-κ B kinase (IKKβ as an effective target for both compound **1** and curcumin, although the latter is less potent. Compound **1** rapidly blocks the nuclear translocation of NF-κB with an IC_{50} of 1.3 μM compared with curcumin with an IC_{50} of 13 μM.[15] In spite of the higher activity of **1** (EF24) and **2** (EF31) by comparison with curcumin, the low bioavailability and fast metabolism of these analogs still remains a critical problem for further development.

Our earlier study showed that **1** induces cell cycle arrest and apoptosis by means of a redox-dependent process in MDA-MB-231 human breast cancer and DU-145 human prostate cancer cells.[13] Compound **1**, containing a dienone moiety, serves as a Michael acceptor to deplete L-glutathione (GSH) and GSSG concentrations in both wild type and Bcl-x_L overexpressing HT29 human colon cancer cells. Comparable chemistry is utilized by a series of novel tyrosine kinase inhibitors developed by Smaill and co-workers, in which a mild Michael acceptor is appended to a quinazoline

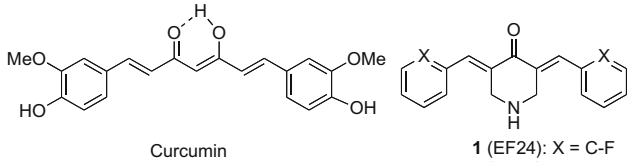

Figure 1. Structures of curcumin, analogs **1** and **2**.

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ring. The compounds engage in a very specific alkylation of Cys-773 in the ATP pocket of the EGFR. The α,β-unsaturated tyrosine kinase inhibitors are currently in Phase I clinical trial.[16,17] In addition, Dimmock et al. have long recognized the affinity of dienones for biological thiols,[18] and isolated ethanethiol and 1,2-ethanedithiol adducts of selected enones.[19] Unexplored, however, are the reversible or partially reversible nature of the compounds, which may offer potential advantages in terms of target suppression and in vivo pharmacokinetics.

The observation of glutathione depletion suggested a means to solubilize the curcumin analogs for evaluation in various cell-based assays. Thus, solubility comparisons indicated that while aqueous dissolution of curcumin is unaffected by GSH, high concentrations of the peptide are capable of drawing **1** into solution. (Fig. 2) The adduction with glutathione is illustrated in Scheme 1. A number of studies have demonstrated that depletion of thiol concentrations prior to treatment with various anti-cancer drugs has increased cell killing compared to the use of drug alone.[20,21]

Synthesis of GSH conjugates. Compound **1** or **2** dissolved in acetonitrile (CH_3CN) was added slowly to 2.1 equiv of GSH in water at room temperature. The yellow color of the dienone disappears as a result of eliminating the extended chromophore and the simultaneous formation of *bis*-adducts **1**- or **2**-$(GSH)_2$. Conjugation of GSH with **2** takes place instantaneously as reflected by the immediate disappearance of the yellow color of the unsaturated ketone, while conjugation with **1** takes several hours to complete. Nevertheless, both reactions provide *bis*-adducts as the only products along with free GSH based on LC/MS (Fig. 3a and b). No *mono*-adducts were detected under these conditions, although *mono*-adduct **3** is surely an intermediate to *bis*-adduct **4**. Accordingly, treatment of **2** with 1.0 equiv of L-glutathione delivers a mixture of *mono*- and *bis*-adducts, **2**-(GSH) and **2**-$(GSH)_2$, with the *mono*-adduct as the major product by MS. However, due to the similar polarities of **2**-(GSH) and **2**-$(GSH)_2$, no clear separation by LC was observed. Thus, a broad peak presumed to be overlapping bands of the two conjugates was observed (Fig. 3c).

In order to further investigate the reaction between thiols and curcumin analogs, several cysteine-containing dipeptides were prepared and their reactions with curcumin analogs, examined.

Synthesis of cysteine-containing dipeptides. The preparation of cysteine-containing dipeptides was initiated with free L-cysteine **5**. Due to the sensitivity of the cysteine molecule toward oxidation and elimination, it was necessary to protect the β-sulfhydryl moiety as well as the amino and/or carboxyl group during synthesis.[22]

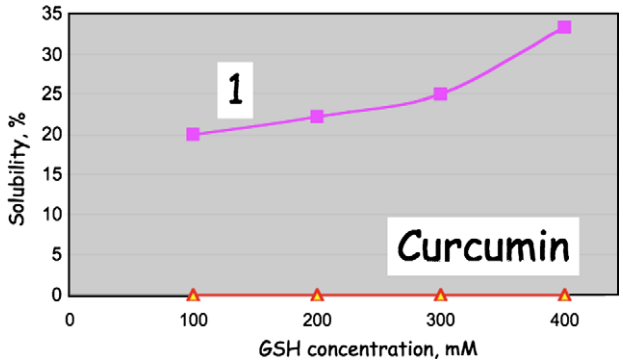
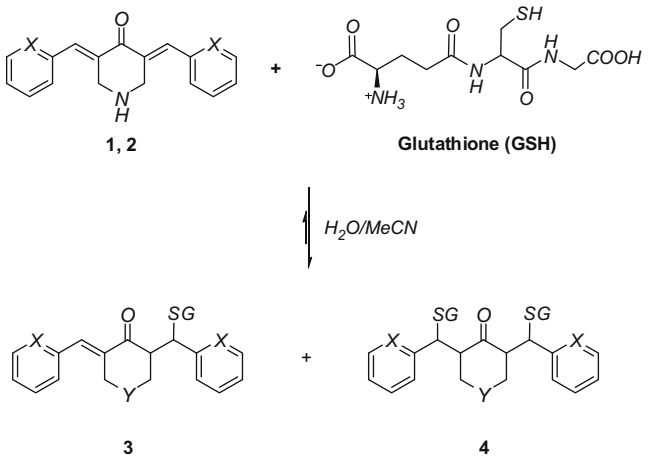


Figure 2. Solubility of **1** and curcumin in GSH-doped aqueous solution. Each point corresponds to 200 mM of compound, and thus a maximum **1**:GSH molar ratio of 1:1 and 1:2 at GSH concentrations of 200 and 400 mM, respectively. Powders of the enones and four GSH concentration were mixed and left at room temperature for 30 min. Insoluble **1** and curcumin were collected by centrifugation, and the pellets content (% compound undissolved) were determined by bioassay after dissolution in DMSO. Incomplete solubility of **1**-$(GSH)_2$ is due to the short 30 min mixing times employed in this experiment.



Scheme 1. Combination of **1** and **2** with GSH to give *mono*-adduct **3** and *bis*-adduct **4**.

Several methodologies for cysteine S-functionalization and protection have been reported previously.[23–26] Dimethyl-thiazolidine (Dmt) has been successfully employed as a sulfhydryl-amino masking group for cysteine in the synthesis of glutathione[27] and in the course of natural product syntheses.[28,29] The protecting unit can be removed under mild conditions to yield the corresponding aminoethanethiol moiety of cysteine. In the present work, Dmt-cysteine **6** was converted into the Boc acetonide derivative **7**. The latter was coupled with the protected amino acid hydrochloride salt **8** using EDCI and HOBt in DMF and further treated with diisopropyl ethyl amine (DIEA) to give Boc-Dmt-cys-amino acid-ester **9** in medium to good yields. Deprotection of the Dmt group with TFA delivered the Cys-Phe-methyl ester TFA salt **12**. Hydrolysis of **9** under basic conditions (NaOH) in dioxane delivered the Boc-Dmt-cys-amino acid **10**. Removal of the Boc group from **10** under acidic conditions (TFA) and subsequent concentration in the presence of EtOH and H_2O (1:1) led to the complete removal of the acetone generated and isolation of free amino and thiol-cysteine-dipeptide TFA salt **11** in excellent yield (95%) (Scheme 2).

Coupling of Cys-dipeptides with 1 and 2. Compounds **1** and **2** were treated with dipeptides Cys-Phe (**11**) and Cys-Gly (**12**), respectively. Reaction between **2** and Cys-Gly is instantaneous when the dienone in CH_3CN is added dropwise to Cys-Gly in water. The yellow color of **2** disappears immediately with the formation of **2**$(Cys\text{-}Gly)_2$ (**13d**), which was confirmed by LC/MS. By comparison, reaction between **1** and Cys-Gly as with GSH is much slower, requiring at least 7 h at room temperature. Both **1** and **2** Cys-Gly conjugates are white, water-soluble powders upon solvent evaporation. Conjugation with higher mass dipeptides, for instance Cys-Phe (**11**), proceeds much more slowly relative to Cys-Gly. Combination of **2** with Cys-Gly occurs instantly, while conjugation with Cys-Phe takes several hours to complete. Nonetheless, the products, **13a** and **13c** are also white, water-soluble powders (Scheme 3).

Previous studies of the interaction between curcumin and glutathione have suggested the operation of Michael addition between the α,β-unsaturated chromophore of curcumin and GSH. FAB-MS and MALDI-MS spectra of glutathionated curcumin have been interpreted in terms of *mono*- and di-glutathionyl-adducts as well as cyclic rearrangement products including feruloyl-methylketone and feruloylaldehyde.[30] A recent investigation by Usta et al. reports that a combination of glutathione and curcumin leads to the formation of two diastereoisomeric monoglutathionyl curcumin conjugates.[31] The structures of both conjugates were identified by LC/MS and one- and two-dimensional ^1H NMR analysis.