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Antitumor effects of curcumin and structurally β-diketone modified analogs on multidrug resistant cancer cells

Daniele Simoni,[a,*] Michele Rizzi,[a] Riccardo Rondanin,[a] Riccardo Baruchello,[a] Paolo Marchetti,[a] Francesco Paolo Invidiata,[b] Manuela Labbozzetta,[c] Paola Poma,[c] Valeria Carina,[c] Monica Notarbartolo,[c] Alessandra Alaimo[c] and Natale D'Alessandro[c]

[a]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Fossato di Mortara 17/19, 44100 Ferrara, Italy*
[b]*Dipartimento Farmacochimico Tossicologico e Biologico, Università di Palermo*
[c]*Dipartimento di Scienze Farmacologiche, Università di Palermo, Via del Vespro 129, 90127 Palermo, Italy*

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Abstract—Using concepts of bioisostery a series of curcumin analogs were synthesized: the diketonic system of the compound was elaborated into enaminones, oximes, and the isoxazole heterocycle. The cell growth inhibitory and apoptosis inducing effects of the new analogs were evaluated by in vitro assays in the hepatocellular carcinoma HA22T/VGH cells, as well as in the MCF-7 breast cancer cell line and in its multidrug resistant (MDR) variant MCF-7R. Increased antitumor activity on all cell lines was found with the isoxazole analog and especially with the benzyl oxime derivative; in the HA22T/VGH cell model, the latter compound inhibited constitutive NF-κB activation.

Drug resistance, either innate or acquired and especially in its multiple form (multidrug resistance, MDR), remains a major and difficult problem to resolve in the therapy of many cancer types. Today, it is recognized that it is most often a multifactorial and heterogeneous process, due to many possible molecular alterations, including the over-expression of multidrug efflux transporters (like, e.g., P-glycoprotein [ABCB1, P-gp]) and of factors (like BCL-2, Bcl-X_L, and Inhibitor of Apoptosis Proteins [IAPs]), which prevent tumor cell death from drugs and other therapeutic stimuli.[1,2]

The multiplicity of the drug resistance determinants poses the question of the optimal strategies to face them; reasonably, multi-targeted agents might be less likely to encounter problems of drug resistance than single-targeted ones. In this respect, there is an increasing evidence that the dietary polyphenols are endowed with a remarkable number of different antitumor mechanisms, yet accompanied by a limited toxicity for normal cells. Among these agents, curcumin (diferuloylmethane), extracted from *Curcuma longa* L. and present in curry spice, has a long story of use in Indian medicine for anti-inflammatory and other therapeutic purposes; it has exhibited definite tumor suppressive and preventive activities in many in vitro or in vivo models (for a review, see, e.g., Ref.3). The ability of curcumin to selectively induce apoptosis in cancer cells contributes to underline its high antitumor potential; moreover, the molecule has been described to efficiently induce cell growth inhibition and apoptosis even in different MDR tumor models.[4–7] Thus, many research groups have considered curcumin as a very good lead compound to design a large variety of analogs as potential new antitumor drugs; nevertheless, the results of the structural modifications have been only seldom investigated within the tumor drug resistance aspect.[4,8]

Curcumin is endowed with a diketone function, which appears to be important for its antitumor activity.[9–11]

For example, depending on the dose, the compound may show either pro-oxidant or anti-oxidant effects, which both may, at least in part, be linked to this structural moiety.[12] In the lower concentration, 'chemopreventive', range, curcumin is a documented anti-

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* Corresponding author. Tel.: +39 0532 455923; fax: +39 0532 455953; e-mail: smd@unife.it

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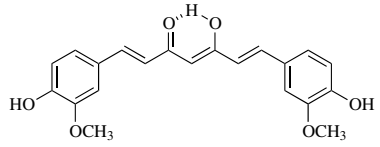
oxidant, although this activity may possibly provide negative interactions with other chemotherapeutic agents.[13] At higher concentrations, the α, β-unsaturated ketone, as a Michael acceptor, can form adducts with the –SH groups and generate reactive oxygen species.[14] This may lead to induction of apoptosis through different possible mechanisms involving endoplasmic reticulum stress, loss of mitochondrial membrane potential, activation of terminal caspases or also other mitochondria- and caspase-independent pathways.[6,15,16] Inhibition of the signaling by the transcription factor NF-κB, and suppression of the Bcl-2 and IAP family proteins, is also considered to be important for the pro-apoptotic activities of curcumin.[16]

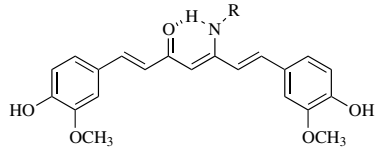
Here we planned to evaluate the consequences of bioisosteric substitutions at the 1,3 dicarbonyl fragment of curcumin with enamino- and oxime functions with regard to its antitumor activity on some cancer cell lines. Curcumin may exist in equilibrium between the diketo and keto-enol forms; the keto-enol form is strongly favored by intramolecular H-bonding.[17] Thus, a series of structurally related β-enaminoketone derivatives **2**–**6** of curcumin were envisaged as suitable bioisostere analogs also stabilized by intramolecular H-bonding (Fig. 1).[18] Additionally, we utilized the bioisosteric substitution of the ketone moieties with oximes to obtain derivatives **8** and **9**.

Finally, the isoxazole derivative **7**, which was used as a precursor of the enaminone **2**, was also included in the pool of derivatives tested in our biological assays. The compound has been previously reported as a good antioxidant and cyclooxygenase (COX) inhibitory agent.[19]

We examined the cytotoxic and pro-apoptotic effects of curcumin and of its new analogs in the breast carcinoma line MCF-7 and its MDR variant MCF-7R. The latter cell line was established treating the wild type cells with gradually increasing concentrations of doxorubicin. The concentration of doxorubicin which causes 50% inhibition of cell growth (IC_{50}) in MCF-7R is 17.1 μM, which is about 92 times higher than the original IC_{50}. Further, in contrast to MCF-7, MCF-7R lacks estrogen receptor alpha expression, is estrogen-insensitive and over-expresses P-gp, different IAPs and COX-2.[20] Curcumin and its derivatives were tested also in the hepatocellular carcinoma HA22T/VGH cell line, which is known to innately produce remarkable amounts of drug resistance and anti-apoptotic factors.[16]



Curcumin (**1**)



2-6

Figure 1. Curcumin exists in solution as an equilibrium mixture of the symmetrical dienone and the keto-enol tautomer stabilized by intramolecular H-bonding. Enaminones are also tautomers stabilized by intramolecular H-bonding.

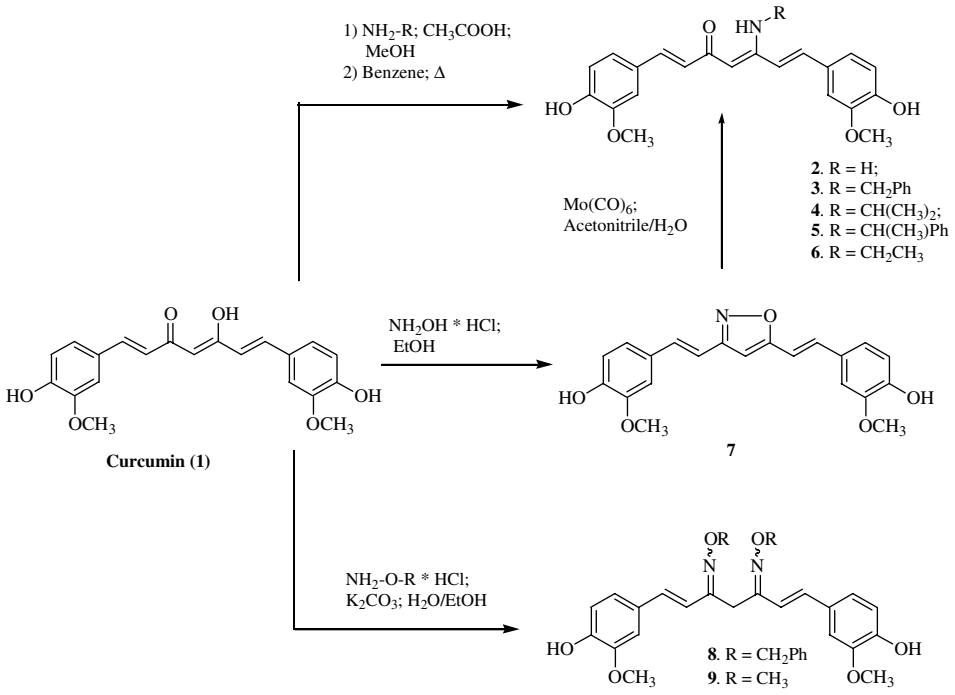
Scheme 1 summarizes the procedures used to prepare the curcumin analogs described in this paper. The enaminone analogs **2**–**6** were prepared in 25–35% yield, adapting literature procedures.[21] The previously prepared acetate salt of the opportune amine was suspended in benzene and to the mixture were added curcumin and acetic acid. The mixture was heated at reflux with water removal using a Dean–Stark apparatus. Spectroscopic NMR data of compounds **2**–**6** in dimethylsulfoxide-d_6 and $CDCl_3$ showed an exchangeable 1H peak at 11.8 together with a CH singlet at 5.6–5.7 ppm as confirmation of an intramolecular hydrogen bond due to enaminone tautomerism.[22]

Since curcumin appears to have the possibility to react with both the 1,3-diketone moiety and the α, β-unsaturated portions, the isoxazole analog **7** was prepared mainly to confirm the exact structure of the synthesized enaminones. Compound **7** was prepared by adding hydroxylamine hydrochloride to a solution of curcumin in ethanol and heating the resulting mixture at reflux temperature for 4 h.[23] Spectral data were in agreement with data reported in the literature.

Ring opening of the isoxazole system of compound **7** by treatment with molybdenum hexacarbonyl ($Mo(CO)_6$) in acetonitrile and some drops of water afforded in appreciable yield (75%) the β-enaminoketone **2**, identical to compound obtained with previous procedure. Similarly, having this information, we assigned the same structure to the derivatives obtained with more complex amino reagents.

The dioxime analogs **8** and **9** were synthesized by the addition of a solution of curcumin and *O*-methyl or *O*-benzylhydroxylamine hydrochloride (2 equiv) in 25% water–ethanol to a solution of potassium carbonate in water. The mixture was refluxed for 25 min, cooled, and concentrated in vacuo to remove most of the ethanol to give a residue. Chromatographic separation from unreacted curcumin led us to obtain a mixture of analogs **8** or **9** in 40% and 32% yield, respectively.[24] Structural evaluation of isolated mixtures was complicated by a multiplicity of isomers deriving from possible different stereochemistry of both oxime functions. A univocal ESI mass determination confirmed the expected molecular weight but, at the same time, HPLC analysis showed nearly unseparable three peaks, and NMR data give signal multiplicity.[25] Moreover, all evidences from NMR spectra in dimethylsulfoxide-d_6 and $CDCl_3$ are suggesting that, differently from compounds **2**–**6**, no intramolecular hydrogen bonding was present in **8** and **9** structures.

Curcumin **1** and its derivatives **2**–**9** were examined for their activity toward the MCF-7 breast cancer cell line



Scheme 1.

and its MDR variant MCF-7R. Through cell growth inhibition assays,[16] we observed that the β-enaminoketone compounds bearing a *N*-alkyl substituent **2**, **4**, and **6** had comparable or somewhat lower cytotoxic activity than curcumin in MCF-7 and in MCF-7R (Table 1). On the other hand, compounds **3** and **5**, bearing an aromatic portion at the amine substituent, were found to be not active. Similar results were observed also in the hepatocellular carcinoma cell line HA22T/VGH. Differently, the results obtained with the isoxazole **7** were of more interest. The IC_{50} and IC_{70} values showed its greater potency, which was about twice that of curcumin both in the MCF-7 and in the MCF-7R cell line. For the dioxime derivatives **8** and **9**, the results of Table 1 showed a difference between the methyl derivative **9** and the benzyl compound **8**, being the latter consistently more active, in particular the most active among all the compounds tested in the cell lines. Apparently, there seems to be an inverse correlation between activity of enaminones versus oximes and their alkyl/aryl substituent nature.

Evaluations of cell death induction by flow cytometry analysis[16] yielded results in good agreement with the cytotoxicity data. In particular, Figures 2 and 3 show that in all the cell lines the order of potency in determining cell death was: compound **8**> compound **7**> curcumin. There was also further evidence that curcumin and compounds **7** and **8** exert in MCF-7R cells antitumor effects comparable to those achieved in MCF-7 (Fig. 3). The results on PI staining of DNA were very well confirmed by flow cytometry assessments of Annexin V binding to cell surface (not shown).

As said before, inhibition of NF-κB signaling may be important for the proapoptotic activities of curcumin. Since, in contrast to MCF-7 and MCF-7R cells, HA22T/VGH, show constitutive activation of NF-

Table 1. Cell growth inhibitory effects of the compounds evaluated after 72 h of treatment by MTS assays

Compound	HA22T/VGH		MCF-7		MCF-7R	
	IC_{50} (μM)	IC_{70} (μM)	IC_{50} (μM)	IC_{70} (μM)	IC_{50} (μM)	IC_{70} (μM)
Curcumin (1)	**17.4 ± 1.2**	**24.6 ± 1.8**	**29.3 ± 1.7**	**51.7 ± 2.2**	**26.2 ± 1.6**	**46.2 ± 3.9**
2	33.0 ± 1.4	41.1 ± 2.8	49.9 ± 4.6	68.1 ± 5.2	54.7 ± 4.6	81.8 ± 6.7
3	>100	>100	>100	>100	>100	>100
4	26.3 ± 2.9	29.8 ± 3.1	70.3 ± 8.0	93.5 ± 7.2	49.8 ± 6.6	79.5 ± 8.4
5	>100	>100	>100	>100	>100	>100
6	26.1 ± 3.3	64.4 ± 5.5	93.0 ± 6.7	>100	67.6 ± 7.4	88.8 ± 5.4
7	**12.8 ± 1.5**	**18.3 ± 1.7**	**13.1 ± 1.6**	**23.2 ± 2.9**	**12.0 ± 2.0**	**24.7 ± 2.7**
8	**5.9 ± 1.2**	**8.4 ± 0.6**	**7.1 ± 0.2**	**9.2 ± 0.6**	**9.3 ± 1.7**	**12.9 ± 1.9**
9	32.9 ± 1.2	40.4 ± 2.6	36.3 ± 2	43.4 ± 1.9	39 ± 5	52.8 ± 6.8

Data are expressed as the concentrations which inhibit 50% (IC_{50}) or 70% (IC_{70}) cell growth and are means ± SE of at least three separate experiments. Data of curcumin (**1**) compared with compounds **7** and **8** have been highlighted with bold characters.

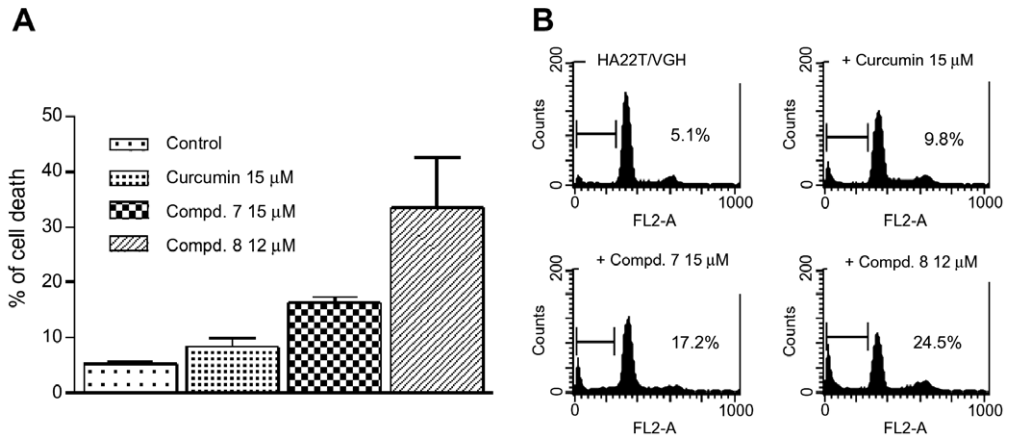


Figure 2. Induction of cell death in HA22T/VGH cells. (A) The cells were incubated with the compounds for 48 h and thereafter cell death was evaluated by flow cytometry analysis of cell DNA stained with propidium iodide. Data are means ± SE of two separate experiments; (B) A representative analysis. The percentages of the events in the preG$_o$-G$_1$ position are indicated in the panels.

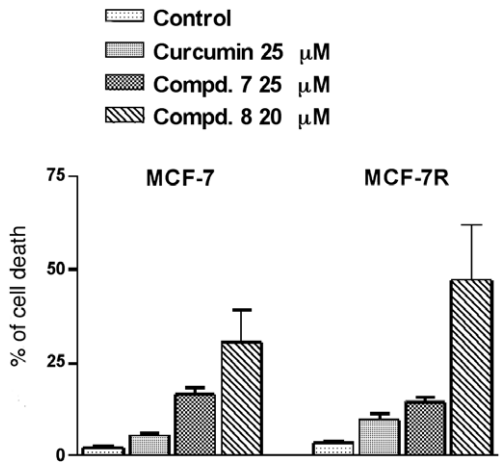


Figure 3. Induction of cell death in MCF-7 and MCF-7R cells. The cells were incubated with the compounds for 48 h and thereafter cell death was evaluated by flow cytometry analysis of cell DNA stained with propidium iodide. Data are means ± SE of three separate experiments.

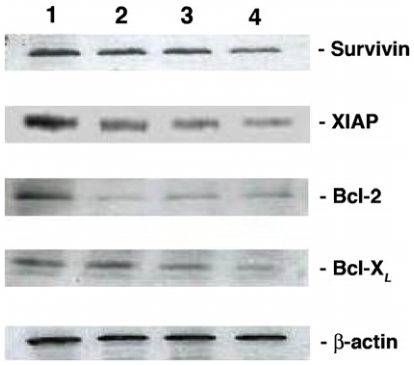


Figure 4. Western blot analysis of the levels of survivin, XIAP, Bcl-2 and Bcl-X$_L$ in HA22T/VGH cells treated for 24 h with the compounds. Lane 1: control; lane 2: curcumin (25 μM); lane 3: compound **7** (25 μM); lane 4: compound **8** (20 μM). A repeat experiment gave very similar results.

κB,[16,20] we examined in the latter cells the effects of curcumin and of compounds **7** and **8** on the DNA-binding activity of NF-κB (p65 subunit). At the concentration of 20 μM, only compound **8** consistently reduced (33.7% and 64.3% of the control after 1.5 and 4 h, respectively) NF-κB activation in HA22T/VGH cells. Further, NF-κB is able to up-regulate different anti-apoptotic factors, including Bcl-2, Bcl-X$_L$ and IAP family members like survivin and XIAP.[16,26] Western blot analyses (Fig. 4) showed that after 24 h of treatment of HA22T/VGH cells, curcumin and compounds **7** and **8** reduced the levels of these factors; the decreases were particularly pronounced in the case of compound **8**.

The data of this work show that small modifications can give access to compounds presenting antitumor activities superior to those of curcumin. Further, they suggest that the diketone fragment of curcumin is not indispensable for these activities. Interestingly, others have reported that replacement of the diketone fragment by an isoxazole or pyrazole ring increases the inhibitory effects of curcumin on COX-2.[19] This enzyme, strongly involved in many aspects of tumor progression and drug resistance, was previously found to be abundantly expressed in our cell lines.[16,20] In other studies, the pyrazole analog of curcumin inhibited with high selectivity and potency the proliferation of bovine aortic endothelial cells, thus candidating as a potential anti-angiogenic agent,[27] or strongly increased the cytotoxicity against various tumor cell lines.[28]

Our data underline that curcumin, as well as the derivatives studied herein, including the potent compounds **7** and **8**, exert unaltered antitumor activity on breast cancer cells with a MDR phenotype due to different mechanisms together. In fact, it is noteworthy that in the HA22T/VGH model the marked pro-apoptotic activity of compound **8** appeared to match with its ability to inhibit constitutive NF-κB activation as well as the expression of anti-apoptotic factors like survivin, XIAP, Bcl-2, and Bcl-X$_L$. Also curcumin and compound **7** decreased, although less strongly, the same factors, but not NF-κB,

suggesting a mechanism different from the interference with the transcription factor.

Overall, the structure of curcumin may represent an important basis for the development of very effective anticancer agents even in MDR tumors, also without retaining the diketone moiety. Moreover, some modifications at this portion have led to obtain more information about different biological activities on several cancer cell lines. A better understanding of these mechanisms may be of interest to evaluate the importance of novel curcumin derivatives within the tumor drug resistance aspect.

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