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ORIGINAL PAPER

Photocytotoxicity of some curcumin derivatives

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Abstract

Curcumin, a well-known active compound with various biological effects also has interesting photochemical properties, including a wide range of photocytotoxic capabilities. This study aims to analyze if these properties are modified when curcumin is complexed with divalent manganese ions and when curcumin is conjugated with triphenylphosphonium (TPP) radicals. Using malignant line cells and strains of Staphylococcus Aureus we studied three compounds including curcumin, manganese-curcumin, and a manganese-curcumin triphenylphosphonium derivative. The viability of the treated cells with or without photoactivation was assayed using tetrazolium dye MTT tests, flow cytometry analysis and dilution antibiogram tests. The results showed that the manganese and triphenylphosphonium (TPP) radical addition generates a significant improvement in photocytotoxicity only for the malignant line cells and has no or even opposite effects regarding the antibacterial properties of the studied compounds.

Keywords: curcumin, triphenylphosphonium radical, phototoxicity, *Staphylococcus Aureus*, manganese complexes.

1. Introduction

Curcumin is an important natural product obtained from a cultivated plant, *Curcuma Longa.* Many plant species have gained the position of major producers for various compounds (S.M. PETRE & al. [1]; P. DOBRE & al. [2]; P. DOBRE & al. [3]), especially for molecules with therapeutic uses (P. ANAND & al. [4]; D.K. AGRAWAL & al. [5]).

The photoactivation of curcumin and its effects on various *in vitro* models have been intensively studied, both on eukaryotic and prokaryotic cells (P. ANAND & al. [4]; D.K. AGRAWAL & al. [5]; A. MARCHIANI & et al. [6]). Due to its special structure, this compound is able to exhibit a large range of biological effects, noteworthy cytostatic and antibacterial effects (P. ANAND & al. [4]; D.K. AGRAWAL & al. [5]; A. SHEHZAD & al. [7]). Many studies also revealed that the majority of the biological effects of curcumin are enhanced after a simple visible light irradiation (K.I. PRIYADARSINI [8]; K.I. PRIYADARSINI [9]). This interesting behavior could be important evidence regarding the mechanism of action through photoactivation, which seems to be supported by oxygen singlet generation (T. QIAN & al. [10]; M. SUBRAMANIAN & al. [11]).

One major drawback regarding the therapeutic use of curcumin in various pathologies is represented by its poor bioavailability, mainly due to its low absorption, rapid metabolism, and quick elimination (P. ANAND & al. [4]; A. SHEHZAD & al. [7]; E. BURGOS-MORON & al [12]). Being a lipophilic compound, the intestinal absorption and stability are affected after absorption, which leads to very low serum concentrations of curcumin, hence to insufficient activity for the target cells (A. NOORAFSHAN & al. [13]; M. ABD EL AZIZ & al. [14]). Many strategies were used in order to improve the chemical stability and to increase the concentration of curcumin in the targeted cells (A. NOORAFSHAN & al. [13]; P.A. SUBRAMANI & al. [15]; S.F. NABAVI & al. [16]). The present study uses two major methods for enhancing curcumin stabilization and properties: metal complexation and the triphenylphosphonium radical attachment.

Metal complexation was done with divalent manganese ions due to the special redox properties of these metal ions. In addition to the chemical stability, this metal complexation also provide other enhancements, like a better cytotoxic effect (S. BANERJEE & al. [17]) and a superoxide dismutase like effect (O. VAJRAGUPTA & al. [18]), suggesting redox improvements compared with normal curcumin.

Using the triphenylphosphonium radical as a covalent attachment to curcumin has at least one important indication. The structure of the radical (lipophilic structure, positive charge) explains the preferential mitochondrial accumulation of the compounds, which today form a new class of so-called mitocans (J. NEUZIL & al. [19]; T. HAHN & al. [20]). Another major advantage for the mitochondrial targeted compounds seems to be the better affinity for the malignant cells. This selectivity could be explained by the differences between the mitochondrial membrane potential of normal and malignant cells, since many solid tumors have a more negative mitochondrial membrane potential compared to their normal counterparts (M. MILLARD & al. [21]; J.S. MODICA-NAPOLITANO & al. [22]).

In conclusion, all the variations presented above (photoexcitation, metal complexation and triphenylphosphonium radical attachment) represent major enhancements for the biological properties of curcumin (Fig. 1, 2, 3). The aim of this study was to verify if these enhancements works together both for cytostatic and for antibacterial effects.

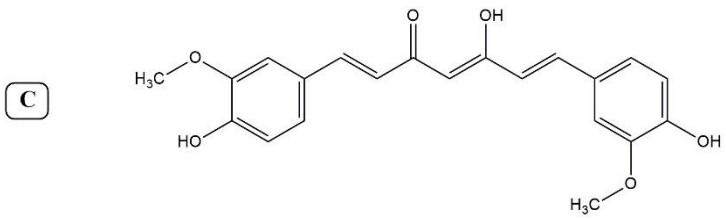


Fig. 1. Curcumin (C).

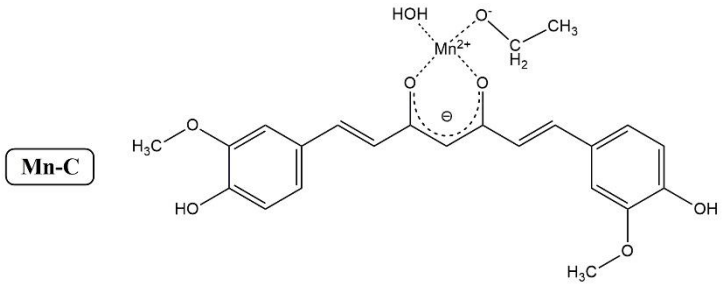


Fig. 2. Divalent manganese complex of curcumin (MnC) (O. VAJRAGUPTA & al. [18]).

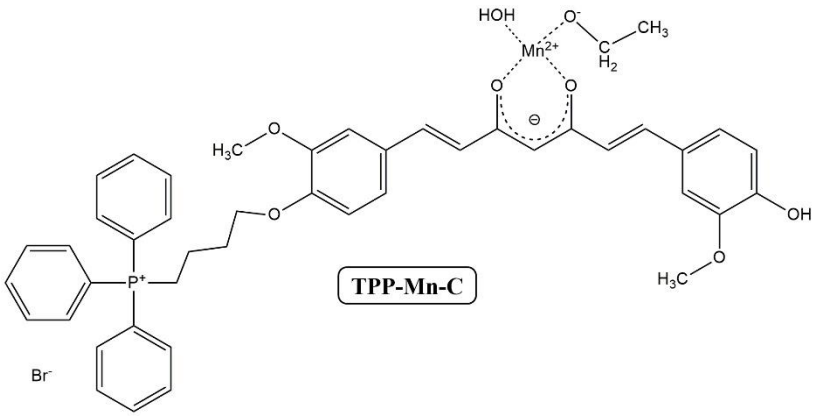


Fig. 3. Divalent manganese complex of triphenylphosphomium-curcumin (TPPMnC) (B. STOICA & al. [23]).

2. Materials and methods

2.1. Tested compounds

Curcumin (C) and manganese acetate were obtained from Sigma-Aldrich (St. Louis, USA) and used without further purification. The manganese complex of curcumin (MnC) and the manganese complex of triphenylphosphomium-curcumin (TPPMnC) were synthetized and characterized in the lab as mentioned previously (O. VAJRAGUPTA & al. [18]; B. STOICA & al. [23]). All the curcumin derivatives were assayed at different concentrations (1 – 1200 μM) from stock solutions made in dimethyl sulfoxide (DMSO), taking into account that the final DMSO concentration is less than 1%. For MnC and TPPMnC a special protocol with cyclodextrin was used, in order to increase solubility (B. STOICA & al. [23]).

2.2. Cell cultures

All the curcumin derivatives were tested on 2 malignant cell lines: HOS (human osteosarcoma cells), and Jurkat cells (T-leukemic cell line). The cell lines were purchased from Cell Lines Service CLS (Eppelheim, Germany). Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2% sodium pyruvate, non-essential amino acids (2 mM), penicillin (100 units/ml), streptomycin (100 mg/ml), and glutamine (4 mM). After achieving 70% confluence at 37^0 C in a humidified atmosphere of 5% CO2 and 95% air, cells were detached with trypsin-EDTA (HOS cells), counted and placed in 96-wells plates at a density of 2 x 10^4 cells/well.

2.3. MTT viability testing

Cellular viability was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) test (J. CARMICHAEL & al. [24]). After pipetting cells in 96-well dishes, an incubation time of 48 hours allowed the contact between cells and compounds at different concentrations. Adding a MTT solution (0.3 mg/mL) to each well for 4 hours at 37^0 C, removing the supernatant and solubilizing with 200 μL DMSO made possible the absorbance reading at 570 nm. All tests were assayed in triplicate.

2.4. Bacteriology studies

A strain of *Staphylococcus Aureus* ATCC 25923 (methicillin-susceptible strain, MSSA) was purchased form American Type Culture Collection (Manassas, VA, USA) and stored in 30% glycerol and frozen at -70^0 C before use. After suspension in Mueller–Hinton broth (MHB) and incubation at 37^0 C for 24 hours, a dilution antibiogram was performed (M. BALOUIRI & al.

[25]): two-fold dilutions of the curcumin derivatives were dispensed in 96-well microtitration plates together with a standardized microbial suspension adjusted to 0.5 McFarland scale. All the tests were performed with and without irradiation (LED source, 2.0 mW/cm2, 20 minutes) and the lowest concentration of antimicrobial agent that completely inhibits growth was defined as MIC.

2.5. Flow cytometry analysis

For the evaluation of cell death, Jurkat cells were labelled with Annexin V-FLUOS (Roche) and 7-AAD (7-Aminoactinomycin D from Sigma-Aldrich), and analyzed by FACS (Annexin/7-AAD labelling assay) (N. SASSI & al. [26]; R.L. HARDING & al. [27]). Briefly, after the treatment with compounds at concentrations in the range of IC_{50} for 24 hours, a 200 µL aliquot of each sample was incubated with 7-AAD (final concentration 1 µg/mL) and Annexin V-FLUOS (1 µL/sample) in the dark at 37^0 C, for 20 minutes. Samples were finally analyzed by FACS (5000 cells for each measurement), using a FACSAria III instrument (BD Biosciences).

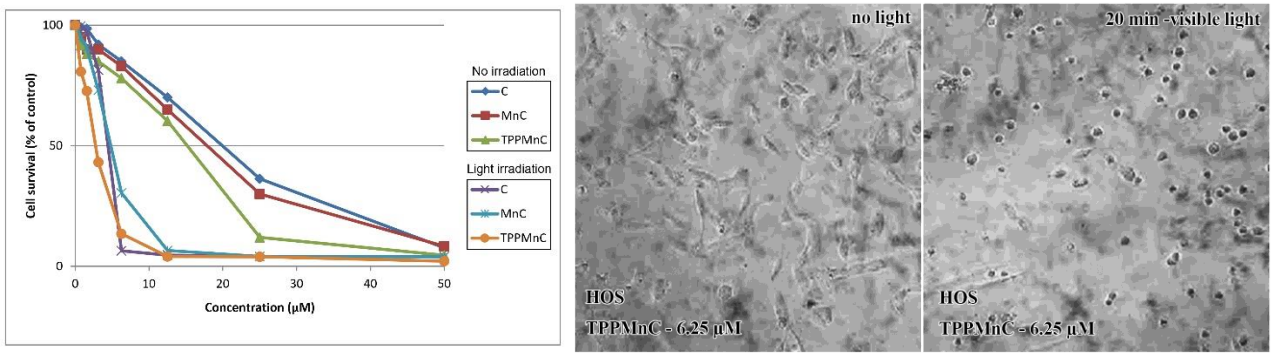
2.6. Photochemical activity

Cell cultures and bacterial strain were illuminated by a 48 white LED matrix device, providing a fluence of 2.0 mW/cm2. All the compounds were mixed with HOS cells ($2x10^4$ cells/well) 2 hours before illumination, at micromolar concentrations (1 – 50 µM) in two 96-well plates and one of the plates was irradiated for 20 minutes (with the light source at 1 cm distance from the plate and a mirror placed on top), avoiding a temperature increase more than 37^0 C. All the experiments were made in triplicate, at 37^0 C, in a humidified atmosphere of 5% CO2 and 95% air. After 48 hours, the cell viability was assessed by the MTT assay.

3. Results and discussions

3.1. Photochemical activity on HOS human osteosarcoma cells

The results revealed that 20 minutes of visible light irradiation increased the cytotoxicity for all the tested compounds (Fig. 4). It is worth noting that the differences between simple curcumin and manganese complexes of curcumin were smaller in these conditions. Interestingly, the manganese complexes Mn-C and TPP-Mn-C revealed a major loss of cytotoxicity when solved in DMSO and kept at room temperature for at least 24 hours, but the photochemical cytotoxicity remained practically unchanged (data not shown).



(A) (B)

Fig. 4. Effect of visible light irradiation on the viability test results for HOS human osteosarcoma cells. (A) MTT viability test results after 20 minutes of irradiation. (B) HOS cells were

photographed after 48 hours following exposure to 6.25 μM TPP-Mn-C without and with light irradiation.

3.2. Flow cytometry analysis

Flow-cytometric analysis with annexin V/7-AAD staining showed that Jurkat cells exposed to curcumin for 24 hours without irradiation undergo an apoptosis process which is concentration dependent (Fig. 5). Both early apoptotic cells (Annexin- V+/7- AAD−) and late apoptotic cells (Annexin- V+/7- AAD+) were present when the concentration of the tested compounds exceeded 6.25 μM. For Jurkat cells, the highest apoptosis percentage was achieved with TPP-Mn-C. After 20 minutes of irradiation, the concentrations required for the induction of apoptosis in 50% of the cells decreased to almost half for all the tested compounds (Table 1).

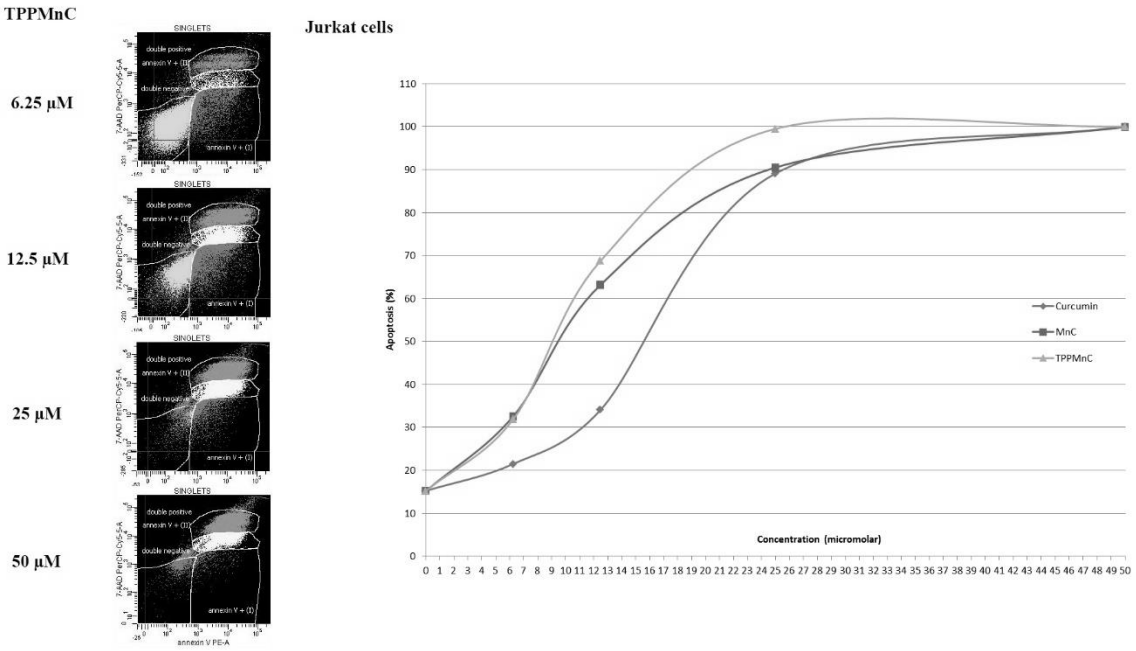


Fig. 5. Assessment of apoptosis by flow cytometry in curcumin-treated Jurkat cells.
Curcumin and manganese complexes of curcumin induced a significant level of apoptosis in Jurkat cells (right side graph). On the left, an example of flow cytometry analysis strategy is depicted. Various stages of apoptosis may be identified: early (annexin V+ (I)), intermediate (annexin V+ (II)) and late (double positive) apoptotic cells. The sum of all these stages was considered for the final calculation. Double negative events are considered viable cells.

Table 1. Effect of light irradiation for the studied compounds on Jurkat cells. Micromolar concentrations needed for 50 % apoptosis induction were calculated from the flow cytometry data.

	No irradiation	Irradiation - 20 minutes
Curcumin (μM)	16.1 ± 1.2	8.2 ± 1.1
MnC (μM)	9.5 ± 1.1	4.8 ± 0.7
TPPMnC (μM)	8.9 ± 0.9	4.3 ± 0.6

3.3. Photochemical activity against *Staphylococcus Aureus*

As depicted in Table 2, the antibacterial properties of curcumin alone against *Staphylococcus Aureus* seems to be modest but are strongly enhanced after 20 minutes of visible light irradiation, results that are consistent with literature data. The presence of divalent manganese ions in unirradiated samples decreased the antibacterial activity both for curcumin and for the triphenylphosphonium variant of curcumin. These antibacterial properties were not improved through irradiation, on the contrary, they are diminished for the triphenylphosphonium variant of the manganese complex.

Table 2. MIC values of the curcumin derivatives evaluated with dilution antibiogram for *Staphylococcus Aureus* with/without visible light irradiation.

	No irradiation	20' irradiation
Curcumin (μM)	300 [a]	5 [b]
MnC (μM)	600	600
TPPMnC (μM)	600	> 1200

- consistent with literature data: [a] (S.Y. TEOW & al. [28]; [b] A.P. RIBEIRO & al. [29]).

4. Conclusions

All the obtained data showed an evident improvement of cytotoxicity for curcumin after manganese complexation and the attachment of triphenylphosphonium radical. The cytotoxic effects were augmented after 20 minutes of light irradiation for all the compounds.

Regarding the type of cellular death, flow cytometry data suggest that the main type of death is apoptosis. A large amount of data already described the induction of apoptosis in various types of cells by curcumin alone (N. HAIL [30]; F. THAYYULLATHIL & al.[31]; J. CAO & al. [32]; S. NAGAI & al. [33]). The results of this study show that the behavior of curcumin manganese complexes is similar and occurred at lower concentrations if the complexes were photoactivated.

All the tested compounds exhibit photochemical proprieties but the manganese complexes Mn-C and TPP-Mn-C showed some advantages compared with curcumin alone after irradiation with visible light. The results could be explained by the metal complexation, which is able to modify the electrons conjugation and density around the keto groups. The β-diketone moiety of curcumin induces intramolecular keto–enol tautomerism (K.I. PRIYADARSINI [9]) which is responsible for at least part of the curcumin photochemical properties. The presence of manganese ions induced changes in this molecular area with benefits in terms of cytotoxicity or stabilization and advantages in terms of photochemistry. Also, the mechanism of action for curcumin as a photosensitizer could be different if compared with the multiple mechanisms of cytotoxicity for unirradiated curcumin. This hypothesis is also supported by the behavioral differences of curcumin and manganese complexes if the DMSO solutions are kept for more than 24 hours at room temperature, when the cyototoxic effect is strongly decreased without light**,** but the photochemical cytotoxic effect is preserved.

All the compounds presented in this study showed the possibility of tuning a natural product from a plant (*Curcuma Longa*) with various chemical procedures. As many other plants, *Curcuma Longa* can be cultivated using different biotechnologies in order to improve the amount

and the quality of the final product (F. IMBREA & al [34]; S. PETRE & al [35]; S. CRISTEA & al [36]).

A significant loss of photochemical properties was found when the antibacterial activity against *Staphylococcus Aureus* was tested. Despite curcumin photochemical efficiency, the manganese complexes lost their photoactivation potential presumably due to their limitations in penetrating the bacterial wall.

All the chemical variations of curcumin presented in this study have shown a promising potential, especially on the photocytotoxic effects on malignant cells, which must be exploited in future studies.

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