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ORIGINAL ARTICLE

Synergistic interaction between a novel mixed ligand copper(II) chelate complex and a panel of anticancer agents in T47D human breast cancer cells *in vitro*

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Summary

Purpose: *We have developed a novel copper(II) chelate complex with a tridentate ONN-Schiff base ligand and the anion of salicylate, which presented a potent cytotoxic activity against a panel of human and murine cancer cell lines. In this experiment we explored the combined effect between Cu(SalNEt$_2$)salicylate (Cu-Sal) complex and the widely used anticancer drugs carboplatin (CBDCA), cyclophosphamide (CTX) and paclitaxel (TXL) against T47D human breast cancer cells. Theoretical (quantum-chemical) study of this complex and its adducts with biological molecules were carried out, aiming at the elucidation of the underlying mechanism of action.*

Materials and methods: *Cells grown in adherence in 96-well microplates were exposed simultaneously to both agents for 48 h. Drug cytotoxicity was assessed via the XTT colorimetric assay. The combined drug interaction was assessed with the median-effect analysis and the combination index (CI).*

Results: *Copper(II) salicylate complex was proved active against T47D human breast cancer cells. Concurrent treatment of cells with Cu-Sal complex and the chemotherapeutic drugs CBDCA, CTX and TXL, mainly showed a synergistic interaction in most concentration ratios.*

Conclusion: *Cu-Sal complex interacts synergistically with tested chemotherapeutic drugs for most schedules of administration, and only occasionally an additive or antagonistic effect was apparent. With the aid of quantum-chemical calculations it was demonstrated that the mechanism of action of this complex involves binding to DNA and RNA. These findings prompt to search for possible interaction of this complex with other cellular elements of fundamental importance in cell proliferation.*

Key words: combination effect, copper complexes, Cu(SalNEt$_2$)salicylate, cytotoxicity, synergy

Introduction

The understanding of the molecular basis of a drug action and the exploration of the chemical interactions involved in the complex processes of drug delivery and reaction with a variety of biological molecules are among the most important goals of current drug design. Since the original discovery of anticancer effects of platinum compounds, numerous studies have been undertaken to determine what relationships exist between chemical structure and antitumor activity with a view to find more active drugs. Transition metal complexes have been studied as potential anticancer chemotherapeutic agents under the assumption that toxic heavy metals are necessary for active complexes. However, a complementary alternative is to consider the properties of coordination compounds involving biologically essential metals such as copper, zinc and iron.

Transition metal complexes with biological activity have gained prominence due to the success of the platinum anticancer drug cisplatin (*cis*-[$PtCl_2(NH_3)_2$]).

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Newer analogs of cisplatin, with reduced side effects, are currently in clinical use or undergoing trials [1]. However, there is still a need for agents active against different tumors, or cisplatin-resistant tumors. To date several Cu(II) complexes have been tested as potential anticancer agents. A promising class of compounds with demonstrated bioactivity are hydrazones of pyridoxal and the structurally related salicylaldehyde. An example is the tridentate ligand salicylaldehyde benzoylhydrazone (H_2sb) whose copper(II) complex [Cu(Hsb)Cl]·H_2O was shown to be a potent inhibitor of DNA synthesis and cell growth and more effective than the metal-free chelator [2].

Structural studies with many copper(II) complexes conclude that a NNS or NNO tridentate ligand system was a common feature of numerous compounds with cytotoxic or cytostatic potency. It is likely that the biological activity is due to the ability of formation of tridentate chelate complexes with biological essential heavy metals as copper [3]. These ligands when coordinated to a metal ion can modify factors like geometry, charge of the complex, lipid solubility etc., and thus enhance or lower their growth inhibitory properties. Transition metal compounds with such ligands are very potent chemotherapeutic reagents.

In accordance to this approach, we have directed our efforts towards the synthesis and biological study of 4-coordinate copper(II) chelate complexes with a tridentate ONN-Schiff base ligand. We have reported results concerning the synthesis, structure elucidation, and antineoplastic activity of some novel copper(II) complexes of the general formula Cu(SalNEt_2)Y, where SalNEt_2 stands for the anion of the N-(2-(diethylamino) ethyl)salicylidenaminato ligand and Y is the anion of a carboxylic or a dicarboxylic acid. The chemical structure of Cu(SalNEt_2)Salicylate complex is demonstrated in Figure 1.

In our previous studies, chelate complexes of Cu(II) of the general formula Cu(SalNEt_2)Y showed exceptional antiproliferative and/or cytotoxic action against HeLa-S3 human cervical cancer cells [4]. In another study, an important synergistic action in various combinations between Cu(SalNEt_2)Y complexes and a panel of chemotherapeutic drugs against various cell lines was observed, increasing cytotoxic activity of the examined drugs [5]. Furthermore, the combination of ascorbic acid with a Cu(II) complex has caused a site-specific disruption of supercoiled DNA in different conditions. The combination of these Cu(II) ions with ascorbic acid produces hydroxyl radicals (OH·) with a known mechanism [6].

In this series of experiments we have focused on one hand, in the study of the possible anticancer activity of Cu(SalNEt_2)Salicylate through *in vitro* experiments, and on the other hand, in the theoretical (quantum-chemical) study on interactions of this complex and its adducts with biological molecules. This complex has shown a potent cytotoxic activity against a panel of human and murine cancer cell lines, inducing inhibition of cellular growth and apoptotic cell death via DNA fragmentation. Cu-Sal complex was found to be more active against leukemic and lymphomatic cell lines (unpublished data).

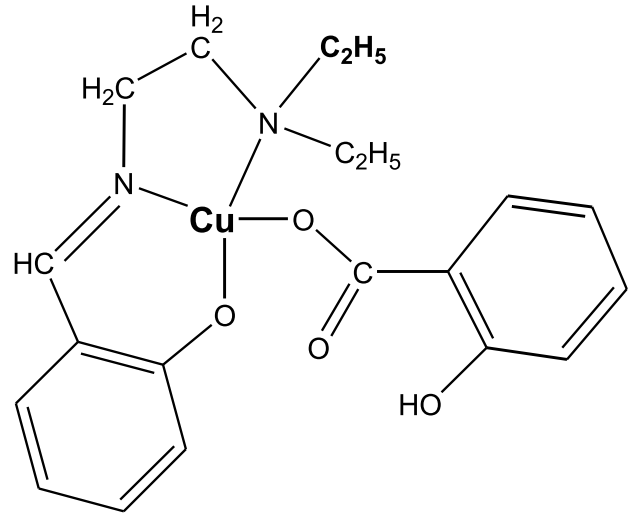

Figure 1. Chemical structure of Cu(SalNEt_2)Salicylate.

The aim of the current *in vitro* study was to explore the combined effect between Cu(SalNEt_2)salicylate (Cu-Sal) complex and 3 widely used drugs in cancer chemotherapy against T47D human breast cancer cells. Cu-Sal complex has been evaluated for its potential to inhibit growth of T47D cells, after simultaneous treatment with carboplatin, cyclophosphamide and paclitaxel. The potency of combinations of these agents was assessed at different treatment schedules and a variety of concentration ratios. Carboplatin is an alkylating agent that is covalently attached to DNA through the formation of stable cross-links between the strands. Taxol acts in M phase of the cell cycle, inducing cell cycle arrest in G_2/M phase, by stabilizing the formation of the mitotic spindle. Cyclophosphamide is an alkylating agent forming stable cross-links with double-stranded DNA and results in DNA replication failure. The chemical structures of the tested drugs are shown in Figure 2.

The ultimate goal was to assess any modulatory effect of Cu-Sal complex on the cytotoxic activity of the chemotherapeutic drugs tested.

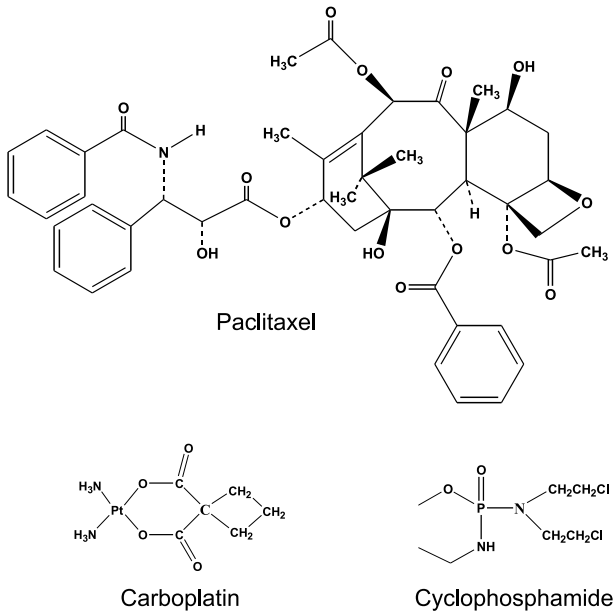

Figure 2. Chemical structures of the chemotherapeutic drugs used.

Materials and methods

The cell line used was T47D (human breast cancer) [7] and was obtained from the Imperial Cancer Research Fund (ICRF), London.

Experimental agent

Cu-Sal complex was initially dissolved in DMSO to a concentration of 2 mM and tested in 9 sextuplicate dilutions starting with a peak concentration of 200 μM. The final concentration of DMSO in cell cultures was less than 0.1%.

Cell culture maintenance

Cells were routinely grown as a monolayer culture in T-75 flasks in an atmosphere containing 5% CO_2 in air and 100% relative humidity at 37° C, and subcultured twice a week. The culture medium used was DMEM (Gibco, Glasgow, UK), supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin.

Drug exposure - XTT cytotoxicity assay

For the experiments, cells were plated in 96-well flat-bottomed microplates at a density of 10,000 cells per well. After 48 h at 37° C cells were exposed simultaneously to both agents for 48 h by the addition of an equal volume (100 μl) of either complete culture medium (control wells), or twice the final drug concentrations diluted in complete culture medium. Drug cytotoxicity was measured by means of XTT colorimetric assay, estimating the survival fractions (SF) as the percent ratio of the absorbance of drug-treated cells to the control absorbance. The XTT assay was carried out as previously described [8].

Calculation of results

For each tested compound a dose-effect curve was produced. Sextuplicate determinations gave a coefficient of variation (CV) of much less than 10%. The inhibition of cellular growth is expressed as the fraction of cells that remains unaffected (fu) (survival fraction, SF). Drug potency was expressed in terms of IC_{50} values calculated from the plotted dose-effect curves (through least-square regression lines).

Median-effect analysis and Combination Index

The combined drug interaction was assessed with the median-effect analysis [9]. When CI <1, =1 or >1, synergism, additivity or antagonism was indicated, respectively. The interaction of the agent was quantitated by the CI method across the entire dose-effect range.

Results

The *in vitro* cytotoxic activity of Cu-Sal complex and its combined effects with the tested chemotherapeutic drugs were promising.

In order to test whether the effect of the Cu(II) complex could be attributed to the salicylate ligand or not, we tested the effect of the ligand alone against T47D cell line. The ligand exhibited only a negligible inhibition of cellular proliferation (data not shown).

The dose-effect curves derived from the combination of Cu-Sal complex with all tested agents are presented in Figure 3.

At mutually non-exclusive assumption, the concurrent treatment of cells with Cu-Sal complex and the aforementioned drugs produced a synergistic result in most concentration ratios (1:1, 1:10, 10:1 and 1:100) (Figure 4). For the combination of Cu-Sal with CBDCA, the CI index illustrated significant synergy (CI: 0.23-0.68) at most of the combination ratios, with a most pronounced effect at concentration ratios of 1:1, 1:10, 10:1 and 1:100 (Cu-Sal:CBDCA). These CI values were generated by the combinations of 0.1 and 1 μM of Cu-Sal with 0.1, 1 and 10 μM of CBDCA (CI: 0.230-

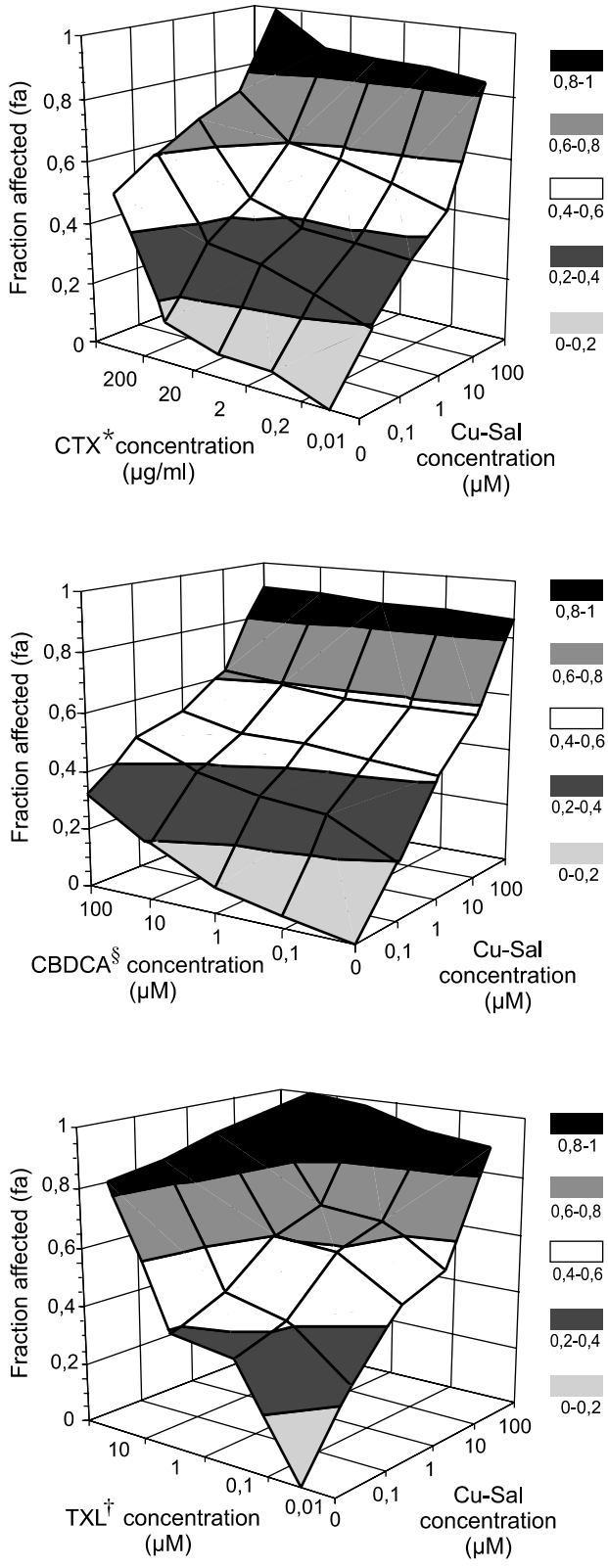

Figure 3. Dose-effect plots for the combination of Cu-Sal complex with 3 chemotherapeutic drugs against T47D cells after 48 h of concurrent administration of the agents. Cytotoxicity was estimated via XTT assay (each point represents a mean of 6 replicate wells). *Fraction affected* is a synonym of the term *inhibition fraction*.

*cyclophosphamide, §carboplatin, †paclitaxel

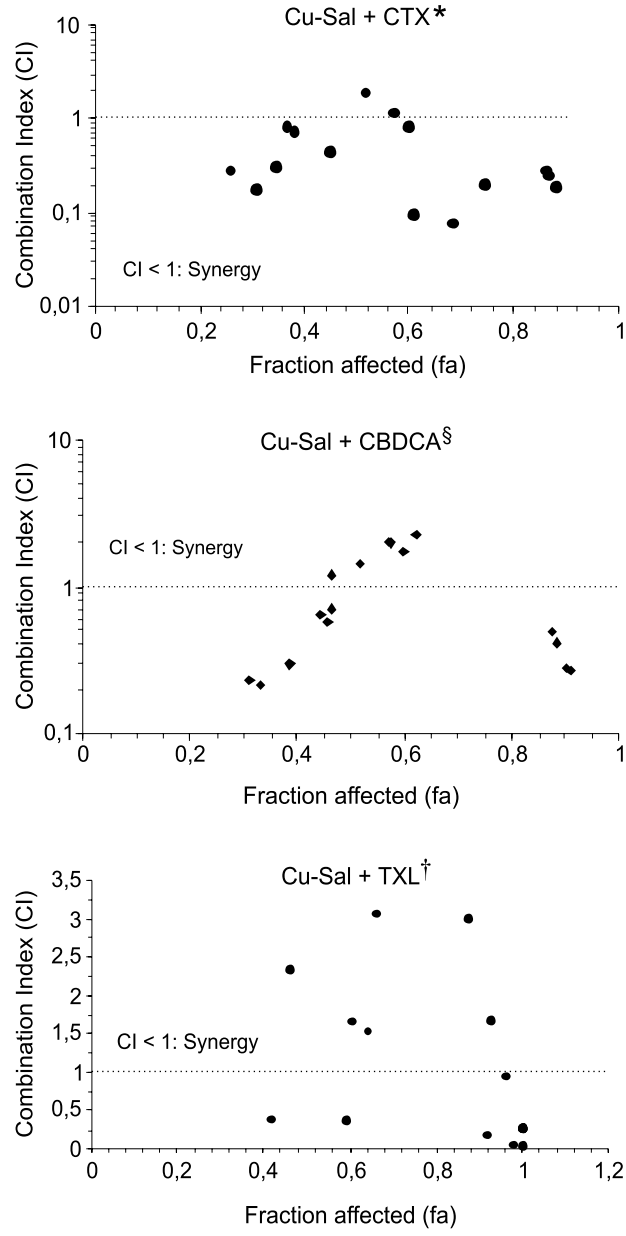

Figure 4. Correlation of the Combination Index (CI) with the Fraction Affected (fa) of T47D cells after 48 h concurrent treatment with Cu-Sal complex and each one of the chemotherapeutic drugs.

*cyclophosphamide, §carboplatin, †paclitaxel

0.685) and the combinations of 100 μM of Cu-Sal with 0.1, 1, 10 and 100 μM of CBDCA (CI: 0.259-0.486). Nearly additive effect for Cu-Sal/CBDCA combination was demonstrated only for 1:100 and 1:1000 concentration ratios (0.1 & 1 μM of Cu-Sal plus 100 μM of CBDCA) (CI ranged between 1.0 and 1.1 for the whole range of fractions affected). Significantly strong synergy was noted for the combination of Cu-Sal with CTX at 13 out of 15 combination schedules (CI values between 0.073 and 0.826). Very strong synergy was

obtained in 2 cases (CI/fa: 0.075/0.68 and 0.096/0.609), strong synergy in 7 combinations (CI: 0.182-0.304), net synergy in 2 (CI: 0.439 and 0.72) and mild synergy in 2 (CI: 0.828 and 0.85). Only for 2 concentration ratios an almost additive (CI/fa: 1.135/0.569) or antagonistic effect (CI/fa: 1.869/0.517) was documented. For the combination of Cu-Sal (0.1, 1, 10 and 100 μM) with TXL, strong synergistic interaction was observed only for 3 concentration ratios (with TXL concentrations up to 0.1 μM), leading to CI: 0.18-0.395 (with fa: 0.418-0.918), whilst almost additive effect resulted from the combination of 10 μM Cu-Sal with 10 μM TXL (CI/fa: 0.947/0.96). On the contrary, all other combinations with TXL concentrations greater than 0.1 μM revealed to be mostly antagonistic (6 out of 10 combination schemes resulted in CI: 1.530-3.069 for fa: 0.463-0.928).

We conclude that the combination of Cu-Sal with all chemotherapeutics tested resulted mainly in synergistic interaction except of concentration ratios including 10 μM Cu-Sal in which an additive to antagonist effect was demonstrated. The degree of synergism was of the following order: Cu-Sal + CTX > Cu-Sal + CBDCA > Cu-Sal + TXL.

The dose reduction index (DRI), which indicates how many folds the tested drug concentrations may be reduced in order to achieve a certain synergistic effect, was constantly above 1 for the combined drugs in all drug combinations (for most concentration ratios in the entire effect level). For Cu-Sal/TXL combination, it was observed that $DRI_{TXL} < DRI_{Cu\text{-}Sal}$.

Discussion

The aim of these experiments was to study the type of the interactive effect between the experimental compound and 3 widely used conventional chemotherapeutic drugs. With a purpose to increase the chemosensitivity of human cancer cells to current chemotherapeutics by modifying their anticancer activity, synergistic, additive or antagonistic drug activities were investigated.

There is a considerable interest in the DNA binding of metal complexes because of their potential applications as DNA probes and as possible antitumor agents [10]. A large number of antitumor drugs interact with DNA and cause scission on the DNA [11].

Coordination of Cu-Sal with DNA, most probably to major or minor grooves, can be achieved through interstrand and intrastrand linkage. The negatively charged ribose-phosphate backbone of the major and minor grooves of double-stranded DNA and RNA constitutes an important target to a variety of positively charged species (such as metals, ligands and protein side chains). It has been suggested that the unique properties of interstrand cross-links of bifunctional transition metal complexes and the derived conformational alterations in DNA, have critical consequences for their antitumor effects. Quantum-chemical calculations can foresee the structures, the energies and other molecular properties, and have been recognized as particularly useful tools for the chemical research. Molecular modeling studies are capable to analyze the mechanistic basis for the formation of adducts with biological molecules. Thus, we have focused our investigations in an effort to study the theoretical interactions of Cu(SalNEt$_2$)salicylate complex and its adducts with DNA, RNA and proteins, and to correlate structure with biological activity. Research on the molecular structure and several other parameters related to chemical reactivity and biological behavior of Cu-Sal complex adducts showed interactions with 5′-GMP and 5′-CMP nucleotides (bidentate attack with the formation of a closed macrochelate ring). The theoretical results supported our hypothesis that the mechanism of action of this complex involves binding to DNA and RNA [12].

The mechanism of the cytotoxic action of the complex is considered to involve a signal transduction pathway, which communicates signals when DNA is damaged to cell cycle mechanisms [13]. DNA-damage checkpoint [14] involves the activation of many genes (ATM, p53, bax, $p21^{WAF1}$, etc) and leads to cell cycle arrest, thus providing cells the critical time needed for DNA-damage repair and survival [15]. TXL-induced G2 arrest is believed to protect cell viability, allowing cells to repair DNA before entering mitosis. On the other hand, Cu(SalNEt$_2$)salicylate complex produces a dose-dependent G1 arrest after 48 h of treatment of Raji and other human cancer cells [16, 17].

The mechanisms of the observed combination effects should be explored by studying the differential expression and regulation of various cyclin proteins in normal and human tumor cells. Additional examination of the interaction pattern of Cu(II) complexes of Cu(SalNEt$_2$)Y type with other cellular elements of fundamental importance in cell proliferation may be informative. Such an investigation will provide more hints about the subjacent mechanism of action.

Conclusion

We conclude that Cu-Sal complex interacts synergistically with the tested chemotherapeutic drugs in

most schedules of administration, and only occasionally an additive or antagonistic effect for these combinations was apparent.

A quantitative structure activity relationship (QSAR) study of the compounds should be considered for the understanding and determination of the responsible factors that produce the observed cytotoxicity of Cu-Sal complex (bioactivity).

In conclusion, we have obtained evidence that the combination of the copper(II) complex with the tested chemotherapeutic drugs appears to be a logic approach to improve clinical antitumor responses and to restrict side effects.

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