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In vitro synergistic effects of some novel Cu(II) complexes in combination with epirubicin and mitomycin C against HeLa-S3 cervical cancer cell line

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The *in vitro* predictive chemosensitivity of a HeLa-S3 human cervical cancer cell line to a new series of Cu(II) complexes as well as their combination with several chemotherapeutic drugs was determined. Antiproliferative activity was evaluated by the XTT assay. Among all tested drugs, HeLa-S3 cells were especially sensitive to epirubicin (EPR) or mitomycin C (MMC) antitumor agents. The combination of EPR or MMC with the complexes resulted in a pronounced synergistic amplification of their antiproliferative effect. This marked synergism was observed in all drug combinations.

Key words: Cu(II) complexes, cytotoxicity assay, epirubicin, HeLa-S3 cells, mitomycin C, synergism, XTT tetrazolium salt.

Introduction

A Cu(II) chelate complex with 2-keto-3-ethoxybutyraldehyde bis(thio-semicarbazone), Cu(II)KTS, has proved to be highly cytotoxic to HeLa cells in tissue culture systems.[1–4] The critical role of copper in the antitumor activity of KTS and presumably other bis(thiosemicarbazones) has already been indicated.[5–9] In this regard, we have attempted to study the potential anti-tumor activity of some recently synthesized Cu(II) complexes of the formula Cu(Sa1NEt$_2$)Y, where Sa1NEt$_2$ stands for the anion of the *N*-(2-(diethylamino)ethyl)salicylidenaminato ligand and Y is the anion of a carboxylic acid.[10] The *in vitro* antiproliferative activity of these complexes against HeLa-S3 human cervical cancer cell line was compared to that of known chemotherapeutic agents, such as bleomycin (BLM), cisplatin, cyclophosphamide (CPM), epirubicin (EPR), 5-fluorouracil (5-FU), mitomycin C (MMC) and vincristine (VCR).

Furthermore, a drug combination experiment was undertaken using the most potent anticancer agents tested—EPR and MMC—along with the aforementioned Cu(II) complexes. Consequently, the modulatory effect of Cu(Sa1NEt$_2$)Y compounds on the antitumor activities of these clinically important anticancer agents was evaluated. All tests were carried out by the utilization of the XTT microculture tetrazolium colorimetric assay through which an assessment of drug-induced cellular growth inhibition was attained.

Materials and methods

Cell line and culture conditions

Cytotoxic activity of experimental and antitumor agents was tested against HeLa-S3 human cervical cancer cell line which is an epithelial-like clonal derivative of the parent HeLa cell line.[11] Cells were routinely grown as monolayer cultures in fresh Dulbecco's modified Eagle's medium (DMEM; Gibco, Glasgow, UK) supplemented with 10% fetal bovine serum (FBS, Gibco) and at antibiotic cocktail (100 U/ml penicillin and 100 U/ml streptomycin) at 37°C in an atmosphere containing 5% CO_2 in air and 100% relative humidity. For routine passages, adherent cells were detached using a 0.05% trypsin (Gibco, 1 : 250)–0.02% ethylenodiamino tetraacetic acid (EDTA) mixture.

Drugs

The experimental compounds tested were four-coordinate Cu(II) chelate complexes with a tridentate ONN-Schiff base ligand and the anion of a carboxylic acid. The chemical structure of the

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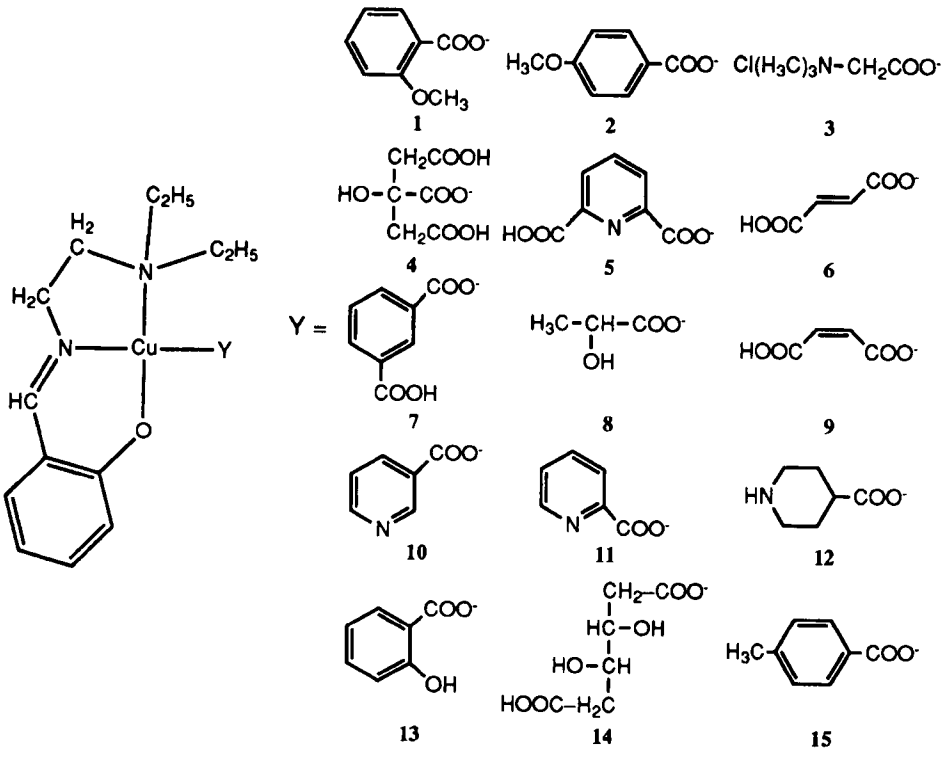

Figure 1. Proposed structure for the Cu(Sa1NEt$_2$)Y complexes (2-anisoato, **1**; 4-anisoato, **2**; betainato, **3**; citrato, **4**; dipicolinato, **4**; fumarato, **6**; isophthalato, **7**; lactato, **8**; maleato, **9**; nicotinato, **10**; picolinato, **11**; piperidinato, **12**; salicylato, **13**; tartrato, **14**; 4-toluato, **15**).

complexes is shown in Figure 1. The compounds were prepared at a stock concentration of 1.0 mg/ml either in phosphate-buffered saline (citrato, dipicolinato, fumarato, lactato, picolinato, piperidinato and 4-toluato derivatives) or in 3.3% dimethyl sulfoxide (DMSO) plus 3% methanol in water (2-anisoato, 4-anisoato, betainato, isophthalato, maleato, nicotinato, salicylato and tartrato derivatives). Commercially available antitumor agents were also prepared at stock concentration of 1.0 mg/ml in water. Drug stocks were sterilized via filtration and stored frozen at −20°C with the exception of cisplatin solution which was stored at room temperature.

XTT cytotoxicity assay

In the current screening protocol, exponentially growing HeLa-S3 cells were inoculated onto 96-well microculture plates (Costar) at an optimal seeding density of 5000 cells per well to assure logarithmic rate of growth throughout the evaluation period of the assay. Cells were inoculated in a volume of 100 μl per well and incubated at 37°C in the presence of complete growth medium (DMEM) minus phenol red. A 100 μl aliquot of complete medium was added to cell-free wells. The microplates containing the cells were preincubated for approximately 48 h to allow cell stabilization prior to addition of agents. Agents at twice the required final concentration in growth medium (100 μl aliquots of each dilution) were applied to triplicate culture wells and microplates were incubated for 24, 48 or 72 h.

Cytotoxicity experiments were carried out in triplicate. The cultures were assayed for cellular growth and viability by XTT microculture tetrazolium assay.[12–16] Briefly, 50 μl of a mixture (10:1) of XTT (1 mg/ml) with 10 mM menadione (MEN) was added to each well, 4 h before the completion of the drug exposure period. Absorbance was then measured on a spectrophotometric plate reader (Anthos Labtec) at 450/620 nm wavelength.

Data calculations

A dose–response profile was generated for each test agent. Data indicating cell growth inhibition were expressed as percent of XTT formazan produced from untreated control cells as determined by the

following equation: % of untreated control XTT formazan = (test OD/control OD) × 100. Drug potency was expressed as the 50% inhibitory concentration (IC_{50}).

For evaluation of the interaction between EPR or MMC and Cu(Sa1N-Et_2)Y complexes, median effect plot analysis was performed.[17–19] The analysis compares the effects of drug combinations to the effects of individual drugs across the entire dose–effect range, indicating if the interaction is synergistic, additive or antagonistic. Statistically significant differences were evaluated by means of Student's *t*-test; $p < 0.02$ was considered to be statistically significant.

Results

The assessment of *in vitro* cytotoxicity was performed by XTT assay after 24, 48 or 72 h of continuous exposure to various agents. In all XTT assays very low background absorbances were generated, resulting in high signal to noise ratios in the range 6.05–14.07. *In vitro* cytotoxicity data for the Cu(Sa1NEt_2)Y complexes as well as for several antitumor agents, expressed as IC_{50} values, are presented in Table 1.

Table 1. Chemosensitivity of HeLa-S3 human cervical cancer cell line to some Cu(Sa1NEt_2)Y complexes and several antitumor agents, assessed by XTT colorimetric assay

	IC_{50} (μg/ml) at duration of continuous drug exposure (h)		
Compound	24	48	72
Cu(Sa1NEt_2)Y derivatives			
2-anisoato	25.2	26.2	20.1
4-anisoato	>40	27.1	18.7
betainato	24.8	19.7	18.6
citrato	>40	>40	>40
dipicolinato	34.9	34.3	33.1
fumarato	>40	>40	>40
isophthalato	>40	>40	31.2
lactato	>40	>40	>40
maleato	>40	>40	32.2
nicotinato	>40	>40	20.1
picolinato	>40	>40	14.1
piperidinato	34.3	28.2	20.1
salicylato	26.5	18.5	16.2
tartrato	>40	>40	15.4
4-toluato	>40	>40	32.3
Antitumor agents			
bleomycin	>20	>20	>20
cisplatin	>20	>20	>20
cyclophosphamide	>20	>20	>20
epirubicin	15.6	5.2	3.4
5-flourouracil	>20	>20	>20
mitomycin-C	1.4	1.3	0.8
vincristine	>20	>20	>20

As depicted in Table 1, HeLa-S3 cells were strongly resistant to BLM, cisplatin, CPM, 5-FU and VCR, with IC_{50} values constantly above 20 μg/ml after 24, 48 or 72 h of continuous drug exposure. However, they showed an intermediate resistance to almost all complexes in concentrations up to 40 μg/ml as indicated by the IC_{50} range of 14.1–38.1 μg/ml. Among the tested complexes, citrato, fumarato and lactato derivatives were the less potent since their IC_{50}'s were higher than 40 μg/ml, 72 h after drug administration. On the contrary, HeLa-S3 cells were found to be most sensitive to EPR and MMC which exhibited the highest cytotoxic activity among all chemotherapeutic agents tested. Figure 2 illustrates the dose–response curves for EPR- and MMC-treated HeLa-S3 cells, obtained 24, 48 or 72 h after drug treatment.

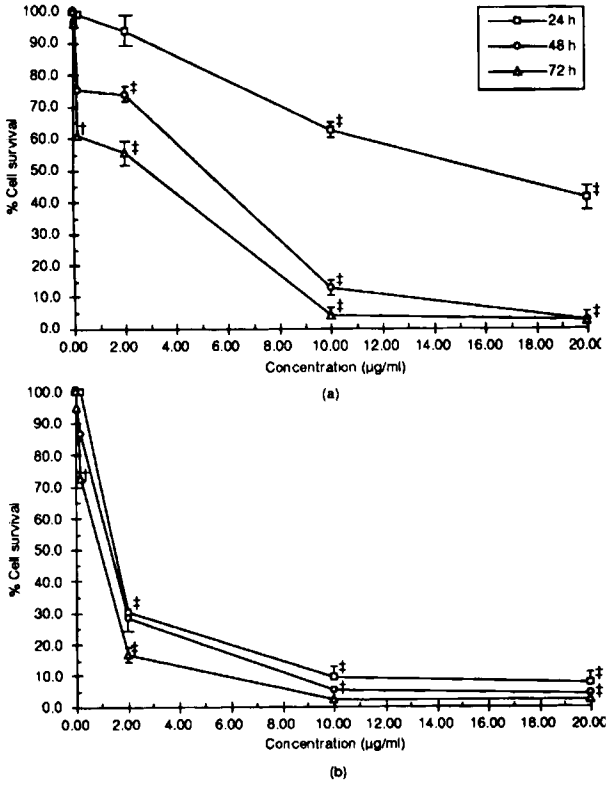

Figure 2. Dose–response curves for (a) EPR- and (b) MMC-treated HeLa-S3 cells (5000 cells per well, 5 day culture duration, 24, 48 and 72 h drug treatment, and 4 h incubation with XTT plus MEN). Data points represent mean values ± SEM from a duplicated experiment calculated from at least six replicate wells subtracting background and determined by the XTT microtitration assay († $p < 0.02$, ‡ $p < 0.001$).

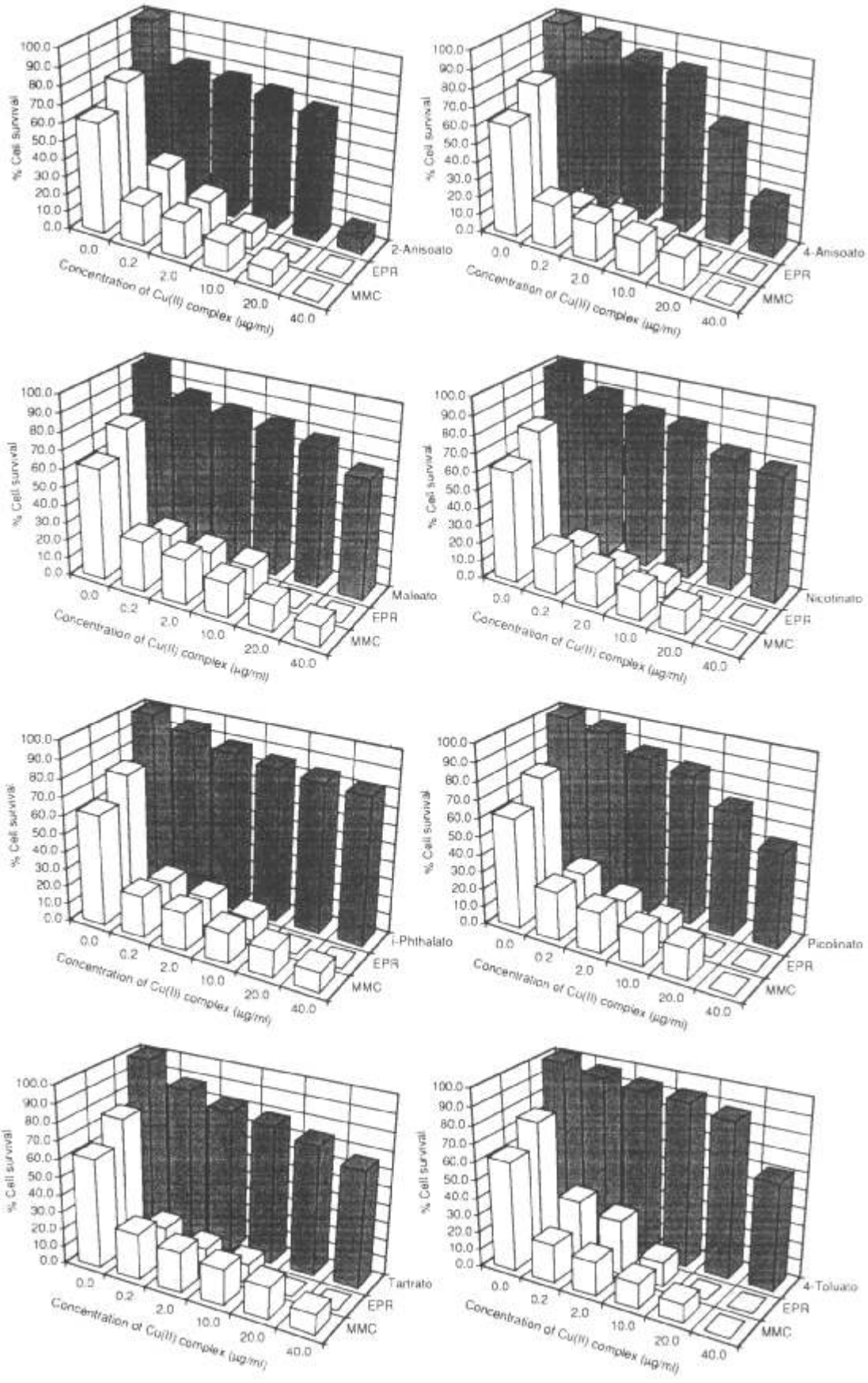
% Cell survival
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
0.0
0.2
2.0
10.0
20.0
40.0
Concentration of Cu(II) complex (μg/ml)
EPR
MMC
2-Anisoato
4-Anisoato
Maleato
Nicotinato
I-Phthalato
Picolinato
Tartrato
4-Toluato

Table 2. *In vitro* cytotoxicity data for betainato and salicylato derivatives when they were administered to HeLa-S3 cells (5000 cells/well)

Derivative	Concentration (μg/ml)	Cell survival (% of control absorbance) at duration of continuous drug exposure (h)		
		24	48	72
Betainato	0.2	95.7 ± 5.4	96.0 ± 3.4	87.7 ± 0.9
	2.0	93.1 ± 0.6	93.3 ± 11.2	77.6 ± 1.8[a]
	10.0	90.9 ± 11.3	81.2 ± 2.1	73.0 ± 1.7[b]
	20.0	58.3 ± 3.0[c]	48.7 ± 0.5[c]	45.4 ± 1.7[c]
	40.0	24.8 ± 4.8[c]	11.1 ± 5.1[c]	6.5 ± 9.2[c]
Salicylato	0.2	98.1 ± 2.8	93.6 ± 2.4	87.8 ± 4.5
	2.0	96.3 ± 3.4	92.4 ± 4.0	86.3 ± 8.6
	10.0	91.7 ± 1.7	79.5 ± 0.0	80.5 ± 2.7
	20.0	60.5 ± 0.6[c]	45.3 ± 2.6[c]	42.6 ± 4.6[c]
	40.0	28.4 ± 0.4[c]	15.5 ± 5.2[c]	12.8 ± 2.8[c]

[a] $p < 0.02$; [b] $p < 0.01$; [c] $p < 0.001$ versus control.

HeLa-S3 cell line proved to be more sensitive to betainato and salicylato derivatives. The growth inhibition data of these complexes expressed as percent of control absorbance values on HeLa-S3 cells treated continuously for 24, 48 or 72 h are presented in Table 2. The sensitivity of HeLa-S3 cells to these agents appeared to be dose dependent, resulting in a significant decrease in viable cells. Among all complexes, only betainato and salicylato derivatives, after 48 h treatment with concentrations above 10 μg/ml, exerted the same survival pattern as after 72 h. However, the picolinato derivative was found to be the most potent when applied at 20 μg/ml concentration, resulting in 8.6% cell survival ($p < 0.001$ versus control) at 72 h. Additionally, for 2-anisoato, 4-anisoato and picolinato derivatives a highly significant growth inhibition ($p < 0.001$) was indicated after 72 h treatment with 40 μg/ml, resulting in 8.3, 7.9 and 7.2% of control values, respectively. Finally, 2-anisoato derivative proved to decrease cell survival more effectively than all other complexes after 48 h exposure at 40 μg/ml (9.0% of control, $p < 0.001$).

Since we have found that EPR and MMC can suppress the growth of HeLa-S3 cells giving IC_{50} values of 5.2 and 1.3 μg/ml, respectively (at 48 h incubation), we examined their antiproliferative efficacy in combination with several Cu(Sa1NEt$_2$)Y complexes. HeLa-S3 cells were treated for 48 h with graded concentrations of eight Cu(II) complexes (concentration range 0.2–40 μg/ml) in combination with EPR (2 μg/ml) or MMC (1 μg/ml). Their combination with the tested complexes resulted in a pronounced synergistic amplification of the antiproliferative effect of each single agent alone, as shown in Figure 3.

According to the chemosensitivity pattern of both chemotherapeutic agents, they demonstrate similar activity against HeLa-S3 cells when administered concurrently with Cu(Sa1NEt$_2$)Y complexes. Thus, EPR and MMC, when administered as a single agent, exhibit percent survival rates of 75.4 and 62.3%, respectively, indicating statistically significant reduction from control value. Subsequently, when combined with a variety of complexes, a highly statistically significant decrease in survival rates was observed in all 16 combination experiments, compared to the treatment of each agent alone. The reduction of these values was in the range of 35–0% for EPR and 27.8–0% for MMC. In addition, median effect analysis revealed induction of maximal and significant synergistic inhibitory effect of the HeLa-S3 cell proliferation, when Cu(Sa1NEt$_2$)Y complexes were administered simultaneously with EPR or MMC, relative to these agents alone. All complexes used in combination experiments enhanced significantly cytotoxicity of the antitumor agents. From all derivatives tested, 4-anisoato appeared to be slightly more potent than the rest, resulting in a synergistic significant decrease in cell viability in all combination schedules used. These results suggest that the above combinations are 3- to 10-fold-times more potent than EPR, MMC or Cu(II) complexes alone.

Figure 3. Antiproliferative effect of several Cu(Sa1NEt$_2$)Y concentrations alone or in combination with 2 μg/ml EPR or 1 μg/ml MMC on HeLa-S3 cell viability (XTT test) after 48 h of continuous drug exposure following a 2 day preincubation period without drugs (5000 cells per well). Data represent the mean of six replicates done in two experiments. For every drug combination $p < 0.001$, indicating statistically significant difference from control.

Discussion

The efficacy of combination therapy in therapeutic strategy for human cervical cancer has reached a plateau that seems hard to overcome. The ongoing quest for new agents or alternative ways to use established treatments has led us to testing a group of recently synthesized Cu(II) complexes against the HeLa-S3 human cervical cancer cell line. Although most of these candidate antitumor compounds were initially regarded as having only minor cytotoxic merits on HeLa-S3 cells, it was worth trying to evaluate them in combination experiments with various anticancer agents. Therefore, a series of experiments was designed to assess the effect of concurrent treatment of HeLa-S3 cells with clinically important antitumor agents and the novel Cu(Sa1N-Et_2)Y complexes. The aim was to explore the modulatory effects of these experimental compounds on the antitumor activities of the chemotherapeutic agents used.

Although EPR and MMC have been demonstrated to be clinically useful anticancer agents, human cervical tumors do not respond completely in all patients. Therefore, combinations of these drugs with other antitumor agents are a logical approach to improve clinical antitumor responses.[20] Actually, our results definitely indicate that EPR and MMC are the best anticancer agents tested for use in combination with a variety of Cu(Sa1NEt_2)Y complexes. However, due to lack of universally accepted terminology and methodology making the analysis of drug combination data complicated,[21] most investigators have relied on either of two approaches to characterize the cytotoxic effects of combination treatment (synergy, additivity, antagonism): the isobologram and the median-effect plot combination indexes.[17,22] In our study we have selected the latter method of analysis.

In our present study we have demonstrated that the Cu(Sa1NEt_2)Y complexes tested had a synergistic effect when combined with either EPR or MMC. This implies that the two agents given together are more effective in suppressing cell growth than would be expected from their individual activities. Yet, there was no drug combination yielding additive or antagonistic cytotoxicity when both drugs were applied concurrently on HeLa-S3 cells for an exposure period of 48 h. However, the mechanism for the observed synergistic interaction between Cu(II) complexes and most active antitumor agents is currently unknown.

We plan to test the same drug combinations on normal cell systems to investigate the relative cytotoxicity and anti-neoplastic activity. Additionally, a series of experiments are underway to reveal the difference between cytostatic and cytotoxic effects of Cu(Sa1NEt_2)Y complexes by measuring DNA synthesis with the BrdU-labeling colorimetric test. Cytostatic action will be judged on the basis of inhibition of DNA synthesis, since it is expedient for a cytostatic drug to have a negligible influence on metabolic activity of target cells.[23,24]

Conclusion

Cu(Sa1NEt_2)Y complexes in simultaneous exposure with EPR and MMC may have the advantage of augmenting the anticancer activity of these antineoplastic agents in a synergistic manner. Median-effect plot analysis revealed no additive cytotoxicity in any drug combination.

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