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Effect of Nickel (II) Complexes with Mannich Bases on Viability and Proliferation of Human Cancer Cells

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The aim of our study was to evaluate the influence of four nickel (II) complexes with ligands containing the antipyrine moiety N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) or N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN) on viability and proliferation of cultured human cancer cells. The following permanent cell lines were used as model systems: MCF-7 (luminal A type breast cancer), SK-BR-3 (Her2 - positive breast cancer), Caco-2 (colorectal adenocarcinoma), HepG2 (hepatocellular cancer), 8MGBA (glioblastoma multiforme). The investigations were performed by MTT test and neutral red uptake cytotoxicity assay (in short-term experiments, 24-72h, with monolayer cultures) and colony-forming method (in long-term experiments, 20 days, with 3D cancer cell colonies). The results obtained reveal that applied at a concentration range of 1 - 200 μg/ml $Ni_2(BAMP)(CH_3COO)_4$ and $Ni_2(BAMP)(Cl)_4$ are more pronounced cytotoxic agents as compared to $Ni(TAMEN)(ClO_4)_2$ and $Ni(TAMEN)(NCS)_2$. Both ligands (BAMP, TAMEN) do not significantly decrease viability and proliferation of the treated cells

Key words: mannich bases, nickel, polynuclear complexes, cytotoxic activity, human cancer cell lines

Introduction

The discovery of antitumor activity of cisplatin and its successful application in clinical oncology stimulated scientists to search for other metal compounds with promising anticancer potential [9, 19].

Nickel (Ni) is important for proper functioning of the immune system, influences hormonal activity and has been considered as an essential micronutrient for humans. In animals, nickel deficiency has been associated with decreased growth, reduced re-

production, changes in glucose and lipid metabolism [2, 6, 28]. Ni(II) complexes with various ligands have been reported to possess antitumor properties in vitro and / or in vivo [3, 7, 15, 16, 27].

Mannich bases, a structurally heterogeneous class of chemical compounds, are products of a condensation reaction (Mannich reaction) of a compound with active hydrogen(s) with an amine (primary or secondary) and formaldehyde (any aldehyde) [5, 17, 24]. Mannich bases express a wide variety of biological activities including antineoplastic potential [5, 24].

It has been found in our previous investigations that Ni(II) complexes with Mannich base N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) significantly reduced viability and proliferation of retrovirus transformed rat sarcoma (LSR-SF-SR) and chicken hepatoma (LSCC-SF-Mc29) cells [3].

The aim of our study was to evaluate the cytotoxic activity of four nickel (II) complexes with ligands containing BAMP or TAMEN (N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane) on viability and proliferation of cultured human cancer cells.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were purchased from Gibco-Invitrogen (UK). Dimethyl sulfoxide (DMSO), neutral red and trypsin were obtained from AppliChem (Germany); purified agar and thiazolyl blue tetrazolium bromide (MTT) were from Sigma-Aldrich Chemie GmbH (Germany). All other chemicals of the highest purity commercially available were purchased from local agents and distributors. All sterile plastic ware was from Orange Scientific (Belgium).

Compounds. Four Ni(II) complexes with ligands containing the antipyrine moiety N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) and N,N'-tetra-(antipyryl-1-methyl)-1,2-diaminoethane (TAMEN) - $Ni_2(BAMP)(CHCOO_3)_4$, $Ni_2(BAMP)(Cl)_4$, $Ni(TAMEN)(ClO_4)_2$, $Ni(TAMEN)(NCS)_2$, as well as both ligands were investigated. The synthesis as well as the physical and chemical characteristics of the compounds were already published [10, 11].

Nickel(II) complexes as well as their ligands BAMP and TAMEN were dissolved in dimethylsulfoxide (the concentration of the compound in the stock solution was 1 mg/ml containing 1-2% DMSO) and then diluted in culture medium.

Cell lines and cultivation. The following human permanent cell lines were used as model systems in our investigations: MCF-7 (luminal A type breast cancer), SK-BR-3 (Her2 - positive breast cancer), Caco-2 (colon adenocarcinoma), HepG2 (hepatocellular cancer) and 8MGBA (glioblastoma multiforme).

The cells were grown as monolayer cultures in a D-MEM medium, supplemented with 5-10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin. The cultures were maintained at 37°C in a humidified CO_2 incubator (Thermo Scientific, HEPA Class 100). For routine passages the cells were detached using a mixture of 0.05% trypsin – 0.02% ethylendiaminotetraacetic acid (EDTA). The experiments were performed during the exponential phase of cell growth.

Cytotoxicity assays. The cells were seeded in 96-well flat-botommed microplates at a concentration of 1×10^4 cells/well. After the cells were grown for 24 h to a subconfluent state (~ 60-70%), the culture medium was removed and changed by media modified with different concentrations (1, 10, 50, 100 and 200 mg/ml) of the compounds tested. Each concentration was applied into 4 to 8 wells. Samples of cells grown in non-modified medium served as controls. After 24, 48 and 72 h of incubation, the effect of

the compounds on cell viability and proliferation was examined by neutral red uptake cytotoxicity (NR) assay [8] and thiazolyl blue tetrazolium bromide (MTT) test [18].

Optical density was measured at 540 nm using an automatic microplate reader (TECAN, Sunrise™, Austria). Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration–response curves were prepared and the effective cytotoxic concentration 50 (CC_{50}) of the compounds causing 50% reduction of cell viability was estimated from these curves. All data points represent an average of three independent assays.

Colony forming method. Tumor cells (10^3 cells/well) suspended in 0.45% purified agar in D-MEM medium containing different concentrations of the compounds examined (ranging from 1 to 250 mg/mL) were layered in 24 well microplates. The presence/absence of colonies was registered using an inverted microscope (Carl Zeiss, Germany) during period of 18-20 days. Colony inhibitory concentration (CIC) at which the compounds tested suppress completely the ability of tumor cells for 3D growth in semi-solid medium was determined.

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test and Origin 6.1™.

Results and Discussion

Short-term experiments (24 – 72 h duration) with monolayer cell cultures have been performed by two methods with different molecular/cellular targets and mechanisms of action: MTT test (based on the ability of mitochondrial dehydrogenases to reduce the yellow water-soluble tetrazolium dye MTT to purple coloured formazan crystals) [25] and neutral red uptake cytotoxicity assay (relies on the accumulation of neutral red into lysosomes of viable cells [23]. The "concentration – response" curves showing the ability of Ni(II) complexes (applied at a concentration range of 1 – 200 μg/ml) to reduce viability and proliferation of the treated cells in a time-and concentration-dependent manner are presented in **Figs. 1-3.** Effective concentrations CC_{50} have been determined only for Ni(II) complexes with BAMP after 72 h incubation period **(Table 1)** because in all other cases cell viability was > 50% **(Figs. 1-3)**. The results obtained reveal that Ni(II) complexes with BAMP exhibit more pronounced cytotoxic activity as compared to Ni(II) complexes with TAMEN. Independently administered, the ligands (BAMP and TAMEN) do not significantly decrease viability / proliferation of the treated cells as compared to the control - the lowest viability has been found to be 94.3% +/- 5.4 (for MCF-7 cells, MTT test, 72 h, $p > 0.05$) and ≥ 91.7% +/- 4.6 (for 8MGBA cells, NR assay, 72h, $p > 0.05$).

Long-term influence (20 day treatment periods) of the compounds investigated on the ability of cancer cells to form 3D colonies in semi-solid medium has been studied. The results obtained demonstrate that among the compounds examined only Ni(II)-BAMP complexes can inhibit completely the 3D growth of the treated cancer cells **(Table 2**). Three dimensional (3D) cell culture systems have been recognized as more reliable models for cancer cell biology studies than 2D (monolayer) cell cultures and provide more realistic and predictable information on their drug sensitivity [14].

The cell lines used as model systems in our study exhibit different degree of sensitivity to the cytotoxic effect of the compounds investigated **(Table 3)** that can be a result of their specific biological characteristics. For example, SK-BR-3 cells are more resistant to the influence of the examined Ni(II) complexes than MCF-7 cells. Although both cell lines were established from human breast cancers, each one of them has its specific molecular profile: MCF-7 cells are estrogen receptor – positive, Her2 nega-

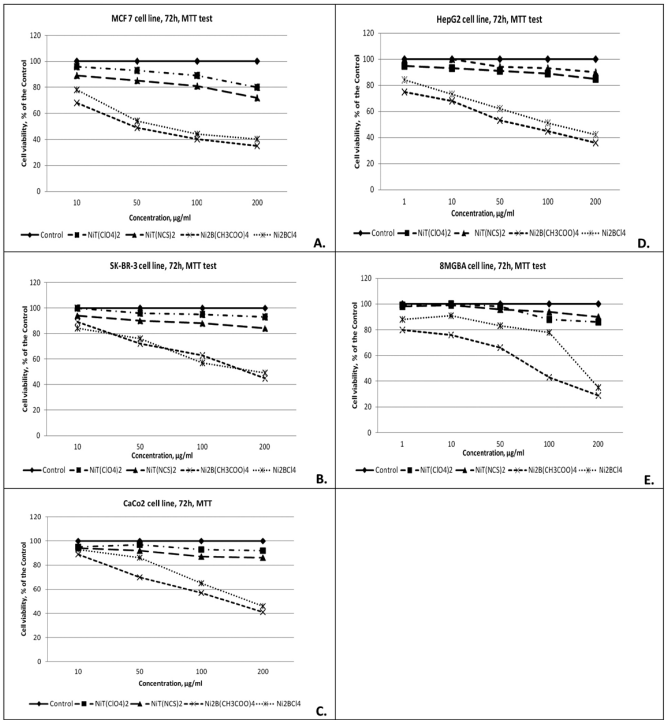

Fig. 1. Concentration-response curves of Ni(II) complexes with BAMP or TAMEN for human MCF-7 (**A**), SK-BR-3 (**B**), Caco-2 (**C**), HepG2 (**D**) and 8MGBA (**E**) cancer cells evaluated by MTT test after 72 h treatment period. B = BAMP, T = TAMEN.

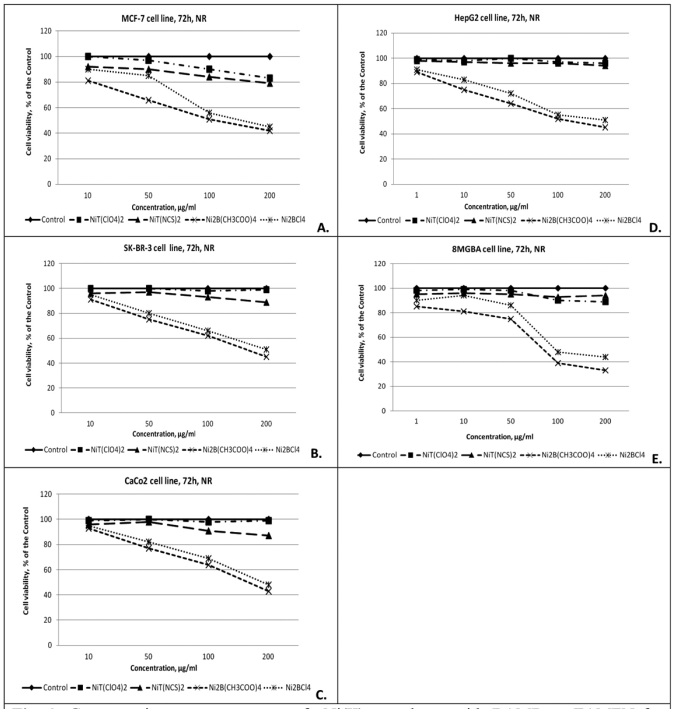

Fig. 2. Concentration-response curves of nickel(II) complexes with BAMP or TAMEN for human MCF-7 (**A**), SK-BR-3 (**B**), Caco-2 (**C**), HepG2 (**D**) and 8MGBA (**E**) cancer cells evaluated by neutral red uptake cytotoxicity assay (NR) after 72 h treatment period. B = BAMP, T = TAMEN.

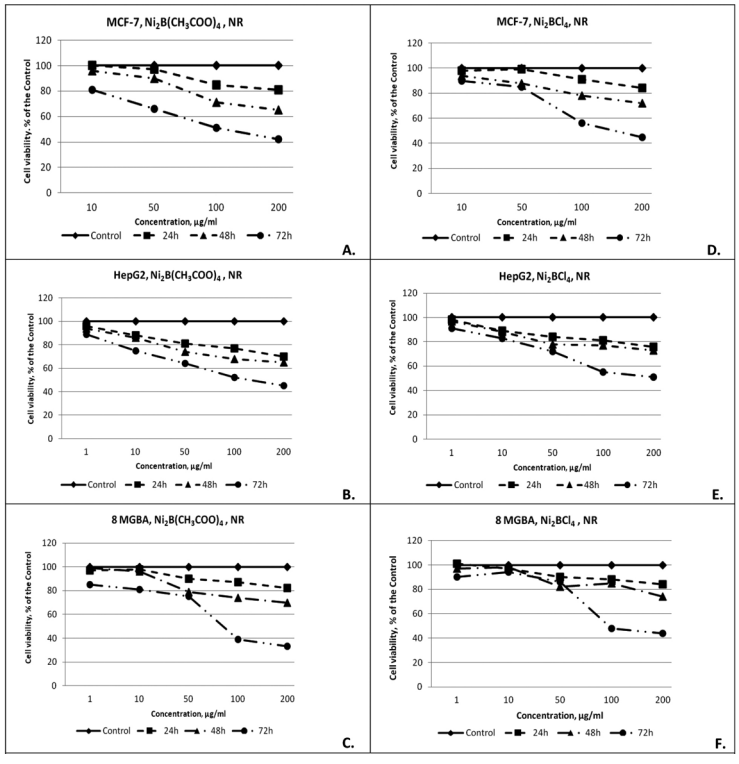

Fig. 3. Concentration-response curves of Ni(II) complexes with BAMP for human MCF-7 (**A, D**), HepG2 (**B, E**) and 8MGBA (**C, F**) cancer cells evaluated by neutral red uptake cytotoxicity assay (NR) after 24, 48 and 72 h treatment period. B = BAMP, T = TAMEN. MTT test after 72 h treatment periods. B = BAMP.

Table 1. Cytotoxicity (CC_{50}, μM, 72h) of Ni(II) complexes with BAMB (B) or TAMEN (T) in human MCF-7, SK-BR-3, Caco-2, HepG2 and 8MGBA cancer cell lines

Compound	Method	Cell line				
		MCF-7	SK-BR-3	Caco-2	HepG2	8MGBA
$NiT(ClO_4)_2$	MTT	n.d.	n.d.	n.d.	n.d.	n.d.
	NR	n.d.	n.d.	n.d.	n.d.	n.d.
$NiT(NCS)_2$	MTT	n.d.	n.d.	n.d.	n.d.	n.d.
	NR	n.d.	n.d.	n.d.	n.d.	n.d.
$Ni_2B(CH_3COO)_4$	MTT	57.2	206.1	172.3	81.8	100.8
	NR	131.0	202.2	198.8	151.7	100.8
Ni_2BCl_4	MTT	94.0	268.3	238.8	149.5	221.6
	NR	155.6 (208.8)	268.3	255.2	268.3	131.3

n.d. - CC_{50} was not determined at 72 h because at all examined concentrations the cell viability was > 50%.

Table 2. Effect of Ni(II) complexes with BAMB (B) or TAMEN (T) on 3D colony-forming ability of human MCF-7, SK-BR-3, Caco-2, HepG2 and 8MGBA tumor cells

	MCF-7	SK-BR-3	Caco-2	HepG2	8MGBA
$NiT(ClO_4)_2$	n.i.	n.i.	n.i.	n.i.	n.i.
$NiT(NCS)_2$	n.i.	n.i.	n.i.	n.i.	n.i.
$Ni_2B(CH_3COO)_4$	≥ 120	≥ 175	≥ 150	≥ 120	≥ 120
Ni_2BCl_4	≥ 135	≥ 235	≥ 200	≥ 135	≥ 135

Colony Inhibitory Concentration (CIC, μM) were determined after 18-20 day treatment period; n.i. = no inhibition

Table 3. Hierarchic orders of human cancer cell lines according to their sensitivity to the cytotoxic activity of Ni(II) complexes with BAMP (B)

Compound	Method	Hierarchic order
$Ni_2B(CH_3COO)_4$	MTT	MCF-7 > HepG2 > 8 MGBA > CaCo-2 > SK-BR-3
	NR	8 MGBA > MCF-7 > HepG2 > CaCo-2 ≥ SK-BR-3
	CFM	MCF-7 = 8MGBA = HepG2 > Caco-2 > SK-BR-3
Ni_2BCl_4	MTT	MCF-7 > HepG2 > 8 MGBA > CaCo-2 > SK-BR-3
	NR	8 MGBA > MCF-7 > CaCo-2 > HepG2 = SK-BR-3
	CFM	MCF-7 = 8MGBA = HepG2 > Caco-2 > SK-BR-3

All hierarchic orders start with the most sensitive cell line (with the lowest CC_{50} or CIC value); MTT = thiazolyl blue tetrazolium bromide test; NR = neutral red uptake cytotoxicity assay; CFM = colony-forming method.

tive, possess wild type p53 and mutant p110 (catalytic subunit of the phosphoinositide-3-kinase - PI3K) whereas SK-BR-3 cells are estrogen receptor - negative, Her2 positive, with mutant p53 and wild type p110 [1, 26]. Higher sensitivity of MCF-7 cells (in comparison to SK-BR-3 cells) to the inhibitory effect of xanafide (a DNA intercalatory agent and topoisomerase II inhibitor) has been reported [1]. In another study MCF-7 cells have been shown to be more sensitive to the cytotoxic effect of aloin (natural anthracycline from Aloe plant) than SK-BR-3 cells [12].

Mannich bases are heterogeneous group of chemical compounds that exhibit a wide range of biological activities including antiinflammatory, antiulcer, anticonvulsant, analgesic, antioxidant, antibacterial, antifungal, antiviral and antiparasitic properties. In addition, some Mannich bases have been proved to regulate blood pressure, to inhibit platelet aggregation or to express antiphyhotic effects. Anticancer potential of Mannich bases have also been reported [5, 24]. It has been found in our previous investigations that Cu(II) and Ni(II) complexes with BAMP and TAMEN decrease viability and prolif-

eration of cultured cancer cells [3, 4]. Cu(II), Co(II) and Ni(II) complexes with the same ligands express antimicrobial activities [20, 21, 22]. Similarly to the results obtained in the present study, Cu(II) complexes with BAMP are more pronounced cytotoxic agents for retrovirus-transformed rat sarcoma (LSR-SF-SR) and chicken hepatoma (LSCC-SF-Mc29) cells as well as human glioblastoma (8MGBA) cells than Cu(II) complexes with TAMEN [4]. The cytotoxic activity of $Ni_2(BAMP)(CH_3COO)_4$ and $Ni_2(BAMP)(Cl)_4$ is also significantly higher than those of $Ni(TAMEN)(ClO_4)_2$ and $Ni(TAMEN)(NCS)_2$, [4]. On the other hand, the same Ni(II) complexes with TAMEN are more effective antibacterial and antifungal agents than Ni(II) complexes with BAMP [20].
In this study we report for the first time the ability of Ni(II) complexes with BAMP ($Ni_2(BAMP)(CH_3COO)_4$ and $Ni_2(BAMP)(Cl)_4$) to suppress the 2D and 3D growth of human cell lines, established from cancers of the breast (luminal A type MCF-7 and Her2-positive SK-BR-3), colon (Caco-2), liver (HepG2) and brain (8MGBA). These are some of the most common and aggressive human malignancies [13] and searching for the effective new treatment modalities for them is among the major challenges of current biomedical sciences. Additional investigations are required to clarify better the antitumor potential of Ni(II) complexes with N,N'-bis(4-antipyrylmethyl)-piperazine (BAMP) as well as their molecular targets and mechanism(s) of action.

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