

ANTITUMOUR EFFECT OF A MIXTURE OF N-PROPYL POLYSULFIDES *IN VITRO*

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ANTITUMORSKI EFEKTI SMEŠE N-PROPIIL POLISULFIDA *IN VITRO*

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ABSTRACT

Copper serves as a limiting factor for multiple steps of tumour progression, including angiogenesis, growth and metastasis. High levels of copper have been found in a wide spectrum of human cancers. Antitumour activities of copper-chelating drugs have been reported in animal models. Organosulfur compounds (diallyl sulfide, DAS; diallyl disulfide, DADS; S-ethylcysteine, SEC; N-acetylcysteine, NAC) derived from garlic exhibit marked copper-chelating activity. We analysed a mixture of fifteen n-propyl polysulfides (DPPS) for potential antitumour activity against several murine tumour cell lines, including colon carcinoma (CT26), mammary carcinoma (4T1) and melanoma cell lines (B16F10), and compared the effects with the antiproliferative effect in highly proliferative murine mesenchymal stem cells (mMSCs). The effects of the mixture of n-propyl polysulfides (100%) on cell viability were determined using MTT assays. Cell apoptosis was analysed using Annexin V-FITC/PI assays.

The results of the MTT assays indicate that this standardized mixture of n-propyl polysulfides has a strong, dose-dependent cytotoxic effect against all three of the tested tumour cell lines (CT26, 4T1, B16F10). The cytotoxic effect of the n-propyl polysulfide mixture against the CT26 and B16F10 cell lines was much stronger than that of cisplatin and was significantly weaker in mMSCs, which are non-cancerous and highly proliferative cells, than in cancer cells. Flow cytometric analysis of CT26 and 4T1 cells revealed that apoptosis was not the dominant mechanism of cell death induced by the n-propyl polysulfide mixture. The n-propyl polysulfide mixture exerted highly cytotoxic activity against murine colon carcinoma and melanoma cell lines, but its antiproliferative activity against mMSCs was significantly lower than that of cisplatin.

Keywords: n-propyl polysulfides, tumour cell lines, cytotoxicity, *in vitro*

SAŽETAK

Bakar učestvuje u različitim fazama progresije tumora, u angiogenezi, rastu i metastaziranju. Povećane vrednosti bakra u serumu i u tkivu tumora, karakteristika su različitih vrsta tumora kod ljudi. U animalnim eksperimentalnim modelima, supstance (lekovi) koje heliraju bakar ispoljavaju anti-tumorski efekat. Helatori bakra su i organosumporna jedinjenja, izolovana iz belog luka. U ovoj studiji analizirali smo potencijalnu anti-tumorsku aktivnost smeše petnaest različitih n-propil polisulfida na nekoliko mišjih ćelijskih linija tumora: karcinom kolona (CT26), karcinom dojke (4T1) i melanom (B16F10). Aktivnost ove smeše na tumorskim linijama, uporedili smo sa antiproliferativnim efektom na mezenhimalne matične ćelije miša (engl. murine mesenchymal stem cells, mMSC). Efekat smeše n-propil polisulfida (100%) na vijabilnost ćelija ispitali smo MTT testom. Apoptozu ćelija smo analizirali koristeći Annexin V-FITC/PI test.

Rezultati MTT testa ukazuju da standardizovana smeša n-propil polisulfida ima jak citotoksični, dozno-zavisni, efekat na sve tri testirane ćelijske linije tumora (CT26, 4T1, B16F10). Smeša n-propil polisulfida ispoljava izraženiji citotoksični efekat na CT26 i B16F10 linije u odnosu na cisplatinu. Citotoksični efekat ove smeše na mMSC je značajno slabiji poredeći sa efektom cisplatine, što ukazuje na selektivnije dejstvo. Analiza CT26 i 4T1 ćelija protočnom citometrijom pokazala je da apoptoza nije glavni oblik smrti ćelija, koju uzrokuje smeša n-propil polisulfida. Smeša n-propil polisulfida ispoljava jaču citotoksičnu aktivnost na ćelijskim linijama mišjeg karcinoma kolona i melanoma i slabiju aktivnost na mMSC u poređenju sa efektom cisplatine.

Ključne reči: n-propil polisulfidi, ćelijske linije tumora, citotoksičnost, *in vitro*



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INTRODUCTION

Copper is an essential trace element for most living organisms. It serves as a structural and catalytic cofactor for enzymes that play crucial roles in various biochemical processes (1). Copper is a cofactor in redox enzymatic reactions required for the normal growth and development of organisms. Since copper has roles in many enzymatic reactions (1, 2), its requirement in different aspects of cancer progression, such as immortalization, angiogenesis, and metastasis, is clear (3, 4). Copper can induce angiogenesis by directly binding to pro-angiogenic factors (such as VEGF) and by stimulating the migration of endothelial cells (5). It also triggers proliferative and metabolic enzymes that increase the ability of cancer cells to metastasize (3). Reduced or elevated levels of copper have been connected to various pathological conditions in humans. Many tumours tend to accumulate high concentrations of copper; the concentrations of copper in serum and tumour tissue are significantly higher in cancer patients than in healthy subjects, and the copper concentration correlates with cancer progression and therapeutic response (6, 7). High levels of copper have been found in a wide spectrum of human cancers, including breast, prostate, colon, lung, and brain cancer (6, 8-10). Furthermore, in comparison with normal tissues, tumours have a greater demand for copper and are more sensitive to reductions in systemic copper levels (11).

Antiangiogenic activities of copper-chelating drugs have been reported in animal models (11) (12). Another study reported impaired oxidative phosphorylation and tumour growth after pharmacological suppression of systemic copper, without concomitant effects on tumour angiogenesis (13). It has also been shown that copper chelation inhibits epithelial-to-mesenchymal transition and decreases the expression of vimentin and fibronectin, thus inhibiting the migratory and invasive properties of cells (14). Organosulfur compounds (diallyl sulfide, DAS; diallyl disulfide, DADS; S-ethylcysteine, SEC; N-acetylcysteine, NAC) derived from garlic exhibit marked copper-chelating activity (15).

Here, we analysed the potential antitumour activity of the mixture of fifteen n-propyl polysulfides against several murine tumour cell lines, including the colon carcinoma (CT26), mammary carcinoma (4T1) and melanoma cell lines (B16F10), and compared these effects with its antiproliferative activity against highly proliferative murine mesenchymal stem cells (mMSCs).

We show that the n-propyl polysulfide mixture exerts highly cytotoxic activity against murine colon carcinoma and melanoma cell lines, while its antiproliferative activity against mMSCs is significantly weaker than that of cisplatin.

MATERIALS AND METHODS

Preparation of drug solutions

The oil mixture of n-propyl polysulfides (100%) and 10 mM cisplatin water solution were diluted in cell culture

medium immediately before use. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, was dissolved (5 mg/mL) in phosphate-buffered saline with a pH of 7.2, and the solution was filtered through a 0.22 mm Millipore filter before use. All reagents were purchased from Sigma Chemicals.

Cell culture

CT26, 4T1, and B16F10 cells were purchased from American Type Culture Collection (ATCC, Manassas, USA). Mouse bone marrow-derived MSCs were purchased from Gibco. Cells were maintained in DMEM (Sigma Aldrich, Munich, Germany) supplemented with 10% foetal bovine serum (FBS, Sigma Aldrich, Munich, Germany), penicillin (100 IU/mL), and streptomycin (100 µg/mL) in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Subconfluent monolayers in the log growth phase were harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA in phosphate-buffered saline (PBS, Sigma Aldrich, Munich, Germany), and the cells were washed three times in serum-free PBS. The number of viable cells was determined by trypan blue exclusion.

Cytotoxicity assay

The effects of the tested compounds on cell viability were determined using the MTT colorimetric technique. All examined cells were diluted with growth medium to 5x10⁴ cells/ml, and aliquots (5x10³ cells/100 ml) were placed in individual wells in 96-well plates. The next day, the medium was exchanged with 100 µL of test compound, which had been serially diluted 2-fold in growth medium to concentrations ranging from 1 mg/ml to 0.008 mg/ml. Both the n-propyl polysulfide mixture and cisplatin were tested in triplicate. Cells were incubated at 37°C and 5% CO₂ for 24 h. After incubation, the supernatant was removed, and 15% MTT solution (5 mg/mL in PBS, 10 µL) in DMEM without FBS was added to each well. After an additional 4 h of incubation at 37°C in 5% CO₂, the medium with MTT was removed, and DMSO (150 µL) with glycine buffer (20 µL) was added to dissolve the crystals. The optical density of each well was determined at 595 nm using a Zenyth 3100 Multimode microplate detector. The percentage cytotoxicity was calculated using the formula: % cytotoxicity = 100 - ((E-B)/(S-B)*100), where B is the background optical density of medium alone, S is the total viability/spontaneous death of untreated target cells, and E is the experimental well. Each of the tested complexes was evaluated for cytotoxicity in three separate experiments.

Apoptosis assay

For the detection of apoptosis, CT26 and 4T1 cells were plated in T25 culture flasks and allowed to grow overnight. After the cells reached subconfluency, the medium was replaced with the test substances (0.01 mg/ml). Treated cells were placed at 37°C in a 5% CO₂ incubator for

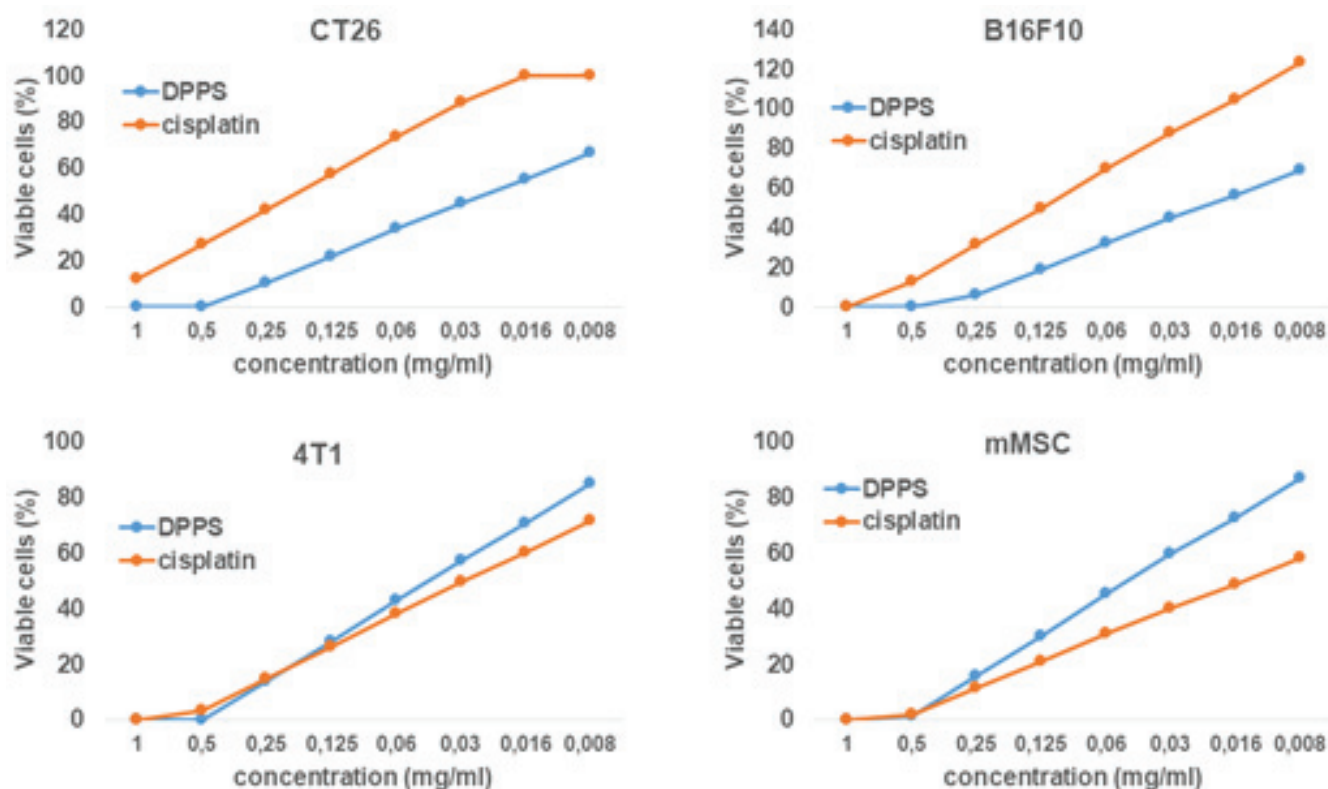


Figure 1. Graphs presenting the survival of CT26, B16F10, and 4T1 cells, as well as MSCs, after 24 h of growth in the presence of DPPS and cisplatin. Data are presented as the mean of three independent experiments.

24 h. The cultured cells were washed twice with PBS and resuspended in 1X binding buffer (10X binding buffer: 0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM CaCl₂) at a concentration 1x10⁶/mL. Annexin FITC and propidium iodide (PI) were added to 100 mL of cell suspension, which was then incubated for 15 min at room temperature (25°C) in the dark. After incubation, 400 mL of 1X binding buffer was added to each tube, and the stained cells were analysed within 1 h using a FACS Calibur (BD, San Jose, USA) and Flow Jo software (Tri Star). Since Annexin V FITC staining precedes the loss of membrane integrity that accompanies the later stage identified by PI, an Annexin FITC-positive, PI-negative staining pattern indicates early apoptosis, while viable cells are Annexin V FITC negative and PI negative. Cells that are in late apoptosis or are already dead are positive for both Annexin V FITC and PI.

RESULTS

Anticancer activity of copper complexes

The results of the MTT assays indicate that the standardized mixture of n-propyl polysulfides has a strong, dose-dependent cytotoxic effect on all three of the tested carcinoma cell lines (CT26, 4T1, B16F10) (Figure 1). The n-propyl polysulfide mixture had almost the same cytotoxic activity as cisplatin against the 4T1 murine mammary carcinoma cell line. Interestingly, the cytotoxic effects of

the n-propyl polysulfide mixture against the murine colon cancer and melanoma cell lines (CT26 and B16F10, respectively) were much stronger than those of cisplatin (Figure 1). Significant cytotoxic effects of the n-propyl polysulfide mixture against CT26 and B16 F10 cells were detected at even the lowest tested concentration (0.008 mg/ml). Importantly, compared with cisplatin, the tested mixture had significantly lower cytotoxicity against mMSCs, which are non-cancerous and highly proliferative cells (Figure 1). The similar effects of the tested mixture and cisplatin on 4T1 cells and the significantly greater effects of the mixture against CT26 and B16F10 cells, as well as the lower cytotoxicity against mMSCs, were confirmed by analysis of the IC₅₀ values (Table 1).

To determine the possible mode of death of the cells treated with the n-propyl polysulfide mixture, flow cytometry analysis of CT26 and 4T1 cells stained with Annexin V and PI after exposure to the test mixture (at a

Table 1. IC₅₀ values (in mM) for 4T1, CT26, and B16F10 cells, as well as mMSCs, after exposure to DPPS and cisplatin for 24 h as determined by MTT assays. The data are presented as the mean ± SD (standard deviation) from three experiments.

Compound	IC ₅₀ (mg/ml)			
	4T1	CT26	B16F10	mMSC
DPPS	0.043±0.006	0.024±0.018	0.023±0.002	0.059±0.002
Cisplatin	0.029±0.004	0.160±0.092	0.123±0.082	0.028±0.006

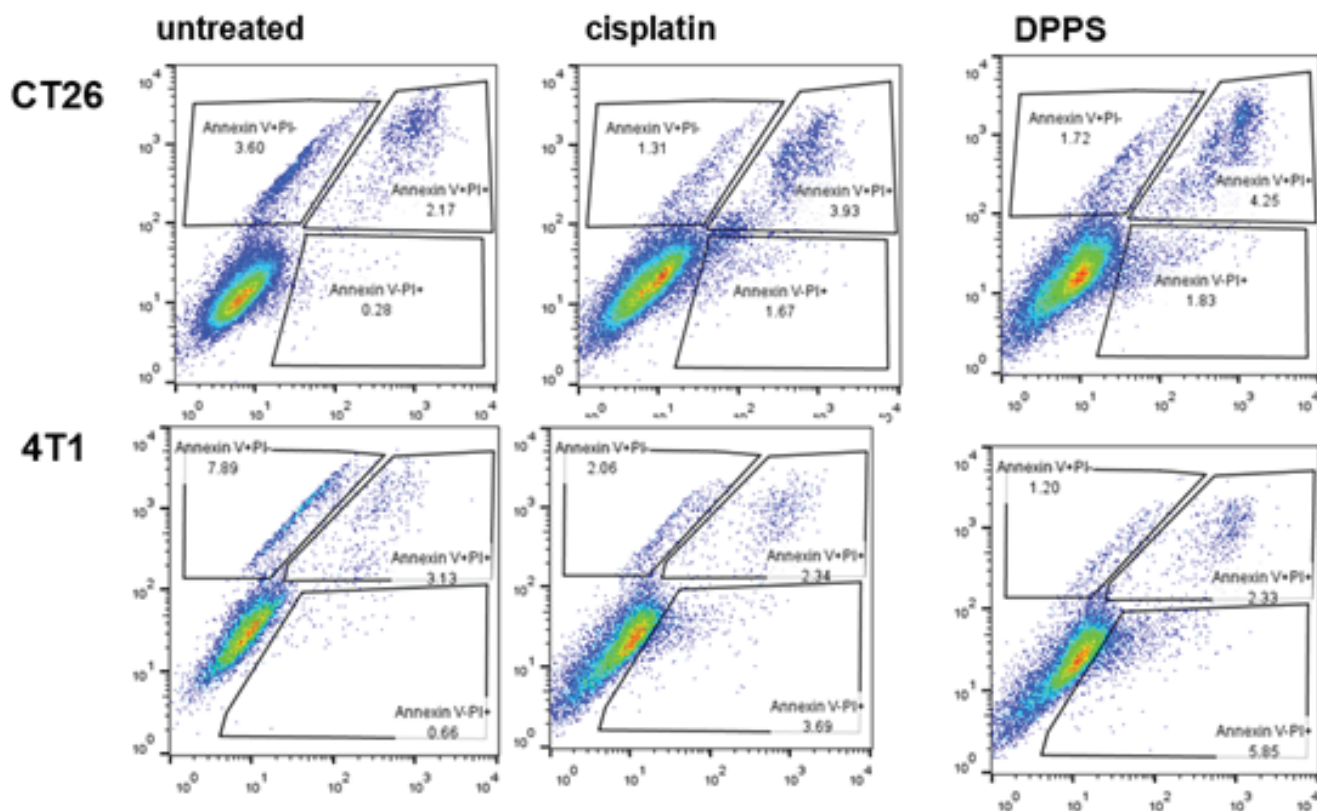


Figure 2. Representative flow plots showing the percentages of early- and late apoptotic and viable CT26 and 4T1 cells after 24 h of treatment with DPPS (0.01 mg/ml).

concentration of 0.01 mg/ml) for 24 h was performed. In agreement with the results of the MTT assays, a stronger cytotoxic effect of the n-propyl polysulfide than of cisplatin, as determined by Annexin V PI staining, was observed in CT26 cells, while better cytotoxicity in 4T1 cells was shown for cisplatin (Figure 2). Furthermore, analysis of the percentages of stained CT26 and 4T1 cells after treatment with the test mixture and cisplatin indicated that apoptosis is not the dominant mechanism of cell death induced by the n-propyl polysulfide mixture. The percentage of PI+ cells (Annexin V+ PI+ and Annexin V- PI+) was higher in the populations of both CT26 and 4T1 cells treated with the test mixture than in those treated with cisplatin (Figure 2).

DISCUSSION

This study shows for the first time a greater cytotoxic effect of an n-propyl polysulfide mixture compared with a standard chemotherapeutic agent (cisplatin) in murine colon carcinoma (CT26), melanoma (B16F10), and mammary carcinoma (4T1) cell lines.

The biological activities of various organosulfur compounds are partially the result of their chelating activity (16). Metal cations, including Cu^+ and Cu^{2+} , may be involved in the formation of chelating complexes (17). Four organosulfur compounds (diallyl sulfide (DAS), diallyl disulfide (DADS), S-ethylcysteine, and N-acetyl-

cysteine) show marked copper-chelating capability. The method used to determine the chelating effects of DAS and DADS on copper is based on restoring the activity of xanthine oxidase, which is inhibited in the presence of copper (15).

Our finding that a mixture of n-propyl polysulfides, which may have chelating activities, reduces the viability of tumour cells agrees with previous findings. The copper chelating agent trientine suppresses tumour development (18, 19). Curcumin, a polyphenol that belongs to the ginger family, chelates copper with high affinity and inhibits cell proliferation, invasion, metastasis, and angiogenesis (20). Administration of curcumin in animal tumour models resulted in the suppression of tumour growth associated with reduced copper concentrations in the serum of the treated groups (20). Furthermore, organic copper-binding compounds, such as clioquinol and pyrrolidine dithiocarbamate, bind copper and form new complexes that function as cancer-specific proteasome inhibitors and apoptosis inducers in human breast cancer cells (21).

More than 95% of the total copper in human plasma is associated with ceruloplasmin, while the remaining plasma copper is associated with albumin and transcuprein (22). It has been shown that the concentration of ceruloplasmin and transcuprein in tumour tissue is increased. Furthermore, tumour tissue can take up copper from the ceruloplasmin fraction of the plasma (22). Copper enters the cell through various transporter molecules in the plasma membrane, known as copper



transporter protein 1 CTR1 (23), and binds to different factors, such as metallothionein, cytochrome oxidase, superoxide dismutase and the cytosolic copper chaperones Cox17 and Atox1 (24). Inhibitors of the copper trafficking proteins Atox1 and CCS significantly reduce the proliferation of cancer cells with no effects on normal cells. Blocking copper trafficking induces cellular oxidative stress and reduces the cellular levels of ATP (25).

Our findings indicate that the reduction in the viability of murine MSCs (noncancerous and highly proliferative cells) in response to the n-propyl polysulfide mixture was smaller than that in response to cisplatin (Figure 1). This finding agrees with previous reports that copper chelating agents selectively kill human colon cancer cells without affecting the viability of noncancerous colon or intestinal cells (26). The selective cytotoxic activity of the n-propyl polysulfide mixture towards tumour cells may be the consequence of more pronounced chelating activity in tumour cells, as they contain higher amounts of copper. The higher percentage of necrotic tumour cells exposed to the n-propyl polysulfide mixture is in line with a previous finding that the organosulfur compound diallyl disulfide induces mainly necrotic death in *Candida albicans* (27).

Based on our findings, further studies should be done to explore the mechanisms of the antitumour action of this n-propyl polysulfide mixture, to elucidate the basis of the selective activity towards tumour cells and to evaluate for *in vivo* effects in animal tumour models.

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