Conjugation of anticancer drugs with novel PEG-containing nanocarrier provides circumvention of drug-resistance mechanisms *in vitro* and protects against general toxicity *in vivo*

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ABSTRACT

The development of targeted drug delivery using conjugated nanoparticles brings more drug molecules to diseased sites, at the same time reducing the negative side effects of systemic drug exposure. In the present study, the binding capability of the newly developed biocompatible PEG-containing polymeric nanocarrier (PNC) was demonstrated. The uptake and cytotoxicity of nanocarrier-immobilized anticancer drugs were enhanced compared to the free drugs. Approximately 10 times lower doses of the PNC complexes achieved similar effects as the free form of the drug on cell cycle arrest, DNA damage, and apoptotic cell death (caspase 7 and PARP cleavage).

We investigated anticancer effects of the compounds ID3882, ID3288 and ID3833, the drugs Doxorubicin (Dox) and Temozolomide (TMZ), and PNC complexes containing the compounds ID3882, ID3288 and ID3833 and Dox. PNC complexes demonstrated reduced general toxicity, and enhanced anticancer effects in drug-sensitive and drug-resistant tumor cells, therefore improving the outcomes of oncotherapy.

Keywords: anticancer drugs, polymeric nanocarrier, drug delivery, tumor cells, apoptosis, tumor-bearing animals, treatment, side effects

1 INTRODUCTION

One of the most important tasks of oncotherapy is the development of novel delivery systems for anticancer drugs that have low general toxicity, high chemotherapeutic potential, and targeted drug delivery to the tumor cells. Polyethylene glycol (PEG) conjugation with a drug delivery platform has been also used to overcome the limitations of the traditional methods for drug delivery.

ID3288, ID3833 and ID3882 compounds were synthesized based on anticancer effects of 4-thiazolidinone derivatives. Our previous findings show that pyrazoline-thiazolidinone-indoline conjugates are the most promising for further pre-clinical studies [1]. These compounds demonstrated antineoplastic effects towards human tumor cells tested at the National Cancer Institute (Bethesda, MD, USA) in 60 cell lines. Of the 60 cell lines, the central nervous system (CNS) SF-539 human cancer cell line was found to be the most sensitive for the ID3288 compound. Four out of 60 tumor cell lines had positive cytostatic effects and 56 out of 60 tumor cell lines had positive cytotoxic effects. While human melanoma cells of the SK-MEL-5 line had sensitivity to ID3833 with none cytostatic effect in 59 and in 59 out and positive cytotoxic effect in 59 tumor cell lines.

We showed in a previous study that apoptosis induced by 4-thiazolidinone derivatives is the dominating effect in mammalian leukemia cells through a mitochondria-dependent pathway. These compounds caused inhibition of cell division and induced G0/G1 arrest in the treated cells [2,3].

We also evaluated the anticancer effect and general toxicity of the PEG-containing polymeric nanocarrier (PNC) complexes with 4-thiazolidinone derivatives (ID3288, ID3833 and ID3882) in cell lines *in vitro* and in experimental animals *in vivo*.

2 EXPERIMENTAL

The novel polymeric nanocarrier (PNC), a water-soluble comb-like polymer poly(VEP-co-GMA)-*graft*-PEG, was

synthesized as we described earlier built on the co-polymer of unsaturated peroxide 5-*tert*butylperoxy-5-methyl-1-hexen-3-yne (VEP) and glycidyl methacrylate (GMA) as the backbone and polyethylene glycolic side chains (Fig. 1). The median diameter of the particles of this polymeric carrier is 61 nm, as determined by transmission electron microscopy, and confirmed with dynamic light scattering on a Zetasizer Nano (Malvern Instruments GmbH, Stuttgart, Germany) and photon correlation spectra using non-invasive back scatter technology [4].

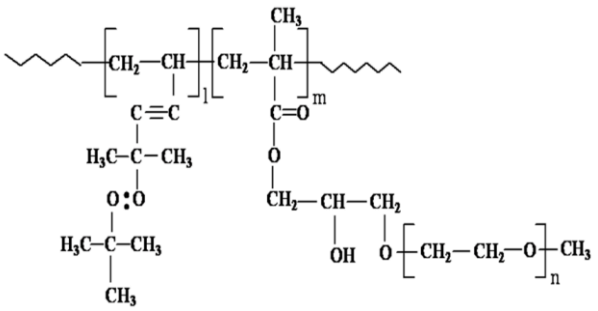

Figure 1. Schematic display of the newly developed biocompatible PEG-containing PNC.

Doxorubicin (Dox) and 4-thiazolidinone derivatives (ID3882, ID3288 and ID3833) were immobilized on the synthesized PNC. The biochemical indicators of the traditional anticancer drug Dox, experimental compounds ID3288, ID3833 and ID3882 and synthetic PNC complexes with Dox and the ID3288, ID3833 and ID3882 compounds were tested.

In *in vitro* study, the compounds, the Dox and the complexes were evaluated with the following cells: (1) murine leukemia L1210, (2) human T-leukemia Jurkat, (3) rat glioma C6, (4) human U251 glioblastoma and (5) murine transformed L929 fibroblasts cells.

The cytotoxicity was measured using colorimetric MTT assay for assessing cell metabolic activity, Trypan blue exclusion test, light and fluorescent microscopy. The pro-apoptotic proteins (cleaved caspase-3, ERK1/2-kinase) were analyzed with Western-blot. Cell cycling and apoptosis were studied with flow cytometry. Cytotoxicity and the pro-apoptotic effects were evaluated *in vivo* also.

Furthermore, the antitumor effects were measured in tumor bearing animals. Dox and the 4-thiazolidinone derivatives were injected into the tail vein of laboratory rats; blood was collected in the same manner. In the serum of the anticancer drug-treated experimental animals, the level of ~~the~~ malonic dialdehyde, and the activities of superoxide dismutase, catalase, and glutathione peroxydase were measured. The small signaling molecules such as OH^*, O_2^-, H_2O_2, NO (measured as $NO_2^-$and NO_3^-), and H_2S were also assessed. The same biochemical indicators of general toxicity used in the *in vitro* experiments were determined in blood serum of rats treated with ID3288, ID3833 and ID3882. The antitumor effect of these derivatives was evaluated in BALB/C mice grafted with murine NK/Ly lymphoma.

All experiments were repeated three times with three parallels in each variant. The analysis of variance was used as the statistical test for comparison of experimental groups. The results are presented as a mean ± SD using the GraphPad Prism 6.0 program (GraphPad Software, Inc., La Jolla, CA, USA), A p-value of <0.05 was considered as statistically significant.

3 RESULTS AND DISCUSSION

3.1 *In vitro* evaluation for vitality and morphology changes in tumor cells treated with 4-thiazolidinone derivatives and PNC complexes

We found that the PNC complex with Dox and 4-thiazolidinone derivatives significantly (by 10 times) decreased the effective cytotoxic dose in targeted tumor cells. At the same time, some free forms of the compounds and drug showed a similar antineoplastic effect towards various mammalian tumor cells. The microscopic investigation of the malignant cells lines demonstrated that some of the immobilized 4-thiazolidinone derivatives increased the apoptosis in the cells (results of microscopic study are not presented). 10% of treated murine leukemia L1210 cells and human T-leukemia Jurkat cells had morphological changes in the nucleus. In 35% of treated murine transformed L929 fibroblasts cells, an increased red fluorescence of acridine orange dye was observed in the lysosomes of the cells. We recorded lower values of the same indicators with free forms of the compounds used. The PNC complex with the compounds increased the cytotoxic effects of ID3882, ID3288 and ID3833, as measured by a reduction in the vitality of treated tumor cells (Fig. 2).

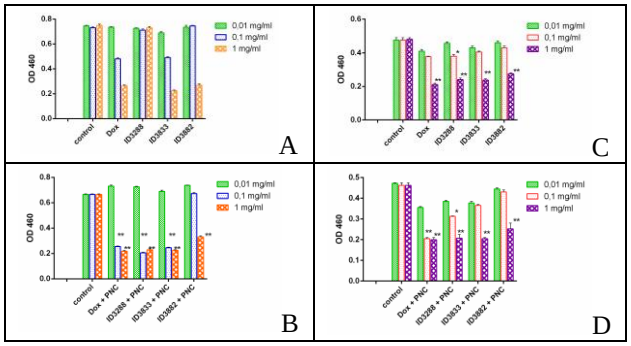

Figure 2. The effects of ID3833, ID3288, ID3882 applied in free form (A, C) and immobilized on the PNC (B, D) on the viability (MTT assay) of murine leukemia L1210 cells (A, B) and human T-leukemia Jurkat cells (C,D) were measured 72 h after treatment. * – P≤0.05; ** – P≤0.01

The results of the MTT assay and Trypan blue exclusion test used for evaluating the vitality and survival of rat glioma C6 and human glioblastoma U251 cells under treatment with ID3882, ID3833, ID3288, Dox and Temozolomide (Dox and TMZ were used as positive controls) showed significant differences in their toxicities in rat glioma C6 and human glioblastoma U251 cells. Based on these results, the following rank of toxicity was established ID3882<ID3833<ID3288≈Dox in rat glioma C6 cells. These results proved the superiority of ID3288 and showed the rank TMZ<Dox≈ID3288 in U251 cells. The classification of pro-apoptotic impairments in the morphology change in glioma U251 cells treated with the studied substances and Western-blot analysis of the cleaved Caspase 3 with studied substances corresponded to the same scale. With the apoptosis mechanisms, an increased cytotoxic effect was observed with the ID3288 derivative. In addition, we found that the cytotoxicity of ID3288 does not involve an increase in ROS production [2].

It should be stressed that the PNC complex enhanced the capability of Dox to induce drug-resistance caused by different molecular mechanisms. The PNC-Dox complex has shown a 3- to 6-fold increase in the intracellular accumulation compared to the free form of Dox in human carcinoma cells MCF-7/adr and SW1573/2R160 sub-lines, that overexpressed the ABCB1 membrane transporter [3]. The enhanced anticancer activity of the PNC-Dox complex caused more rapid and more efficient delivery of Dox into targeted tumor cells.

3.2 Biochemical indicators of cardio-, hepato- and nephrotoxicity *in vivo* using 4-thiazolidinone derivatives

The activities of α-amylase, γ-glutamyltransferase, lactate dehydrogenase, alkaline phosphatase, creatine kinase, aspartate aminotransferase, and alanine aminotransferase are specific indicators of toxicity in an organism. These enzymes were measured in blood serum of the experimental animals and used as indicators for toxic effects, specifically for nephrotoxicity. In addition, the levels of total protein, urea, creatinine, glucose, iron, calcium, sodium and chloride ions were measured. The intravenous (IV) injection of the studied compounds increased the activity of alanine aminotransferase, creatine kinase, alkaline phosphatase and α-amylase compared to the control animals. Dox injection was accompanied by a 4-fold increase in γ-glutamyltransferase activity, and injection of ID3833 led to a 2.5-fold elevation of enzyme activity.

The PNC complex with antineoplastic 4-thiazolidinone derivatives substantially decreased the activity of the investigated enzymes compared to the effect of the compounds in free form. The most evident decreases were detected for α-amylase, γ-glutamyltransferase and lactate dehydrogenase activities. The PNC complexes with the studied compounds compared with their free forms showed the same concentrations of total protein, urea and creatinine as in sera from control animals.

The tumor toxicity of the free form of compounds and the PNC complexes with the same compounds *in vitro* in human tumor cell lines and *in vivo* in experimental rats had a positive correlation with stronger toxic effects of free form compounds. Dox possessed the highest anti-neoplastic activity and had the highest general toxic effect in the treated animals. All rats injected with Dox died in 10 days, while the animals injected with 4-thiazolidinone derivatives did not die in 20 days or longer. In the experimental animals, the PNC complex with the studied compounds had less toxic effect compared to the free form of compounds. The synthetic PNC complex with the studied compounds led to a stable water-soluble drug delivery system and reduced significantly the cardio-, hepato- and nephrotoxic effect of the anticancer drugs.

3.3 Evaluation *in vivo* of the oxidative stress induced by 4-thiazolidinone derivatives in rats

Investigation of the optimal balance between the anticancer activity of novel and/or traditional drugs and the oxidative stress is required to study the side effects. The expression of negative side effects strongly depends upon free radical oxidation or reactive oxidant species (ROS) balanced processes and the activity of the antioxidant system (AOA). We have established that the levels of ROS induced by the 4-thiazolidinone derivatives (ID3288, ID3882 and ID3833) were significantly lower than the level induced by Dox (Fig. 3).

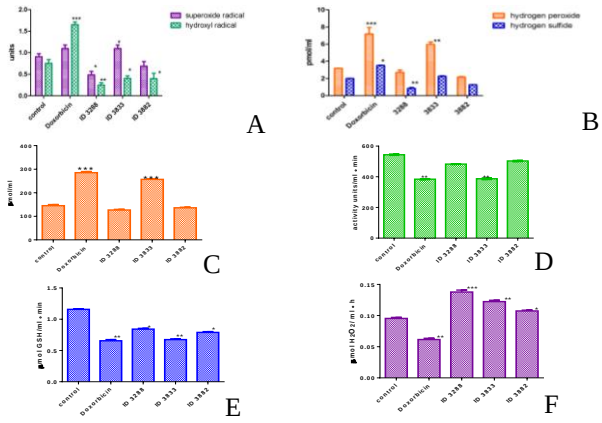

Figure 3. Concentrations of superoxide radical and hydroxyl radical (A), hydrogen peroxide and hydrogen sulfide (B), malonic dialdehyde (C), and activity of superoxide dismutase (D), glutathione peroxidase (E) and catalase (F) in blood serum of rats injected with Dox and the ID3288, ID3882 and ID3833 compounds.
* – P≤0.05; ** – P≤0.01, *** – P ≤0.001 (difference compared to control).

We measured both ROS and malonic dialdehyde as indicator of all ROS. We also investigated the activity of the antioxidant defense system using enzymes (superoxide dismutase, catalase, glutathione peroxidase, NO-synthase and NO-reductase). The antioxidant defense system was reduced more significantly by Dox than by the 4-thiazolidinone derivatives, at the same time, the catalase activity was elevated [2].

We have demonstrated that an increased level of ROS and a decreased activity of AOA positively correlated with the level of general toxicity of Dox, ID3288, ID3882, and ID3833. The proper modulation of ROS and AOA may be a useful strategy for decreasing negative toxic effects and their consequences as side effects of anticancer drugs.

3.4 Antitumor and associated effects of 4-thiazolidinone derivatives in mice with NK/Ly lymphoma

The novel PNC complex with Dox cured the mice with NK/Ly lymphoma or L1210 leukemia at a low dose (0.1 mg/kg), while free form Dox at same dose only extended the survival time of mice. A much higher dose of Dox (1.0 mg/kg) was needed to cure tumor-bearing animals. The PNC distinctly enhanced the antineoplastic activity of Dox. Consequently, less negative side effects were observed in tumor-bearing mice treated with the PNC Dox complex compared to mice treated with the free form of Dox.

The antitumor effects of the ID3833, ID3288, and ID3882 compounds and Dox were studied in mice injected with NK/Ly lymphoma intraperitoneally, that grew there and caused ascites. Ascites is a fluid in the peritoneal cavity that is not normal and is induced by inflammation or malignancies. Lymphoma development was controlled by measuring the volume of ascites (VA), and the tumor cells in the ascites. ID3833 was as effective as Dox in treating mice NK/Ly lymphoma, however ID3288 demonstrated weaker effect and ID3882 did not show antitumor action in this experimental tumor model. The number of different cells in mice blood, as well as the activity of aminotransferases monitored at the 14^{th} and 21^{st} days of the experiment were also measured and demonstrated high efficiency of tumor treatment by Dox, ID3833 and ID3288, while the effect of ID3882 was insignificant. Nevertheless, the 4-thiazolidinone derivatives extended the lifespan of the NK/Ly lymphoma-bearing mice in the order: ID3882<ID3288<ID3833≈Dox. After tumor inoculation and treatment with ID3833 or Dox, mice stayed alive with a reduction of lymphoma for more than 60 days.

During treatment, after 14 days in mice blood the activity of aminotransferases increased, but after 21 days it returned to the level in the control animals. After 21 days, Dox strongly reduced the number of red blood cells, compared to an insignificant effect of the 4-thiazolidinone derivatives, and normalized the increased number of blood neutrophils. Dox also increased the number of lymphocytes, while the studied compounds (ID3833 ID3288 ID3882) did not. It was demonstrated that in the treatment of murine lymphoma the compounds possess anticancer potential and are less toxic for organs of the animals (heart, liver and kidney) compared to Dox toxicity.

4 CONCLUSIONS

Novel PNC was prepared for drug delivery, the biocompatibility was demonstrated *in vitro* and *in vivo* and PNC reduced general toxicity in laboratory animals (rats and mice). The PNC complex with experimental anticancer compounds (ID3882, ID3288 and ID3833) and Dox both *in vitro* and *in vivo* enhanced the anticancer effects. These PNC complexes were equally effective in treatment of drug-sensitive and drug-resistant tumor cells. Therefore, the novel PNC is a good candidate for targeted drug delivery and could improve the treatment of malignant tumors with traditional drugs and/or with experimental anticancer compounds.

BioEthics Committee Approval. This study and the experimental procedures performed on animals were approved by the Ethical Committee of Danylo Halytsky Lviv National Medical University (Ukraine), Protocol N4 from 18.04.2016.

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