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Effect of Anticancer Agents on Viability and Proliferation of 2D and 3D Cultures of Rat Sarcoma Cells, Transformed by Rous Sarcoma Virus Strain Schmidt-Ruppin

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In this study we evaluate the effect of antitumor agents cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells, transformed by avian Rous sarcoma virus strain Schmidt-Ruppin, that express v-src oncogene. The investigations were performed by short-term experiments (24-72h) with MTT test and neutral red uptake cytotoxicity assay, and long-term experiments (25 days) with 3D colony-forming method. The results reveal that antitumor agents decrease cell viability and proliferation in a time- and concentration-dependent manner. The compounds completely inhibit 3D cell colony-forming ability of the treated cells applied at concentrations ≥ 18.5 μM (for epirubicin), ≥ 33 μM (for cisplatin) and ≥ 75μM (for oxaliplatin). According to the literature available the cytotoxic effect of cisplatin, oxaliplatin and epirubicin in virus-transformed cells and their "interactions" with v-src gene are not clarified yet. The presented cell model and experimental data are an important initial step in this direction.

Key words: Rous sarcoma virus/v-src, rat sarcoma, 2D and 3D cell cultures, antitumor agents, cytotoxicity, model systems

Introduction

Permanent cell line LSR-SF-SR was established from a transplantable rat sarcoma induced by Rous sarcoma virus (strain Schmidt-Ruppin) [2] known to induce tumors in various avian and mammalian species [24]. LSR-SF-SR cells are a suitable model system in the field of experimental oncology, oncovirology, tumor immunology and oncopharmacology because of at least three reasons: i) the cells express oncogene

v-src – the first retroviral oncogene to be discovered. V-src and its cellular counterparts (proto-oncogenes) encode membrane-associated non-receptor tyrosine kinases and are involved in pathogenesis of cancers in avian species, animals and humans [18]; ii) the cell line offers the opportunity to test the putative anticancer activity of new compounds (synthetic compounds, natural products) in retrovirus-transformed tumor cells; iii) Sarcomas are a heterogeneous group (including more than 100 subtypes) of rare malignancies of mesenchymal origin, which represent about 1% of known tumor diseases. With complex treatment (surgical resection of the tumor and chemotherapy – mainly doxorubicin-based regimens), the five-year survival is 60-80%. Metastases are found in 10% of patients at the time of diagnosis, and 25% of patients develop metastases after treatment of the primary tumor. Despite the successes in the treatment of sarcomas, the development of promising new therapeutic strategies in this area is among the main challenges facing modern biomedical science [19, 25, 27].

The possible application of LSR-SF-SR cells in antitumor drug discovery and development requires information about their biological characteristics, including sensitivity to the cytotoxic action of commercially available antineoplastic agents. The aim of our study was to evaluate the effect of cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells. Platinum-based drugs cisplatin and oxaliplatin are widely used in clinical oncology for the treatment of a wide range of human neoplasms [13]. Cisplatin is one of the most frequently prescribed antitumor agents in veterinary practice [16, 29]. Epirubicin (anthracycline medication smilar to doxorubicin) is included in the treatment of human breast cancer [17]. Moreover, these antitumor agents are included in some combined therapeutic regimens for soft tissue sarcomas (STS). For example treatment with combination of etoposide, ifosfamide and cisplatin has been suggested to be effective in patients with previously treated soft tissue sarcomas [20]. Oxaliplatin-dacarbazine neoadjuvant/adjuvant chemotherapy results in improved prognosis of patients with advanced limb STS in comparison with vincristine, epirubicin, cyclophosphamide combination therapy [31]. According to the literature available the influence of cisplatin, oxaliplatin and epirubicin on viability and proliferation of virus-transformed cells as well as their relationships with v-src gene are not clarified yet. Presenting for the first time data about cytotoxic effect of these antitumor agents in LSR-SF-SR cells is an important initial step in this direction.

Materials and Methods

Materials and Supplies

Antitumor agents cisplatin, oxaliplatin and epirubicin as well as dimethyl sulfoxide (DMSO) and trypsin were purchased from AppliChem (Germany). Purified agar, thiazolyl blue tetrazolium bromide (MTT) and 3-Amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral red) were obtained from Sigma-Aldrich Chemie GmbH (Germany). Dulbecco's modified Eagle's medium (D-MEM) and fetal bovine serum (FBS) were provided from Gibco-Invitrogen (UK). The antibiotics (penicillin and streptomycin) for cell cultures were from Lonza (Belgium). Ethylendiaminotetraacetic acid (EDTA) and 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).

Cell model system andcultivation

The cell line LSR-SF-SR (transplantable sarcoma in rat, induced by Rous sarcoma virus, strain Schmidt-Ruppin) was obtained from the Cell culture Collection of the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences [2].

The cells were routinely grown as monolayer (2D) cultures in D-MEM medium, supplemented with 5-10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The cultures were maintained at 37°C in humidified CO_2 incubator (Thermo scientific, Hepa class 100). The passaging was performed using a mixture of 0.05% trypsin and 0.02% EDTA.

Cytotoxicity assays

The cells were seeded in 96-well flat-bottomed microplates for cell culturing 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 (0.1-100 μg/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 thiazolyl blue tetrazolium bromide (MTT) test and neutral red uptake cytotoxicity (NR) assay.

The MTT test was carried out as described by Mossman [21]. Briefly, after three hours of incubation with MTT solution (5 mg MTT in 10 mL D-MEM) at 37°C in a humidified CO_2 incubator, the cells were washed by phosphate saline buffer (PBS, pH 7.2; 0.2 mL/well) followed by extraction with a mixture of absolute ethanol and DMSO (1:1, vol/vol) to dissolve the blue formazan.

The NR assay was based on the method of Borenfreund and Puerner [10]. A medium containing NR (50 μg/mL, 0.1 mL) was added to each well. The plate was placed in the CO_2 incubator for 3 h for the uptake of vital dye. Thereafter, the medium with NR was removed and the cells were washed with PBS (0.2 mL/well), followed by the addition of 0.1 mL 1% acetic acid solution containing 50% ethanol to extract the dye from the cells.

Optical density was measured at 540 nm / 620 nm (MTT) and 540 nm (NR) using an automatic microplate reader (TECAN, Sunrise™, Austria).

3D Colony forming method

The method was performed in order to obtain information about cytotoxic activity of the compounds examined for a longer period of time (namely 25 days) in conditions that reproduce better the 3D growth of tumor / tumor cells *in vivo*. The investigations were carried out as it was described earlier [15]. LSR-SF-SR rat sarcoma cells (10^3 cells/well) suspended in 0.45% purified agarose in D-MEM medium containing different concentrations of the compounds examined (ranging from 0.1 to 100μg/mL) were layered in 24 well microplates. The presence/absence of 3D cell colonies was registered using an inverted microscope (Carl Zeiss, Germany) during period of 25 days.

Statistical analysis

Statistical analysis was done using one-way analysis of variance (ANOVA) followed by Dunnett post-hoc test (*GraphPadPrizm, GraphPadSoftware Inc., USA,2000*) and Origin 6.1™.

Results

The short-term experiments carried out by MTT test (the gold standard for cytotoxicity assays) and neutral red uptake cytotoxicity technique revealed that applied at a concentration range of 0.1 to 100 μg/ml for 24h, 48h and 72h respectively, anticancer agents cisplatin, oxaliplatin and epirubicin decreased in a time- and concentration-dependent manner viability and proliferation of the treated LSR-SF-SR rat sarcoma cells. Relative cell viability, expressed as a percentage of the untreated control (100% viability), was calculated for each concentration. Concentration–response curves were prepared (**Fig. 1**) and the compounds effective cytotoxic concentrations – CC_{50} (μM) and CC_{90} (μM) causing respectively 50% and 90% reduction of cell viability as compared to the untreated control were estimated from these curves (Tables 1 and 2). On the basis of their cytotoxic activity (CC_{90}, μM) determined by MTT test after 48 h and 72 h of treatment, the compounds examined were graded as follows (starting with the compound with the highest cytotoxic activity according to Fig. 1, **Tables 1** and **2)**: cisplatin > epirubicin > oxaliplatin (48h) and cisplatin > oxaliplatin > epirubicin (72h).

The long-lasting cytotoxic effect of cisplatin, oxaliplatin and epirubicin on viability and 3D growth of rat sarcoma cells was examined by 3D colony-forming method. The first colonies composed of 12-16 non-treated control cells appeared after 4-6 days of cultivation. The appearance and development of 3D cell colonies were recorded for

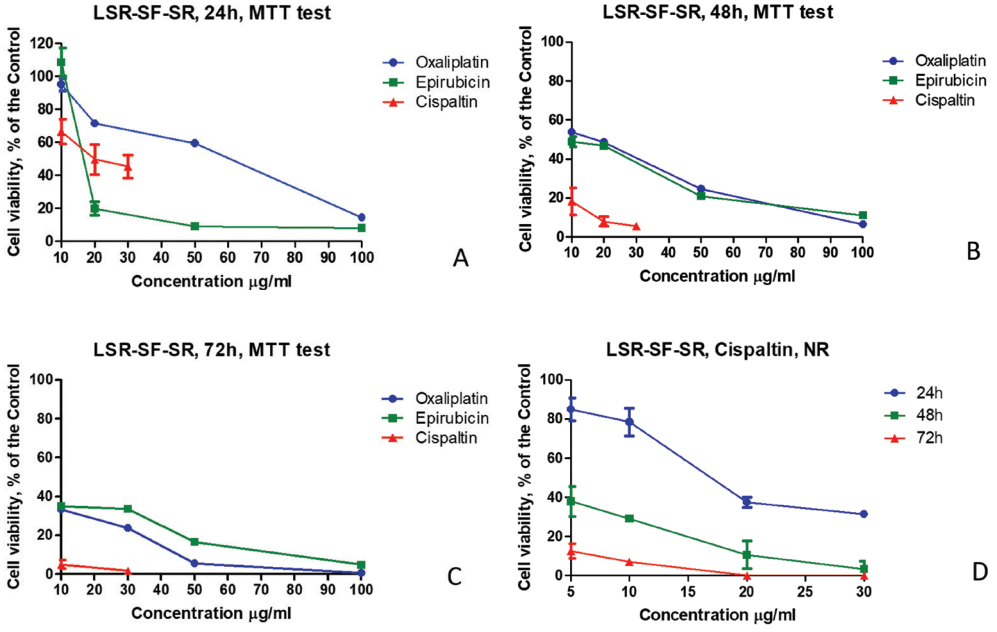

Fig. 1. Effect of anticancer agents on viability and proliferation of LSR-SF-SR rat sarcoma cells. Influence of cisplatin, oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR cells determined after 24h (A), 48h (B) and 72h (C) of treatment by MTT test. Cytotoic effect of cisplatin in rat sarcoma cells examined by neutral red uptake cytotoxicity assay (NR) after 24h, 48h and 72 h (D). All data points represent an average of three independent assays.

25 days. The compounds examined were found to inhibit completely 3D growth of rat sarcoma cells administered at concentrations ≥ 18.5 μM (for epirubicin), ≥ 33 μM (forcisplatin) and ≥ 75μM (for oxaliplatin). 3D cell colonies were observed although with a smaller number and / or size compared to the untreated control when antitumor drugs were administered at lower concentrations.

Table 1. Effect of cisplatin on viability and proliferation of LSR-SF-SR rat sarcoma cells

Treatment interval (h)	**24**		**48**		**72**	
Method	**MTT**	**NR**	**MTT**	**NR**	**MTT**	**NR**
CC_{50}, μM	66.7	56.8	10.9	8.3	4.8	5.3
CC_{90}, μM	n.d.	n.d.	59.9	66.7	16.7	24.4

MTT = MTT test; NR = Neutral red uptake cytotoxicity assay; n.d. – not determined

Table 2. Effect of oxaliplatin and epirubicin on viability and proliferation of LSR-SF-SR rat sarcoma cells

Anticancer agent	**Oxaliplatin**			**Epirubicin**		
Treatment interval (h)	**24**	**48**	**72**	**24**	**48**	**72**
CC_{50}, μM	151.7	43.4	9.8	30.7	16.8	7.3
CC_{90}, μM	n.d.	228.7	113.4	85.0	184.0	143.6

CC_{50} (μM) and CC_{90} (μM) were determined by MTT test; n.d. – not determined

Discussion

In this study we report for the first time data about sensitivity of retroviral-transformed rat sarcoma (LSR-SF-SR) cells to the cytotoxic activity of three of the most widely clinically applied antitumor agents. Cisplatin, oxaliplatin and epirubicin have been found to decrease viability and 2D/3D growthof LSR-SF-SR cells using methods with different molecular/cellular organelle targets and mechanisms of action (MTT test and NR assay) as well as 3D colony forming method. The MTT test is based on the functional activity of dehydrogenase enzymes in mitochondria, while neutral red dye penetrates the intact lysosomes of healthy cells. Cisplatin shows the highest cytotoxic activity in short-term experiments, and epirubicin most effectively inhibits the 3D growth of sarcoma cells in long-term experiments (25 days). Compared to the traditional monolayer (2d) cell cultures, 3D cell cultures more adequately represent biology and behavior of tumors/ tumor cells, especially their chemosensitivity.

Among the advantages of the 3D colony-forming method we use is that it allows the influence of the compounds examined on the survival and proliferation of the treated cells to be monitored for a long period of time (25 days in the study presented) providing valuable information about stability of their cytotoxic effect. From clinical point of view the presence of even a single 3D cell colony surviving after treatment can lead to relapse and metastasis *in vivo*. That is why our interest was focused on effective concentrations in which the compounds completely suppresses formation of 3D cell colonies [8].

The cell line LSR-SF-SR has a number of valuable biological characteristics that make it a suitable model system for research purposes, such as: i) easy cultivation as 2D and 3D cell cultures;

ii) the cells can be successfully implanted into laboratory animals (for example immunocompetent Wistar rats), leading to the development of tumor formation at the site of inoculation after a typically 7-20 day latency period. This allows *in vivo* studies to be carried out on various aspects of tumorigenesis, including tumor immunology and testing of antitumor activity of various agents and strategies [3-5].

iii) the cells express v-src gene. Src is a member of a superfamily of membrane-associated nonreceptor protein tyrosine kinases that are stimulated by receptors of growth hormones, cytokines and adipokines. Src proto-oncogene plays key roles in cell adhesion, growth, division, migration, and survival signaling pathways and is known to be disregulated in many types of human and animal cancers. Src has been recognized as a promising target for innovative antitumor treatment strategies [26,30].

LSR-SF-SR rat sarcoma cells have been used as model systems to evaluate the cytotoxicity and potential antitumor activity of compounds with various structure and chemical /physicochemical properties including alkaloids [7], photosensitizers [28], disulfiram [14], ammonium vanadate [1], basic salts of zinc(II) and copper(II) [9], metal complexes with different ligands such as ionophore antibiotic monensin [22], non-steroidal anti-inflammatory drugs [11,12], Mannich bases [6], etc.

Comparison of the data on the cytotoxic activity of these substances [1,6,7,9, 11,12,14,22,23,28] with the results obtained in the present study indicate that some of the newly synthesized metal complexes studied (e.g. those of the ionophore antibiotic monensin) are more effective compared to cisplatin – currently the most widely clinically used anticancer agent. Thus, after 72h incubation period, the CC_{50} of monensin and La(III), Nd(III), Mn(II) and Ca(II) complexes with this ionophore antibiotic have been calculated (by MTT test) to be 4.4, <0.23, <0.23, 2.1 and 2.2 μM respectively [22]; [23]; whereas CC_{50} of cisplatin determined at the same conditions (72h, MTT test) is 4.8 μM.

Conclusions

Our study presents original data on the sensitivity of retrovirus-transformed rat sarcoma cells (the cell line LSR-SF-SR) to the cytotoxic effect of three commercially available antitumor agents widely used in human and veterinary clinical practice (cisplatin, oxaliplatin and epirubicin). LSR-SF-SR cells are valuable model system for the needs of experimental oncology and oncopharmacology as they are easily maintained, capable of growing as 2D and 3D cultures *in vitro* as well as in laboratory animals (including immunocompetent rats) *in vivo.* Most importantly, LSR-SF-SR cells express a representative of src gene family (v-src gene) that perform important life-supporting functions and when dysregulated they take part in pathogenesis of a wide range of tumors human and animal cancers. The results obtained are a step forward in better clarification of the biological characteristics of LSR-SF-SR cells and their application in our attempts to identify new antitumor treatment strategies, especially directed against cancer cells expressing the src oncogene, and to elucidate better their mechanism of action.

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