INTRODUCTION

The activation of antitumor immunity is an important component in the treatment of immunocompromised cancer patients. Recent studies clearly show that an increase in tumor cell immunogenicity is capable of restoring antitumor immune responses, ultimately leading to tumor rejection in mouse tumor models [1-3]. Although cytotoxic anticancer drugs and so-called targeted agents act mostly through direct effects on cancer cells, many of these agents have additional effects on the immune system. Chemotherapeutic agents and radiation can increase the immunogenic properties of tumor cells by enhancing MHC class I expression, thereby increasing their vulnerability to cytotoxic T lymphocytes (CTL). Some chemotherapeutic agents increase the expression of NKG2D ligands, thus facilitating tumor cell lysis by NKG2D-expressing lymphocytes (including natural killer cells, NKT cells, and CTLs). Radiotherapy or chemotherapy increase the expression of death receptors (in particular Fas/CD95 and TNF-related apoptosis-inducing ligand [TRAIL] receptors), enabling lysis of the tumor cells by Fas/CD95 ligand and TRAIL-positive immune effectors [4]. In addition, the cancer cell death elicited by radiotherapy and some chemotherapeutic agents such as anthracyclines and platinum compounds is immunogenic [5-7].

Immunogenic cell death is characterized by the early surface exposure of chaperones including calreticulin and heat shock proteins, which affect dendritic cell maturation and the uptake and presentation of tumor antigens. It has also been shown that immunogenic cell death is characterized by the late release of high mobility group box 1 (HMGB1) proteins [8, 9]. HMGB1 is a member of a subfamily of the HMG proteins. HMG proteins are constitutively expressed in the nucleus of eukaryotic cells. Like the other members of this protein family, HMGB1 plays an important role in DNA architecture and transcriptional regulation. HMGB1 is released passively during cellular necrosis by almost all cells which have a nucleus and signals ongoing damage to neighboring cells. As HMGB1 has multiple
downstream signaling responses due to activation of different receptors, it also induces cell-specific responses when it stimulates immune system cells. HMGB1 induces dendritic cells maturation as measured by the increased expression of many cell surface markers as well as the secretion of inflammatory cytokines. Monocytes and neutrophils stimulated with HMGB1 have an increased capacity for adhesion and release numerous cytokines and inflammatory mediators. T cells stimulated with HMGB1 release cytokines and appear to demonstrate increased proliferation, survival, and Th1 functional polarization [10-12]. Recent data suggest that it is precisely innate and cognate immune responses elicited by anti-cancer agents that are required for an optimal therapeutic outcome, underscoring the clinical relevance of immunogenic cell death [13, 14]. However, it is known that most anticancer agents target tumor cells and rapidly proliferating immune system cells without any distinction and could be cytotoxic for non-malignant cells.

NSC631570 (Ukrain™) is a semisynthetic compound of thiophosphoric acid and alkaloids from the plant Chelidonium majus. It exerts its cytotoxic and cytostatic effects on cancer cells due to its selective accumulation in tumor tissue and it activates apoptosis in malignant cells but not in normal cells [15-18]. In addition, it is able to modulate immunological reactivity in vitro and to alter immunocyte functions in vivo [15, 19, 20]. Previous results from our laboratory have shown that monotherapy with NSC631570 resulted in strong tumor growth inhibition in mice with melanoma B16. However, the therapeutic effect was more expressed in mice bearing the high-metastasizing, low-immunogenic tumor variant [21, 22].

This study aimed to investigate the immunogenicity of melanoma B16 after treatment with NSC631570.

MATERIALS AND METHODS

Experimental animals, experimental tumor models and cell lines

The experiments used female C57BL/6 mice, 2–3 months old and weighing 18-2 g. bred in the vivarium of the Educational and Scientific Center “Institute of Biology” of Taras Shevchenko National University of Kiev (Ukraine). All animal procedures were carried out according to the rules of the local Ethics Committee. Transplantable B16 melanoma cells of C57BL/6 mice were kindly supplied by the Bank of Cell Cultures and Transplantable Experimental Tumors of R. E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine. Different B16 melanoma sublines with distinct metastatic potential were established from transplantable B16 melanoma of C57BL/6 mice as described previously [21]. MM-4 cells, exhibiting a relatively low metastatic potential, were established from the primary lesion of subcutaneously injected B16 cells. MM-4M2 cell lines, established by two sequential passages of lung metastases of MM-4 cells after intravenous injection, are highly metastatic. Cells were cultured in vitro in Dulbecco modified Eagle medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37°C in 5% CO2.

Experimental design

All experiments were performed at least three times (for in vitro experiments in duplicate, triplicate or quadruplicate). For in vivo experiments animals were randomized by weight and put into five groups (8 animals per group): two experimental groups (MM-4 and MM-4M2-bearing mice treated with NSC631570), two positive control groups (mice transplanted with MM-4 and MM-4M2), and a negative control group (intact animals). Tumor cells (2 x 10⁶) were inoculated intravenously at the volume of 0.1 ml of physiologic solution.

Treatment regimen

Starting from the second day after inoculation of tumor cells and seven times every third day, NSC631570 was administered intravenously (5 μg/g). The total course dose per animal was 700 μg. Animals from the control groups received solvent at the same volume according to the same regimen.

Evaluation of the antitumor effect

Body weight was determined twice a week during the study. Mice were sacrificed on the 24⁰ day after tumor induction (24 h after the last dose of NSC631570); livers, thymi and spleens were dissected and weighed. Splenic index and thymic index were calculated respectively.

\[
\text{Thymic index} = \frac{\text{Thymus weight}}{\text{Body weight}}
\]

\[
\text{Splenic index} = \frac{\text{Spleen weight}}{\text{Body weight}}
\]

The number of tumor nodules on the surface of five lung lobules of each mouse was determined using a binocular with dimension glass.

The antitumor effect was characterized by the growth inhibition index GII, calculated by the formula:

\[
\text{GII} = \frac{(N_c - N_i)}{N_c} \times 100\%
\]

-Nc average numbers of tumor nodules in the lungs of control animals
-Ni average number of tumor nodules in the lungs of experimental animals

Determination of cell viability

Cell viability was determined by the MTT test [23] and confirmed by the Trypan blue exclusion test [24]. MTT (5 mg/ml) was dissolved in RPMI-1640 without phenol red. The solution was filtered through a 0.2 µm filter and stored at 2-8°C for frequent use. To determine the effects

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of NSC631570 (obtained from Nowicky Pharma, Vienna, Austria) and cis-Diammineplatinum(II) dichloride (Sigma) on cell viability, cells were either treated with NSC631570 or with cis-Diammineplatinum(II) dichloride at the different concentrations (1.6, 3.2, 6.4, 12.5, 25, 50, 100 and 200 µg/ml) for 24 h and 48 h periods. For the determination of cell viability, the medium was discarded and MTT solution was added and incubated for 3 h. At the end of the incubation period the MTT solution was removed and the cells and dye crystals were dissolved by adding dimethylsulfoxide (DMSO). Absorbance was measured at 570 nm using a microplate reader and the results were expressed as a percentage of the absorbance of the samples in comparison to the control.

**Flow cytometry analysis**

Cell apoptosis and necrosis were assessed by the Annexin V-FITC apoptosis kit (Imgenex, San Diego, CA, USA) according to the manufacturer’s instructions. MM4 and MM4-M2 cells were treated with NSC631570 and cisplatin as mentioned above. After the treatments, the cells were incubated with 5 μl Annexin V and 5 μl propidium iodide (PI) for 10 min at room temperature in the dark. Cells from each sample were then analyzed by FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed using CELLQuest software (BD; Franklin Lakes, NJ, USA).

**RNA isolation and TAP1 and TAP2 mRNA quantitation in cultured cells (RT-PCR)**

For RT-PCR studies, B16 melanoma cells were cultured for 48 h with or without NSC63570 (1.6 and 25 µg/ml). Total RNA was isolated from homogenized cells using the ‘RiboSorb’ kit (AmpliSens, Moscow, Russia). Extracted RNA was transcribed into cDNA using M-MuLV reverse transcriptase (Fermentas; Vilnius, Lithuania) following the recommendations of the manufacturer. The cDNA concentration was analyzed with absorbance at 260 nm. Equal aliquots of cDNA samples were taken for semiquantitative analysis of the expression level and primers for the GAPDH gene were used in the same reaction vessel to circumvent false negative results. For each reaction the PCR mixture contained Taq Buffer with (NH₄)₂SO₄, 2.5 µM Mg²⁺, 0.2 µM each dNTP, 1U Taq polimerase (Fermentas), 50 µM each primer from a pair, and 2 µl cDNA. The following primers were used (fw, forward, rv, reverse):

- fw TAP1: 50GGACTGTCAGCAGCGGCAACC-30
- rv TAP1: 5'CAGGATGCAGTGGCCAGGGCG-3'
- fw TAP2: 5'GCCAAGGTCATCCATGACAACTTTGG-3'
- rv TAP2: 5'GCCAAGGTCATCCATGACAACTTTGG-3'
- fw GAPDH: 5'GCCAGTCTGATGACCCCTGAGG-3'
- rv GAPDH: 5'GCCAGTCTGATGACCCCTGAGG-3'

PCR included an initial denaturation step (94°C, 4 min) and 30 amplification cycles (93°C, 35 s; 60°C, 35 s; 72°C, 35 s). PCR products were visualized by electrophoresis in 2% agarose on RAF-buffer, using a UV-transilluminator and ethidium bromide staining. Semiquantitative analysis of the expression level was performed using TotalLab TL120 (Nonlinear Dynamics Inc., Newcastle, United Kingdom). The GAPDH mRNA/cell was unaffected by NSC631570 and served as an internal control. For Fig.3, expression was normalized to GAPDH. Each PCR data point for TAP1 and TAP2 was divided by the respective individual GAPDH results. Mean and SD were calculated with normalized values, and statistical significance was evaluated by the Student two-tailed t-test.

**Western blot**

The tumors were surgically removed. Cell lysates were prepared by snap freezing the samples in liquid nitrogen and incubating them upon thawing for 45 min in ice-cold lysis buffer, which consisted of sterile PBS supplemented with 1% IPEGAL and 15% protease inhibitor cocktail (1 tablet/7.5 ml H₂O₂; Boehringer, Mannheim, Germany). After removal of unlysed cell remnants and nuclei by centrifugation in an Eppendorf micro-centrifuge (5 min, 10,000 rpm), the protein concentrations were determined by BCA Protein Assay kit (Pierce; Rockford, IL, USA) and 10 µg equal amounts of protein were loaded into a 15% polyacrylamide gel. Proteins were resolved and transferred to an Immobilon-P membrane (Millipore; Billerica, MA, USA) using semi-dry transfer (Bio-Rad; Hercules, CA, USA). After incubating the membrane in a blocking buffer, the membrane was incubated with TAP1 polyclonal antibodies (Santa Cruz Biotechnology; Santa Cruz, CA, USA). For a loading control, the levels of expression of the β-actin protein were detected in each sample using mouse β-actin monoclonal antibodies (Sigma). Immunoreactive bands were visualized by chemiluminescence using hors eradish peroxidase (POD)-conjugated IgG antibodies and ECL kit (Amersham; Uppsala, Sweden) according to the instructions of the manufacturer.

**ELISA assays**

HMGB1 expression was determined in mouse serum and cell culture supernatant using a specific anti-HMGB1 ELISA (Shino-Test Corporation; Tokyo, Japan) following the manufacturer’s protocol. Briefly, 100 µl sample diluent was added to each well. Next, 10 µl of cell culture supernatant or mouse serum was added to each well and incubated at 37°C for 24 h. Wells were washed five times with wash buffer and incubated for 2 h at 25°C with 100 µl of POD-conjugate solution. Wells were washed a further five times in wash buffer and incubated for 30 min at room temperature with substrate solution. The reaction was stopped by adding 100 µl of stop solution to each well and the absorbance was read at 450 nm (the background was subtracted by
measuring absorbance at 570 nm) [25]. The levels of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-2 were evaluated in serum of MM-4 and MM-4M2-bearing untreated mice and tumor-bearing mice treated with NSC631570 by ELISA following the manufacturer’s protocol (Gen-Probe Diacolonc, San Diego, CA, USA). The levels of IL-12, tumor necrosis factor (TNF)-α and IL-10 were also evaluated using appropriate test-systems (R&D System, Biomedica Medizinprodukte; Vienna, Austria). Wells were read on a 96-well plate reader at 450 nm and at 620 nm to subtract background absorbance.

**Cytokine determination from tumor nodules**

The cytokine profiles in tumors were determined by ELISA [26]. On day 24 after tumor inoculation, non-necrotic tumors were harvested, cut into small pieces and a tumor extract was prepared in ice-cold lysis buffer, which consisted of sterile PBS supplemented with 1% IPEGAL and 15% protease inhibitor. After removal of unlysed cell remnants and nuclei by centrifugation in an Eppendorf micro-centrifuge (5 min, 10,000 rpm), tumor nodule homogenates were evaluated for the production of IL-12, GM-CSF, TNF-α and IL-10 using appropriate test-systems (R&D System). Tumor-derived cytokines were corrected for total protein by Bradford assay (Sigma) and the results are expressed as pg/mg of total protein. The sensitivity of the IL-10 ELISA was 15 pg/ml. For IL-12 and GM-CSF the sensitivities were 7.8 pg/ml and for TNF-α 10.9 pg/ml.

**Statistical analysis**

The statistical significance of the experimental results was determined by Student’s t-test. Pearson’s test was used to determine the correlation coefficient ($R^2$) between HMGB1 release and percentage of cell death (total, apoptosis and necrosis) (GraphPad Prism). For all analyses, $P < 0.05$ was accepted as a significant probability level.

**RESULTS**

**Cell death level and HMGB1 release from dying B16 melanoma cells in vitro upon treatment with NSC631570**

We tested the cell viability after treatment with NSC631570 at the different concentrations for 24 and 48 h, using low-metastatic MM-4 and high-metastatic MM-4M2 tumor cell variants. Cisplatin was used in our experiments as a positive control. High-metastatic cells were characterized by a higher rate of spontaneous cell death after 24 h of culturing. We found that upon the use of the drugs at low concentrations (1.6-12.5 μg/ml), cisplatin more effectively induced the death of melanoma cells than NSC631570 after treatment for 24 h (Fig.1ab). Used at the high concentrations (25-200 μg/ml) both cisplatin and NSC631570 exerted comparable cytotoxicity towards B16 melanoma cells. High-metastatic cells were more sensitive to cytotoxic action of NSC631570 used at low concentrations (1.6-12.5 μg/ml): the level of cell death in MM-4 cells treated with the drug was on average 3 times higher than that in MM-4-M2 cells.

As for HMGB1 release, we found that its level in the culture supernatants of cells treated with NSC631570 was significantly higher than that after treatment with cisplatin as detected by ELISA. Pearson’s correlation analysis was used to determine the correlation coefficient ($R^2$) between the concentration of HMGB1 in the cell supernatants and the percentage of cell death in vitro in B16 melanoma cells treated with the cytotoxic drugs. The levels of HMGB1 in the cell probes treated with NSC631570 exhibited strong correlation for MM-4 cells ($R^2 = 0.96$, $P < 0.05$; Fig.1a) and for MM-4M2 cells ($R^2 = 0.9$, $P < 0.05$; Fig.1b) with the levels of cell death. Unlike in the cell probes treated with cisplatin where there was no correlation between HMGB1 level and cell death level. Moreover, in the supernatant of cells treated with cisplatin at high concentrations, the HMGB1 level was extremely low up to full absence.

Prolonged culturing of low-metastatic melanoma cells resulted in increase of spontaneous cell death (Fig.1cd). Unlike long-term culturing of high-metastatic cells lead to the decrease of spontaneous cell death. B16 melanoma cells with different biological properties responded in different ways to prolonged exposure to both cytostatic drugs. The level of cell death and the level of HMGB1 in the culture supernatants of low-metastasizing melanoma cells after prolonged treatment with cisplatin and NSC631570 (Fig.1c) did not differ significantly from those after treatment for 24 h (Fig.1a). In probes of high-metastasizing cells treated with cisplatin at the low concentrations (1.6-6.4 μg/ml) for 48 h (Fig.1d) we observed nearly 4 times higher level of cell death than that after treatment with the drug for 24 h (Fig.1b). The level of HMGB1 in these probes was also considerably higher than after treatment for 24 h. It should be pointed out that the level of HMGB1 in the probes of MM-4M2 cells treated with cisplatin at high concentrations was extremely low as before.

The levels of cell death in MM-4M2 cells treated with NSC631570 at different concentrations for 48 h did not differ significantly from those after treatment for 24 h. The mean level of HMGB1 in the supernatant of these cells treated with NSC631570 was significantly higher than that after treatment with the preparation for 24 h and significantly higher than that in MM-4 cell probes. The precise mechanism of cell death induced by NSC631570 as well as by cisplatin is not clear enough yet. Cells treated with these cytotoxic drugs die mostly in two general ways: apoptosis and necrosis. Apoptotic as well as necrotic cells can release HMGB1 proteins.
Therefore our next step was to assess whether the release of HMGB1 depends on cell death mode. Apoptotic cells were predominated among dead MM4 and MM-4M2 cells after treatment with both cytotoxic drugs independently of exposure time (Fig.2). The number of necrotic cells in the pool of dead MM4-M2 cells after treatment with both cytotoxic preparations was more than that in the pool of dead MM4 cells. The necrosis rate in MM4-M2 cells treated with NSC631570 at the concentration of 12.5-200 µg/ml for 24 h was much higher than that in the probes of these cells treated with cisplatin (Fig.2cd). Relative amount of necrotic cells in the probes of MM-4M2 cells treated with NSC631570 for 24 h was significantly higher than that in probes of MM-4 cells (Fig.2bd). It should be pointed out that the portion of necrotic cells in the pool of dead untreated MM4-M2 cells was higher than that in the probes of untreated MM4 cells after 24 h culturing. However, culturing for 48 h resulted in an increase of spontaneous death of MM-4 but not MM-4M2 cells. In melanoma cells treated with NSC631570, HMGB1 correlated higher with the number of apoptotic cells than with the number of necrotic cells (Table 1). In the tumor cells treated with cisplatin, we registered weak correlation between HMGB1 and relative amount of necrotic cells in some cases.

Treatment of B16 melanoma cells with NSC631570 increases expression of transporters for antigen presentation

Semi-quantitative RT-PCR demonstrated that treatment of high-metastasizing MM-4M2 cells with NSC631570 at the non-apoptogenic concentration increased expression of mRNA encoding TAP2 by nearly 3-fold (P < 0.05; Fig.3b) and at the apoptogenic concentration did not increase TAP2 mRNA. In contrast, treatment with NSC631570 at the different concentrations did not influence TAP2 mRNA expression in low-metastatic MM-4 cells.

TAP1 mRNA expression in MM-4 cells was also not affected by NSC631570 treatment. Surprisingly, we found a significant increase in the number of mRNA for TAP1 in high-metastatic low-immunogenic MM-4M2 cells after treatment with NSC631570 at the concentration of 1.6 µg/ml, whereas only a trace quantity of the TAP1 mRNA was found in untreated cells (Fig.3a).

TAP1 protein expression in cell lines was also assessed by Western blot (Fig.4). Normal TAP1 expression was observed in MM-4 cells. Strongly reduced expression was registered in MM-4M2. TAP1 expression in MM-4M2 cells was significantly increased after exposure to NSC631570 at the concentration of 1.6 µg/ml for 48 h but was unchanged in MM-4 cells.

**Figure 1.** Treatment with NSC631570 induces dose-dependent cell death accompanied by dose-dependent HMGB1 release in B16 melanoma cells with different biological properties. Low-metastasizing (a; MM-4) and high-metastasizing (b; MM-4M2) B16 melanoma cells were treated with NSC631570 for 24 h; (c) MM4 cells were treated with the preparation for 48 h; (d) MM-4M2 cells were treated with the preparation for 48 h. Untreated cells and cells treated with cisplatin (Cis) were used as a control. Release of HMGB1 was assessed in the cell culture supernatant by ELISA. The Pearson correlation analysis was used to determine the correlation coefficient (R²) between the concentration of HMGB1 in the cell supernatant and the percentage of cell death in vitro in melanoma B16 cells treated with NSC631570, P < 0.05.

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Figure 2. NSC631570 induces apoptosis as well as necrosis of low-metastasizing MM-4 and high-metastasizing MM-4M2 melanoma cells. MM-4 (abef) and MM-4M2 (cdgh) cells were treated with NSC631570 at the different concentrations for 24 h (a-d) and 48 h (e-h). After culturing, cells were stained with annexin V (AnnV)/propidium iodide (PI) and analyzed by flow cytometry. Data are averages of quadruplicate ± SEM; *P < 0.05 compared with MM-4M2 cells treated with cisplatin for 24 h (c); ^P < 0.05 compared with MM-4 cells treated with NSC631570 for 24 h (b).
Figure 3. Treatment with NSC631570 increases the expression of TAP mRNA in B16 melanoma cells. Low-metastatic (MM-4) and high-metastatic (MM-4M2) B16 melanoma cells were treated for 48 h with NSC631570 at the concentrations of 1.6 and 25 µg/ml. Semi-quantitative RT-PCR was performed using primers specific for TAP1 and TAP2. Total cellular RNA from MM-4 and MM-4M2 cells was extracted and subjected to RT-PCR analysis. Amplification of the GAPDH cDNA served as an internal control. TAP were normalized to GAPDH (GAPDH expression was not affected by NSC631570 treatment). Treatment with NSC631570 resulted in an increase in TAP1 (a) and TAP2 (b) mRNA expression in high-metastasizing B16 melanoma cells. These data were obtained in four independent experiments *P < 0.01 compared with untreated control.

Table 1. Pearson's correlation between HMGB1 levels and the number apoptotic or necrotic tumor cells in melanoma cell probes treated with NSC631570. Data are presented as coefficient; P value (RA = relative amount).

<table>
<thead>
<tr>
<th>Variables</th>
<th>HMGB1 concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Culturing for 24 h</strong></td>
<td></td>
</tr>
<tr>
<td>Treatment with NSC631570</td>
<td></td>
</tr>
<tr>
<td>RA of apoptotic MM-4 cells</td>
<td>0.864; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of necrotic MM-4 cells</td>
<td>0.496; P &lt; 0.001</td>
</tr>
<tr>
<td>RA of apoptotic MM-4M2 cells</td>
<td>0.973; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of necrotic MM-4M2 cells</td>
<td>0.968; P &lt; 0.05</td>
</tr>
<tr>
<td>Treatment with cisplatin</td>
<td></td>
</tr>
<tr>
<td>RA of apoptotic MM-4 cells</td>
<td>0.111; P &lt; 0.001</td>
</tr>
<tr>
<td>RA of necrotic MM-4 cells</td>
<td>0.244; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of apoptotic MM-4M2 cells</td>
<td>0.547; P &lt; 0.001</td>
</tr>
<tr>
<td>RA of necrotic MM-4M2 cells</td>
<td>0.863; P &lt; 0.001</td>
</tr>
<tr>
<td><strong>Culturing for 48 h</strong></td>
<td></td>
</tr>
<tr>
<td>Treatment with NSC631570</td>
<td></td>
</tr>
<tr>
<td>RA of apoptotic MM-4 cells</td>
<td>0.986; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of necrotic MM-4 cells</td>
<td>0.975; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of apoptotic MM-4M2 cells</td>
<td>0.987; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of necrotic MM-4M2 cells</td>
<td>0.658; P &lt; 0.05</td>
</tr>
<tr>
<td>Treatment with cisplatin</td>
<td></td>
</tr>
<tr>
<td>RA of apoptotic MM-4 cells</td>
<td>0.174; P &lt; 0.001</td>
</tr>
<tr>
<td>RA of necrotic MM-4 cells</td>
<td>0.737; P &lt; 0.05</td>
</tr>
<tr>
<td>RA of apoptotic MM-4M2 cells</td>
<td>0.091; P &lt; 0.001</td>
</tr>
<tr>
<td>RA of necrotic MM-4M2 cells</td>
<td>0.375; P &lt; 0.001</td>
</tr>
</tbody>
</table>

In vivo efficacy of therapy with NSC631570 in mice with transplanted B16 melanoma: release of HMGB1, serum level of cytokines, intratumoral cytokine profile and tumor regression

In vivo growth of melanoma B16 with different biological properties was associated with a slight increase in liver weight, statistically significant only in MM-4M2-bearing mice (Table 2). In animals with this tumor variant we also observed statistically significant splenomegaly. Both MM-4 and MM-4M2-bearing groups of mice tolerated the treatment well and exhibited normal behavior, as determined by activity level and grooming behavior throughout the study. Body weights as well as liver and thymus weights were unaffected by NSC631570 treatment (Fig.5). A moderate decrease in spleen weight was registered in mice bearing MM-4 after treatment with NSC631570. Therapy with NSC631570 elicited a strong tumor growth inhibition in mice with both variants of melanoma B16 (Fig.6). However, the therapeutic effect was more expressed in mice bearing the MM-4M2. GII in the group of mice bearing MM-4 after treatment with NSC631570 was on average 55.1%, as opposed to 89.9% in mice bearing MM-4M2 and treated with the drug.

In vivo efficacy of therapy with NSC631570 in mice with transplanted B16 melanoma: release of HMGB1, serum level of cytokines, intratumoral cytokine profile and tumor regression
Serum levels of HMGB1 were determined by ELISA in tumor-bearing mice 3 days after the treatment with NSC631570, as controls, mice were injected with saline (Fig. 7). In addition, we estimated the serum levels of HMGB1 in intact animals. Levels of HMGB1 were increased in the serum of mice bearing either MM-4 (about 7 times as compared to intact animals, P < 0.05) or MM-4M2 (about 5 times as compared to intact animals, P < 0.05). Treatment with NSC631570 resulted in an increase of serum levels of HMGB1 in mice bearing either MM-4 (about 3 times as compared to untreated animals, P < 0.05) or MM-4M2 (about 6 times as compared to untreated tumor-bearing mice, P < 0.05). We could not analyze the correlation between the GII and serum level of HMGB1 since some of the treated animals (about 30%) in both experiments had no tumor nodules in the lungs.

As shown in Fig. 8, on the 24th day after tumor induction (3rd day after last NSC631570 administration), tumor bearing control animals had an increased serum level of GM-CSF (28.3 ± 1.32 pg/ml in MM-4-bearing and 22.6 ± 0.76 pg/ml in MM-4M2-bearing animals) as compared to the normal level (16 ± 3.1 pg/ml). Treatment with NSC631570 resulted in a slight increase in this cytokine in serum of MM-4-bearing mice (34 ± 2.05 pg/ml), and a more expressed increase in MM-4M2-bearing animals (41 ± 3.12 pg/ml).

The IL-2 level in serum of tumor-bearing control animals was decreased (15.6 ± 4.32 pg/ml in MM-4-bearing and < 15.6 pg/ml in MM-4M2-bearing animals) as compared with intact control mice (26.4 ± 3.9 pg/ml). A decreased level of IL-2 in B16 melanoma-bearing animals was found to be significantly influenced by the administration of NSC631570. At the conclusion of the experiment the concentration of IL-2 in the serum of MM-4-bearing mice was twice as high as that in untreated animals.

Table 2. The effect of treatment with NSC631570 on organ weight in melanoma B16-bearing mice (n = 24 for each group)

<table>
<thead>
<tr>
<th>Animal group</th>
<th>Liver weight (mg)</th>
<th>Spleen weight (mg)</th>
<th>Splenic index</th>
<th>Thymus weight (mg)</th>
<th>Thymic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact animals</td>
<td>631.3 ± 23.4</td>
<td>79.5 ± 8.9</td>
<td>3.42 ± 0.5</td>
<td>34.5 ± 3.6</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Untreated MM-4-bearing mice</td>
<td>702 ± 36.2</td>
<td>75.2 ± 12.8</td>
<td>3.4 ± 0.6</td>
<td>39.5 ± 7.6</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>MM-4-bearing mice treated with NSC631570</td>
<td>692.8 ± 37</td>
<td>93.6 ± 7.9</td>
<td>4.1 ± 0.4</td>
<td>46 ± 10.5</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Untreated MM-4M2-bearing mice</td>
<td>713.5 ± 29.9*</td>
<td>159.2 ± 19.2*</td>
<td>7.4 ± 1.2*</td>
<td>46.5 ± 12.6</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>MM-4M2-bearing mice treated with NSC631570</td>
<td>687.9 ± 43.3</td>
<td>81.4 ± 26.7</td>
<td>3.7 ± 0.7**</td>
<td>47.5 ± 8.2</td>
<td>2.2 ± 0.3</td>
</tr>
</tbody>
</table>

P < 0.05 vs *intact animals, and **untreated animals.

Figure 5. Effect of treatment with NSC631570 on body weight of tumor-bearing mice. Body weight was determined twice a week during the study.

Figure 6. The antitumor effect of NSC631570 on B16 melanoma cells in vivo. The therapy started on day 2 after inoculation of tumor cells. NSC631570 was administered intravenously (5 μg/g) seven times every third day. Mice were sacrificed on the 24th day after tumor induction. The number of tumor nodules on the surface of five lung lobules of each mouse was determined using a binocular microscope with dimension glass. *P < 0.01 compared with untreated control (n = 24 for each group).

Figure 7. Treatment of B16 melanoma-bearing mice with NSC631570 accompanied by HMGB1 release in animal serum. Serum levels of HMGB1 were determined by ELISA 3 days after treatment cessation. P < 0.01 compared with *intact animals, and **untreated control (n = 16 for each group).
In MM-4M2-bearing animals, the serum level of IL-2 was about 3.7 times higher (37.21 ± 4.12 pg/ml) as compared to untreated mice. The serum level of IL-12 was slightly increased in MM-4M2-bearing mice as compared to intact animals. The levels of this cytokine in the serum of MM-4-bearing animals did not differ from that in intact control mice. Treatment of MM-4-bearing mice with NSC631570 did not affect the serum level of IL-12. Treatment of MM-4M2-bearing animals resulted in a significant increase in the serum level of IL-12.

The levels of circulating TNF-α were significantly increased in mice bearing both MM-4 and MM-4M2 tumors in comparison with intact animals. Treatment with NSC631570 did not significantly affect the serum level of this cytokine in tumor-bearing mice.

In our experiments the level of circulating IL-10 was significantly increased in tumor-bearing mice (in MM-4-bearing animals by 5 times and in MM-4M2-bearing by 3.6 times). The increased level of IL-10 in B16 melanoma-bearing animals was found to be significantly decreased by the administration of NSC631570; in MM-4-bearing animals by 1.8 times and in MM-4M2-bearing by 3.5 times.

Compared with untreated controls, the treatment group of MM-4-bearing animals receiving NSC631570 had a modest yet significant increase in type 1 cytokines (TNF-α, by 1.4 times; GM-CSF, by 2 times; and IL-12, by 1.6 times) with a concomitant slight decrease in the immunosuppressive mediator IL-10 by 1.3 times at the tumor sites. In comparison with untreated control animals and treated MM-4-bearing groups, MM-4M2-bearing animals treated with NSC631570 produced the most significant increases in type 1 cytokines and the most substantial decline in the tumor production of IL-10. Compared with tumors from the untreated group, MM-4M2-bearing mice treated with NSC631570 demonstrated significant reductions in IL-10 (1.8-fold), but an increase in TNF-α (1.7-fold), GM-CSF (3.5-fold), and IL-12 (2-fold) (Fig.9).

Figure 8. The effect of NSC631570 on serum TNF-α, GM-CSF, IL-12, IL-2 and IL-10 levels in B16 melanoma-bearing animals. Serum levels of cytokines in MM-4-bearing animals (a) and MM-4M2-bearing animals (b) were determined by ELISA 3 days after treatment cessation. P < 0.05 compared with *intact animals, and **untreated controls (n = 24 for each group).

Figure 9. NSC631570 therapy leads to an increase of T-helper 1 cytokines and a decrease in immunosuppressive molecules in tumor tissues. Tumors were harvested for cytokine analysis 3 days after treatment cessation. Non-necrotic tumors were cut into small pieces and a tumor extract was prepared in ice-cold lysis buffer. After removal of unlysed cell remnants and nuclei, tumor nodule homogenates were evaluated for the production of IL-12, GM-CSF, TNF-α and IL-10 using appropriate test-systems. Tumor-derived cytokines were corrected for total protein and the results are expressed as pg/mg of total protein. *P < 0.05 compared with untreated control; (a) the levels of cytokines in tumor tissue of MM-4-bearing mice; untreated tumor-bearing mice (n = 24), tumor-bearing mice treated with NSC631570 (n = 8); (b) the levels of cytokines in tumor tissue of MM-4M2-bearing mice; untreated tumor-bearing mice (n = 24), tumor-bearing mice treated with NSC631570 (n = 24).
DISCUSSION

In our in vitro experiments treatment of melanoma B16 cells with NSC631570 at apoptogenic concentrations induced dose-dependent tumor cell death accompanied by dose-dependent release of innate alarm in HMGB1, more in MM-4M2 than in MM-4 cells. The levels of HMGB1 in the cell probes treated with NSC631570 exhibited strong correlation with the levels of cell death. Unlike in the cell probes treated with cisplatin (used in the experiments as a positive control due to its ability to induce immunogenic tumor cell death [27]) there was no correlation between HMGB1 level and cell death level. It is known that the tumor suppressing activity of cisplatin mainly depends upon DNA damage, leading to apoptotic cell death through p53 stabilization. In addition, both types of cell death (apoptosis and necrosis) have been found in the same population of cisplatin-treated cells [28, 29]. The mechanisms of NSC631570-mediated tumor cell apoptosis have not yet been extensively investigated. Tubulin expression in pancreatic ductal adenocarcinoma (PDAC) cells indicated an antiproliferative effect of NSC631570 on the basis of alterations in mitotic spindle microtubule dynamics, leading to abnormal mitosis [30]. In HeLa cervical cancer cells and some other tumor cells the drug induces apoptosis independently from death receptor signaling via a pathway involving mitochondrial damage, cytochrome c release in the cytoplasm and caspase-activation that is partially sensitive to overexpression of Bcl-2, Bcl-xL and a dominant negative caspase-9 [18, 31]. These data led us to speculate that the intensity of HMGB1 release from dying tumor cells after treatment with NSC631570 depends on the mode of cell death induction and may correlate either with apoptosis or non-apoptotic cell death. To check our assumption we examined the ratio of apoptosis and necrosis in MM4 and MM4-M2 cells treated with NSC631570 and cisplatin at apoptogenic concentrations (12.5-200 μg/ml). The correlation between the HMGB1 and necrosis levels was strong and moderate in melanoma cells treated with NSC631570 and weak in the cells treated with cisplatin. These results suggest that the level of HMGB1 release from dying tumor cells after treatment with cytotoxic drugs depends not only on the mode of cell death. As cisplatin is a nonspecific drug and reacts not only with DNA but also with proteins [32], we cannot rule out the possibility that cisplatin at the high concentrations can damage HMGB1 proteins whereas there were no data indicating any protein-damaging action of NSC631570.

Prolonged exposure to both cytostatic agents led to more extensive death of B16 melanoma cells. High-metastasizing tumor cells were more sensitive to prolonged action of cisplatin and NSC631570: the level of cell death and the level of HMGB1 in the culture supernatants were considerably higher than after treatment for 24 h. The possible reason for this effect in cells treated with cisplatin is that the cytotoxic action of cisplatin depends on the duration of treatment and the late responses possibly initiated by cisplatin-DNA adducts requires more than 24 h to be observed [33]. We have previously reported that high-metastasizing, low-immunogenic MM-4M2 cells are characterized by higher sensitivity to various apoptosis inducers in comparison with MM-4 [22]. Probably, it was one of the reason of the high receptivity of these cells to cisplatin’s cytotoxic action. However, the level of HMGB1 in the probes of MM-4M2 cells treated with cisplatin at the high concentrations for 48 h was extremely low as before. The levels of HMGB1 in the supernatants of these cells treated with NSC631570 for 48 h were nearly 2 times higher than those after treatment with the preparation for 24 h and significantly higher than those in MM-4 cell probes. The findings let us to suppose that the ability of NSC631570 to induce tumor cell death accompanied by strong dose-dependent HMGB1 release can be used for cancer immunotherapy by using dendritic cells, as a high level of HMGB1 can potentiate the immunotherapeutic effect of the autologous tumor lysate-pulsed dendritic cells vaccine.

It is known that several chemotherapeutic drugs modulate antitumor immune responses, despite not inducing classic immunogenic tumor cell death. Used in nonlethal/sublethal doses, cytotoxic agents alter tumor phenotype and render the tumor more sensitive to immune effector cells [34]. Changes of tumor cell phenotype after such manipulations include elevated expression of MHC class I [35]. The classical MHC class I pathway functions to present largely endogenous peptides to cytotoxic T lymphocytes, and is understood to restrict the antitumor T cell response. It has been shown that many tumors express less MHC class I on their surface compared to the normal tissue from which they have arisen. Human melanoma cells are often deficient in MHC class I molecules and this appears to play a role in their expansion and escape from immunosurveillance. Alteration of MHC class I expression results in a lack of tumor-associated antigen processing and low immunogenicity [36]. MHC class I down regulation may result from structural defects in MHC genes, down regulation of MHC gene transcription, defects in β2-microglobulin synthesis or defects in MHC molecule assembly. Defects in antigen processing and presentation machinery impair the assembly of MHC class I molecules and thus decrease their cell surface expression and stability. Analysis of melanoma cell lines has revealed a heterogeneous down regulation of low molecular mass polypeptide...
(LMP)−2, LMP−7, transporter in antigen processing (TAP)1 and TAP2 [37]. In our previous investigation RT-PCR analysis revealed almost total inhibition of mRNA transcription of the peptide transporter TAP1 in high-metastasizing MM−4M2 cells compared with low-metastasizing MM−4 cells. Meanwhile TAP2 mRNA levels did not differ significantly in melanoma B16 cells with different biological properties. That is why we consider MM−4M2 cells as low-immunogenic [21]. It is known that mutations in transporters associated with antigen processing correlate with increased metastatic potential and reduced host survival in several malignancies [38]. It is known that correction of TAP1 and/or TAP2 defects in B16 mouse melanoma enhanced the cell surface expression of MHC class I molecules and significantly reduced the rate of subcutaneous tumor growth and pulmonary metastatic burden [39]. In this study, in addition to investigating the immunogenicity of B16 melanoma cell death after treatment with NSC631570 we examined the effect of the preparation used at the minimal non-apoptogenic (1.6 μg/ml) and minimal apoptogenic (25 μg/ml) concentrations on TAP expression in treated tumor cells. TAP1 mRNA expression in MM−4M2 cells was significantly increased after exposure to NSC631570 at the minimal non-apoptogenic concentration. Elevated level of TAP1 protein in MM−4M2 cells after treatment with the drug was also confirmed by Western blot.

Considering that NSC631570 induces HMGB1 release from dying melanoma cells that could affect antitumor immune response and thereby influence tumor growth in tumor-bearing mice, we investigated the levels of circulating HMGB1 as well as cytokine profile in the serum and tumor tissue of mice bearing B16 melanoma with different biological properties after treatment with the drug. Treatment with NSC631570 elicited a strong tumor growth inhibition, more expressed in MM−4M2-bearing mice. It was shown that NSC631570 down regulates matrix metalloproteinase (MMP)−2 and MMP−9 in pancreatic cancer cells, suggesting that the preparation may decrease cancer cell invasion [40]. This property of the preparation could be one of the reasons for the greater therapeutic efficacy in mice with melanoma B16 variant with high metastatic potential. Therapy with NSC631570 was associated with a moderate decrease of splenomegaly in MM−4M2-bearing mice, probably caused by extramedullary hemopoiesis, typical for high-metastatic tumors [41].

Treatment with NSC631570 induces tumor regression accompanied by strong HMGB1 release in serum of both MM−4- and MM−4M2-bearing mice, more expressed in mice with high-metastasizing, low-immunogenic tumors. However, dying tumor cells may be not the only source of elevated serum level of HMGB1. It is known that HMGB1 translocation occurs in macrophages that have been stimulated by Toll-like receptor (TLR) ligands as well as cytokines etc [42]. Taking into account the ability of NSC631570 to modulate macrophage function [19, 20], it seems probable that the increased level of serum HMGB1 in tumor-bearing mice after treatment with the drug can be the result of both tumor cell death and macrophage activation. In our previous investigations we have shown that NSC631570 did not affect HMGB1 release from intact murine macrophages in vitro [43].

The results of our investigation indicate the inhibition of cell immunity in mice bearing B16 melanoma with different biological properties, more expressed in the case of MM−4M2-bearing animals. Increase of the level of serum HMGB1 after treatment with NSC631570 was accompanied by increase in GM-CSF and IL−2 in serum of mice with both of melanoma B16 variants, increase in IL−12 in MM−4M2-bearing animals and significant decrease in IL−10 in serum of tumor-bearing mice.

Considering the ability of the preparation to accumulate selectively in tumor tissue and influence tumor microenvironment cells, we evaluated cytokine production from tumor sites. The tumor sites were evaluated for the presence of TNF−α, GM-CSF, IL−12 and IL−10 by ELISA. Increased levels of type 1 cytokines were registered in tumor tissue of treated animals. Tumor tissue of animals treated with NSC631570 was also characterized by decline of the level of IL−10, more substantial in MM−4M2-bearing mice.

These results show that treatment with NSC631570 causes immunogenic tumor cell death and associated with the stimulation of local and systemic immune responses in tumor-bearing mice. Thus, treatment of melanoma B16 cells with NSC631570 at apoptogenic concentrations induces dose-dependent tumor cell death accompanied by dose-dependent HMGB1 release in vitro, more in MM−4M2 than in MM−4 cells. In addition, the preparation at the non-apoptogenic concentration causes an increase in TAP expression, more evidently in the case of high-metastatic melanoma cells.

Treatment of melanoma-bearing mice with NSC631570 was also accompanied by a strong release of HMGB1 in their serum. This effect was more expressed in MM−4M2-bearing mice, in which greater therapeutic efficacy was registered, and was associated with an increase in type 1 cytokine levels in tumor tissue and serum. Our results taken together suggest that immunomodulation is an important component of the mechanism of action of NSC631570. In addition to the direct induction of tumor cell apoptosis the preparation has a positive influence on tumor immunogenicity.
REFERENCES


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