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Additivity, antagonism, and synergy in arsenic trioxide-induced growth inhibition of C6 glioma cells: Effects of genistein, quercetin and buthionine-sulfoximine

Ellen Klauser, Michael Gülden, Edmund Maser, Sabine Seibert, Hasso Seibert *

Institute for Toxicology and Pharmacology for Natural Scientists, University Hospital Schleswig-Holstein, Campus Kiel, Brunswiker Str. 10, D-24105 Kiel, Germany

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ABSTRACT

Arsenic trioxide (ATO) induces clinical remission in acute promyelocytic leukemia and growth inhibition in various cancer cell lines *in vitro*. Recently, genistein and quercetin were reported to potentiate ATO-provoked apoptosis in leukemia and hepatocellular carcinoma cells. Genistein acted via enhanced ROS generation and quercetin via glutathione depletion. Searching for potential strategies for the treatment of malignant gliomas in this study the capacity of these flavonoids to sensitize rat C6 astroglioma cells for the cytotoxic action of ATO was investigated. ATO inhibited cell growth in a concentration- and time-dependent manner. This effect was accompanied neither by enhanced radical generation nor lipid peroxidation and was not attributed to apoptosis. ATO treatment concentration-dependently increased glutathione levels. Genistein enhanced radical generation. Combined with ATO it inhibited cell growth additively. Additivity was also obtained after cotreatment with ATO and H_2O_2. Quercetin acted antagonistically on ATO-induced growth inhibition. Quercetin increased glutathione levels. In contrast, buthionine-sulfoximine (BSO) depleted cellular glutathione and acted synergistically with ATO. In conclusion, in C6 cells neither genistein nor quercetin are suited as sensitizing agent, in contrast to BSO. Depletion of cellular glutathione content rather than an increase of ROS generation plays a central role in the enhancement of ATO-toxicity in C6 cells.

1. Introduction

Gliomas represent the most common primary brain tumors in adults. Their malignancies are graded from I to IV by the WHO. Malignant gliomas (grade III: anaplastic form of astrocytoma, oligodendroglioma and oligoastrocytoma, and grade IV: glioblastoma multiforme, GBM) are known to invade the surrounding normal brain tissue (Louis, 2006; Louis et al., 2007). Because of this infiltrative nature, they can escape the most curative surgical approaches. Moreover the tumors are resistant to radiotherapy and most of the chemotherapeutic agents. The survival rate of patients with malignant gliomas is very low. GBM is associated with the worst prognosis (median life expectancy less than 1 year) (Laks et al., 2010; Lefranc and Kiss, 2006). Therefore, effective new treatment strategies are needed for malignant gliomas.

Arsenic trioxide (ATO) is successfully used for the treatment of acute promyelocytic leukemia (APL) since the year 2000 (Antman, 2001). APL is a unique form of leukemia. It is characterized by a reciprocal chromosomal translocation and the generation of the PML/RARα fusion protein. ATO induces complete remission in APL patients who are refractory to conventional chemotherapy (Soignet et al., 1998, 2001). The exact role of ATO in APL is still under investigation. Two important mechanisms have been presumed: Low concentrations of ATO (0.1–0.5 μM) induce differentiation in APL cells via degradation of the PML-RARα fusion protein, while higher concentrations (0.5–2.0 μM) cause apoptosis in promyelocytic and other malignant cells (Dilda and Hogg, 2007).

Based on these positive experiences numerous studies have been initiated aimed at finding out whether other types of blood cancer as well as solid tumors also could be treated with ATO (Chen et al., 2006; Han et al., 2010; Maeda et al., 2004). It is generally believed that for such applications it could be necessary to sensitize the tumor cells for the cytotoxic action of ATO in order

Abbreviations: APL, acute promyelocytic leukemia; ATO, arsenic trioxide; BSO, DL-buthionine-(S,R)-sulfoximine; DCF, 2′-7′-dichlorofluorescein; EC, effective concentration; GSH, glutathione; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; ROS, reactive oxygen species.

* Corresponding author. Tel.: +49 431 597 3166; fax: +49 431 597 3558.
E-mail address: Seibert@toxi.uni-kiel.de (H. Seibert).

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to keep the therapeutic doses as low as possible to avoid severe side effects. During treatment of APL patients ATO concentrations in plasma amount to 1 to 2 μM (Dai et al., 1999). Different sensitizing strategies are under investigation. Recently, Sánchez et al. (2008) have reported that genistein potentiates arsenic trioxide-induced apoptosis in human leukemia cells, and that this effect is mediated by an increased generation of reactive oxygen species (ROS). Jiang et al. (2010) obtained similar results in human hepatocellular carcinoma (HCC) cell lines. Genistein synergized with ATO to suppress the growth of HCC cells and to induce apoptosis accompanied by an enhanced generation of ROS.

Genistein (4′,5,7-trihydroxyisoflavone) belongs to a large group of non-nutrient plant compounds, called flavonoids. It is the major isoflavone in the human diet and most found in soy and soy products (Horn-Ross et al., 2000). Genistein is structurally similar to mammalian estrogens and is able to mimic them (Klein and King, 2007). In a wide range of *in vitro* studies on different tumor cell lines the flavonoid is shown to modulate cell proliferation (Ramos, 2007), to trigger apoptosis per se (Schmidt et al., 2008) and to potentiate apoptosis induction by chemotherapeutics (Banerjee et al., 2005, 2007). Although genistein was formerly described as ROS scavenging agent (Chan and Yu, 2000; Orie et al., 1999), also a pro-oxidant action of the isoflavone was recently shown, e.g. in cultures of C6 astroglioma cells (Rüweler et al., 2008).

The aim of the present study was to investigate whether genistein – as observed in human leukemia and HCC cells – can enhance the efficacy of ATO in inhibiting the growth of C6 glioma cells. Furthermore, the effects of genistein should be compared with those of quercetin, a flavonoid which is well known for its anti-oxidative activity (Ishige et al., 2001; Rüweler et al., 2008; Seibert et al., 2011) and which, recently, was reported to potentiate the apoptotic action of ATO in leukemia cell lines via depletion of cellular GSH (Ramos and Aller, 2008). In order to elucidate the role of cellular glutathione levels for the sensitivity to ATO in C6 cells the effects of combined treatments with ATO and buthionine sulfoximine (BSO) should be studied. BSO is well known for its synergistic action with ATO in growth inhibition of various tumor cell lines (Dai et al., 1999; Davison et al., 2003). A further objective was to clarify whether ATO alone or in combination with genistein and quercetin, respectively, acts via enhancement of oxidative stress (radical generation, glutathione levels, lipid peroxidation) and whether it induces apoptotic cell death.

2. Materials and methods

2.1. Chemicals

ATO, the flavonoids (genistein, quercetin), DL-buthionine-(S,R)-sulfoximine (BSO), staurosporine, hydrogen peroxide (H_2O_2), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), Hoechst 33342 and propidium iodide were obtained from Sigma–Aldrich (Steinheim, Germany). A stock solution of ATO (10 mM) was prepared in 0.1 N NaOH and stored at 5 °C; stock solutions of genistein (50 mM) and quercetin (50 mM) were prepared in dimethyl sulfoxide (DMSO). All these solutions were diluted in culture medium or Hank's balanced salt solution (HBSS) to the desired final concentrations. The final concentration of the solvents was always 0.2% (v/v). BSO and H_2O_2 were dissolved directly in culture medium or HBSS. Stock solutions of propidium iodide (10 μg/ml) and Hoechst 33342 (5 μg/ml) were prepared in HBSS, while H_2DCF-DA was dissolved in DMSO (stock solution) and diluted in HBSS to its final concentration.

2.2. Cell culture

The rat C6 astroglioma cell line (Benda et al., 1968) was obtained from the American Type Culture Collection (ATCC, No. CCL-107, passage 37). Frozen stocks were routinely thawed, grown in 25-cm^2 tissue culture flasks in an incubator with a humidified atmosphere of 10% CO_2/90% air at 37 °C, and passaged once a week. The culture medium was Dulbeccós Modified Eagle Medium (DMEM, c.c.pro, Neustadt, Germany) containing 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES, Merck, Darmstadt, Germany), supplemented with fetal bovine serum (FBS, 5%, v/v) and antibiotics (100 μg/ml gentamicin and 1.25 μg/ml fungizone) all from Gibco (Paisley, Scotland). Cells used for ROS detection, were cultivated in phenol red-free DMEM from PAN (Aidenbach, Germany) with supplements as above.

2.3. Determination of cell growth inhibiting potency

To determine the potency to inhibit cell growth, exponentially growing C6 cell cultures were exposed to various concentrations of ATO and the other test compounds alone and in combination with ATO, respectively, starting 24 h after plating 2×10^4 cells into the 96-well plates. Six wells per condition were used and two groups of six wells per plate served as control. The medium (200 μl/well) used was pyruvate-free DMEM with supplements as described in Section 2.2. Within each single experiment cells cultured in four to five separate 96 well plates were exposed to six concentrations of ATO alone, the possible "sensitizer" alone, and combinations of ATO and selected concentrations of the second compound, respectively. After 72 h the medium was aspirated and the wells were washed thrice with phosphate buffered saline (PBS). Adherent cells were lysed with 0.5 N NaOH and the protein content per well was determined by the method of Lowry et al. (1951) modified for microtiter plates. Bovine serum albumin (1.5–24 μg/well) served as standard. Absorption at 630 nm was read with a microtiter plate photometer (Bio-Tek ELX800UV). The mean protein content of the test group wells was expressed as percentage of untreated controls.

Under the conditions used the doubling time in control cultures amounted to 28 ± 1.8 h (n = 7) resulting in a sixfold increase of the cell protein per well during the 72 h exposure period. Growth inhibition measured as reduction in cell protein per well compared to the untreated controls after 72 h of exposure assesses cytotoxic as well as cytostatic action. Cytostatic action alone (cell cycle arrest) decreases the final cell number to less than 20% of control, cytotoxic action prevents cell growth and, additionally, induces detachment and loss of the initial number of viable cells.

The potency of the compounds to inhibit cell growth was characterized by EC_{20}-, EC_{50}- and EC_{80}-values, defined as those concentrations reducing the final cell protein content to 80%, 50% and 20%, respectively, of the untreated controls. These values were determined by fitting a Hill equation (Y = Top/(1 + 10[(log(EC_{50}/X)) × HillSlope])) to the concentration–effect data of each single experiment using GraphPad Prism®.

2.4. Analysis of combination effects

The concentration dependence of the growth inhibiting effect of ATO was determined in the presence of various but fixed concentrations of selected agents. To analyse the kind of combination effects they were compared to mixture effects calculated from the concentration–effect relationships of the individual compounds assuming non-interaction according to the concept of concentration addition (Loewe and Muischnek, 1926; Loewe, 1953). Synergism is characterized by stronger effects and antagonism by weaker effects than predicted from the additivity assumption.

Concentration additivity can be tested by the method of isoboles which was claimed to be valid independent of the form of the concentration–effect curves (Berenbaum, 1985). Isoboles are the lines connecting iso-effective combinations of concentrations of two compounds. Additivity is given and isoboles are straight lines if:

$$\frac{C_A}{EC_{x,A}} + \frac{C_B}{EC_{x,B}} = 1 \quad (1)$$

where C_A and C_B are the concentrations of the compounds in the mixture and $EC_{x,A}$ and $EC_{x,B}$ are the concentrations of the compounds that, when used alone, exert the same effect intensity x (e.g. 50%) as the mixture. In case of synergistic and antagonistic combination effects, respectively, the left side will be either smaller than 1 and the isoboles concave or the left side will be larger than 1 and the isoboles convex. The isoboles for 20%, 50% and 80% effect connecting the individual EC_{20}-, EC_{50}- and EC_{80}-values, respectively, of the two substances combined were constructed and used for analysis.

2.5. Fluorescence microscopy

To determine numbers of apoptotic and necrotic cells C6 cells were sub-cultured in 10-cm dishes, by inoculating 1×10^6 cells in 10 ml/dish. Cultures were exposed to ATO and the other test compounds alone and in combination for 24 h in pyruvate- and phenol red-free DMEM (8 ml) starting 96 h after plating. After the incubation period cells and culture medium were collected, combined and centrifuged (3000 RPM, 15 min, 5 °C). Cell pellets were re-suspended in 700 μl of cold HBSS and aliquots were stained with propidium iodide (10 μg/ml) and Hoechst 33342 (0.5 μg/ml). Additionally, aliquots of the cell suspension were taken to determine the total cell protein content. Stained cells were placed on glass slides and nuclear morphology and the numbers of propidium iodide positive cells were examined using a Zeiss fluorescence microscope equipped with a Canon digital camera. Images were taken with a 40× objective lens. At least one hundred cells were analyzed per sample.