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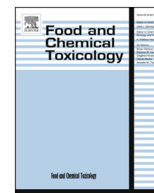

Chrysin and silibinin sensitize human glioblastoma cells for arsenic trioxide

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Arsenic trioxide (ATO) is highly efficient in treating acute promyelocytic leukemia. Other malignancies, however, are often less sensitive. Searching for compounds sensitizing arsenic resistant tumours for ATO the plant polyphenols, chrysin and silibinin, and the ATP binding cassette (ABC) transporter inhibitor MK-571, respectively, were investigated in human glioblastoma A-172 cells. The sensitivity of A-172 cells to ATO was characterized by a median cytotoxic concentration of 6 μM ATO. Subcytotoxic concentrations of chrysin, silibinin and MK-571, respectively, remarkably increased the sensitivity of the cells to ATO by factors of 4–6. Isobolographic analysis revealed synergistic interaction of the polyphenols and MK-571, respectively, with ATO. Sensitization by chrysin was associated with depletion of cellular glutathione and increased accumulation of arsenic. In contrast, silibinin and also MK-571 increased the accumulation of arsenic more strongly but without affecting the glutathione level. The increase of arsenic accumulation could be attributed to a decreased rate of arsenic export and, additionally, in the case of silibinin and MK-571, to an increasing amount of irreversibly accumulated arsenic. Direct interaction with ABC transporters stimulating export of glutathione and inhibiting export of arsenic, respectively, are discussed as likely mechanisms of the sensitizing activity of chrysin and silibinin.

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1. Introduction

The poison arsenic trioxide (As_2O_3, ATO) is highly efficient in the treatment of acute promyelocytic leukemia (APL) (Au and Kwong, 2008). This successful use of ATO as anti-cancer agent prompted numerous pre-clinical investigations and clinical trials on the use of ATO for the treatment of other blood and solid cancers. The studies on solid cancers, however, often revealed a limited clinical efficiency of ATO as a single agent (Emadi and Gore, 2010) and an insensitivity of cancer cells towards the clinically achievable arsenic concentrations of 0.5–2 μM (e.g. Maeda et al., 2004; Baumgartner et al., 2004; Han et al., 2010; Emdad et al., 2011).

Some promising results with ATO in combination therapy (Evens et al., 2004; Emadi and Gore, 2010) lead to an intensive search for compounds acting additively or even synergistically in combination with ATO, thus, allowing to lower the effective concentrations of ATO to clinically achievable and safe levels. Compounds investigated in combination with ATO were other chemotherapeutic drugs and modulators of cell signalling (Takahashi, 2010), modulators of the cellular redox state and the GSH system (e.g. Shimizu et al., 1998; Dai et al., 1999; Kito et al., 2002; Maeda et al., 2004; Han et al., 2008; Klauser et al., 2014) and a variety of secondary plant metabolites especially polyphenols (e.g. Ramos and Aller, 2008; Sánchez et al., 2009; Dizaji et al., 2012; Ma et al., 2011). Previously we have screened various plant polyphenols for their synergistic activity in combination with ATO in rat and human glioma cells (Klauser et al., 2014; Klauser, 2014). In continuation of this work here we report that the two plant polyphenols, chrysin and silibinin, sensitize human glioblastoma A-172 cells for the growth inhibiting and cytotoxic action of ATO.

Chrysin (Fig. 1A) is found in the blue passion flower (*Passiflora caerula*) and many other plants, in honey and in propolis. Chrysin has been reported to prevent tumour growth in several preclinical

Abbreviations: ABC, ATP-binding cassette; ATO, arsenic trioxide; APL, acute promyelocytic leukemia; BSO, L-buthionine sulfoximine; GSH, reduced glutathione; GSSG, oxidised glutathione; GSx, total glutathione (GSH + 2 GSSG); BCRP, breast cancer resistance protein; MRP, multidrug resistance protein; P-gp, P-glycoprotein.

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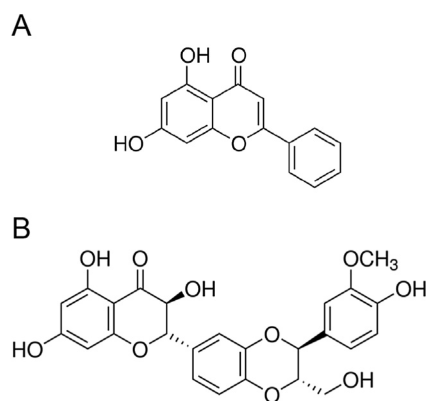

Fig. 1. Chemical structure of (A) chrysin (5,7-dihydroxyflavone) and (B) silibinin (2,3-dihydro-3-(4-hydroxy-3-methoxy-phenyl)-2-(hydroxymethyl)-6-(3,5,7-trihydroxy-4-oxobenzopyran-2-yl)benzodioxin).

studies with animal models (Kasala et al., 2015) and to enhance the potency of cancer chemotherapeutics in resistant and non-resistant tumour cell lines from different tissues (Brechbuhl et al., 2012; Gyémant et al., 2005; Kachadourian et al., 2007; Katayama et al., 2007; Zhang et al., 2004). In a human leukemia cell line chrysin was reported to sensitize for the cytotoxic action of ATO (Ramos and Aller, 2008).

Silibinin (silybin, Fig. 1B) is the most active and abundant constituent of silymarin, a flavonoid extract from the milk thistle (*Silybum marianum*). Silymarin has traditionally been used as hepatoprotective agent in liver cirrhosis and chronic hepatitis (Loguercio and Festi, 2011) and for many years silibinin is successfully used to prevent severe liver damage after intoxication by the death cap mushroom *Amanita phalloides* (Mengs et al., 2012). Silibinin has been reported to show promising anticancer activity *in vitro* and in vivo (Deep and Agarwal, 2007), to synergize the effect of various cancer chemotherapeutics in prostate, breast and lung cancer cells *in vitro* (Raina and Agarwal, 2007) and to increase the cytotoxicity of ATO in human glioblastoma U87MG cells (Dizaji et al., 2012).

The objective of the present study was to investigate whether chrysin and silibinin are capable of sensitizing A-172 cells towards the cytostatic/cytotoxic action of ATO and, if so, to determine their sensitizing potency and efficacy. Furthermore some insight into the mechanism of sensitization should be gained. Sensitization may be regarded as reversal of resistance. Resistance of tumour cells often results from mechanisms that limit the accumulation of cytostatic drugs or enhance their detoxification, for instance via conjugation with glutathione (GSH). Acquired resistance to arsenic in various cell lines was indeed shown to be associated with increased capacity to synthetize GSH (Thompson and Franklin, 2010), with elevation of the intracellular GSH levels (Lee et al., 1989), increased expression/activity of glutathione S-transferase (Lee et al., 1989; Romach et al., 2000; Liu et al., 2001), increased expression of ATP-binding cassette (ABC) transporters (Romach et al., 2000; Liu et al., 2001) and a decreased cellular arsenic accumulation concomitantly with an increased arsenic efflux rate (Wang et al., 1996; Lee et al., 1989; Romach et al., 2000; Liu et al., 2001). On the other side depletion of cellular glutathione using L-buthionine sulfoximine (BSO) to inhibit glutathione synthesis sensitized cells *in vitro* to ATO (Shimizu et al., 1998; Dai et al., 1999; Liu et al., 2001; Kito et al., 2002; Maeda et al., 2004; Han et al., 2008; Klauser et al., 2014). Therefore, in order to elucidate the cause of sensitization, the effects of both polyphenols on arsenic accumulation and release by the cells and their glutathione content were assessed. Furthermore, to reveal the importance of ABC transporters the effects of the ABC transporter inhibitor MK-571 (Matsson et al., 2009) on cytotoxicity and accumulation of arsenic in A-172 cells was investigated.

2. Material and methods

2.1. Chemicals

Arsenic trioxide (ATO), the plant polyphenols chrysin and silibinin (see Fig. 1) and the ABC-transporter inhibitor MK-571 were purchased from Sigma-Aldrich, Steinheim, Germany. All other chemicals used were from usual sources and of analytical grade.

2.2. Cell culture

The human glioblastoma cell line A-172 (Giard et al., 1973) was purchased from Cell Line Service (CLS, Eppenheim, Germany; CLS no. 300108, passage no. 27). Frozen stocks were routinely thawed and grown in 75 cm^2 tissue culture flask at 37 °C in a humidified atmosphere of 10% CO_2/90% air and subcultered once a week. The culture medium was Dulbeccos Minimal Essential Medium (Sigma-Aldrich, Steinheim, Germany) with 4.5 g/l glucose, supplemented with 20 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes), foetal bovine serum (FBS, 5% v/v, Sigma-Aldrich), gentamicin (100 μg/ml) and fungizone^R (1.25 μg/ml) both from Gibco (Paisly, UK). Experiments were performed with passages no. 32–46.

2.3. Determination of cell growth inhibiting and cytotoxic potency

The cell growth inhibiting and cytotoxic potency of the test substances was determined in 96-well microplate cultures (growth area = 0.32 cm^2/well) of A-172 cells. Cultures were started by seeding 1×10^4 cells per well (3.1×10^4 cells/cm^2) in 200 μl of culture medium. After 24 h cells were exposed to various concentrations of ATO and the other test compounds alone or in combination with ATO, respectively.

Stock solutions of ATO (10 mM) were prepared in 0.1 N NaOH and diluted with culture medium to give concentrations of ≤20 μM. Chrysin and silibinin were initially dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The final concentration of DMSO was always 0.2% (v/v). MK-571 was directly dissolved in culture medium. Each plate received six concentrations of a test compound or a combination of test compounds (six wells per condition). Two groups of six wells served as control receiving culture medium without test substance but in case of chrysin and silibinin 0.2% (v/v) DMSO. When performing combination experiments four to five separate 96-well plates were exposed to ATO alone, the possible “sensitizer” alone, and combinations of ATO and selected concentrations of the second compound, respectively.

Growth inhibition and cytotoxicity were determined after 24 h and 72 h, respectively, as loss of cell protein per well compared to the controls. Exposure was terminated aspirating the medium and washing thrice with phosphate buffered saline (PBS). 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 served as standard. Absorption at 630 nm was read with a microtiter plate photometer (Titertek, Multiskan PLUS MK II). The mean protein content of the test group wells was expressed as percentage of the untreated controls.

The concentration-effect data of each single experiment were fitted to a Hill function to derive the median effective concentration, the EC_{50}.

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