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Modulation of multidrug resistance in cancer cells by chelidonine and *Chelidonium majus* alkaloids

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ABSTRACT

Cancer cells often develop multidrug resistance (MDR) which is a multidimensional problem involving several mechanisms and targets. This study demonstrates that chelidonine and an alkaloid extract from *Chelidonium majus*, which contains protoberberine and benzo[c]phenanthridine alkaloids, has the ability to overcome MDR of different cancer cell lines through interaction with ABC-transporters, CYP3A4 and GST, by induction of apoptosis, and cytotoxic effects. Chelidonine and the alkaloid extract inhibited P-gp/MDR1 activity in a concentration-dependent manner in Caco-2 and CEM/ADR5000 and reversed their doxorubicin resistance. In addition, chelidonine and the alkaloid extract inhibited the activity of the drug modifying enzymes CYP3A4 and GST in a dose-dependent manner. The alkaloids induced apoptosis in MDR cells which was accompanied by an activation of caspase-3, -8,-6/9, and phosphatidyl serine (PS) exposure. cDNA arrays were applied to identify differentially expressed genes after treatment with chelidonine and the alkaloid extract. The expression analysis identified a common set of regulated genes related to apoptosis, cell cycle, and drug metabolism. Treatment of Caco-2 cells with 50 μg/ml alkaloid extract and 50 μM chelidonine for up to 48 h resulted in a significant decrease in mRNA levels of P-gp/MDR1, MRP1, BCRP, CYP3A4, GST, and hPXR and in a significant increase in caspase-3 and caspase-8 mRNA. Thus, chelidonine is a promising model compound for overcoming MDR and for enhancing cytotoxicity of chemotherapeutics, especially against leukaemia cells. Its efficacy needs to be confirmed in animal models.

Introduction

Greater celandine; *Chelidonium majus* L. (Papaveraceae) is a perennial plant native to Europe and Western Asia. In Traditional Chinese Medicine (TCM), it is used against pain, as an antitussive (for bronchitis and whooping cough), anti-inflammatory, and detoxicant. Moreover, in eastern Asia it was valued as a remedy against cancer, jaundice, gout, toothache, and peptic ulcers. European herbal traditions regard greater celandine as a valuable internal remedy to treat appetite loss, stomach cramps, and gastrointestinal problems as well as bile duct and liver disorders (hepatitis, jaundice). Topically, the plant was used against several skin disorders including warts, eczema, and corns (Van Wyk and Wink 2004). At present, the alkaloid extract of *C. majus* is the major constituents of immunomodulatory preparations and is applied in the therapy of several types of abnormal growths, probably owing to the antimitotic and cytotoxic properties (Gilca et al. 2010). Both crude extracts of *C. majus* and its purified alkaloids exhibit a wide variety of biological activities (anti-inflammatory, immunomodulatory, choleretic, hepatoprotective, analgesic, antitumoral, antimicrobial, and antiviral properties) which can explain the traditional uses of *C. majus* (Gilca et al. 2010). The dried aerial part of *C. majus* is an official drug in several pharmacopoeias, e.g. in the European Pharmacopoeia (Ph.Eur.5), German Pharmacopoeia (DAB-10), and Hungarian Pharmacopoeia (Ph. Hg. VIII) (Sarkozi et al. 2006). In addition, the alkaloid extract prevents glandular stomach carcinogenesis in rats *in vivo* treated with N-methyl-N′-nitro-N nitrosoguanidine (MNNG) (Kim et al. 1997).

The main secondary metabolites of *C. majus* are alkaloids (more than 20) such as benzo[c]phenanthridines, both quaternary (chelerythrine, sanguinarine) and tertiary alkaloids (chelidonine), protopine and its derivatives (allocryptopine), and

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protoberberines (berberine, coptisine) (Barnes et al. 2007; Gilca et al. 2010). Generally, some of these alkaloids intercalate DNA (berberine, sanguinarine), and in consequence inhibit DNA and RNA polymerase, topoisomerase, telomerase, and even ribosomal protein biosynthesis or bind to tubulin/microtubules (chelidonine, chelerythrine), thus acting as spindle poisons (Wink 2007). These properties might be responsible for the observed anticancer effect, which can be due to reduced telomerase activity (Noureini and Wink 2009), cell death by apoptosis (Noureini and Wink 2009; Philchenkov et al. 2008), and arrest of mitosis (Noureini and Wink 2009; Wink 2007).

Several herbal constituents, often used by cancer patients, are employed as complementary and alternative medicines (CAMs). They can overcome MDR of cancer cells (reviewed in Eichhorn and Efferth 2012) by interacting with many molecular and cellular targets (Wink 2012). Moreover, natural products can modulate or inhibit ABC-transporter activity and/or expression. Recently, the effects of many natural occurring polyphenols, terpenoids, and alkaloids on ABC-transporters were reviewed (Eichhorn and Efferth 2012; Wink et al. 2012).

The present study focused primarily on the effect of the alkaloid extract and of one of the major active alkaloids, chelidonine, on multidrug resistance in cancer cells. The multifactorial properties of chelidonine and the alkaloid extract on the modulation of ABC-transporters, of metabolic enzymes (CYP3A4 and GST), and induction of apoptosis were addressed. In addition, cDNA arrays were employed to identify differentially expressed genes after 48 h treatment by chelidonine and the alkaloid extract. Moreover, pathways associated with these expression changes were identified using the Ingenuity Pathway Analysis and real-time PCR was employed to confirm changes of gene expression after alkaloid treatment.

Materials and methods

Preparation of alkaloid extracts

The aerial parts of flowering *C. majus* were collected in late spring 2009 in Heidelberg (Germany); 100 g of dry plant material was extracted with 1 l MeOH and filtered. The total MeOH extract was dried over anhydrous sodium sulphate and evaporated to dryness under vacuum at 45 °C. The cell culture experiments were carried out with freshly prepared extracts.

Characterization of the alkaloid extract by LC–MS

The MeOH extract (20 mg/ml) was separated by reversed-phase HPLC by injecting 5 μl via a Rheodyne system. Separation was achieved using a RP-C18e LichroCART 250 × 4 mm, 5 μm column (Merck, Darmstadt, Germany). The mobile phase consisted of HPLC grade water with 0.5% formic acid (A), and acetonitrile (B). A Merck-Hitachi L-6200A system (Merck, Darmstadt, Germany) was used with a gradient programme at a flow rate of 1 ml/min as follows: from 0 to 75% B in 45 min, then to 100% in 5 min. Mass spectrometry conditions: a Quattro II system from VG with an ESI interface was used in positive ion and negative ion mode under the following conditions: drying and nebulizing gas was nitrogen (N_2). Capillary temperature: 120 °C; capillary voltage: 3.50 kV. Lens voltage was 0.5 kV, cone voltage 30 V. Full scan mode was in the range of *m*/*z* 200–800 for which the instrument was set to the following tune parameters: nebulizing and drying gas pressure was 350 l/h and 3.5 l/h respectively. Data were processed using MassLynx® 4.0 software (Waters).

Cell lines

Caco-2 cells (DSMZ Nr. ACC 169), HepG-2 (DSMZ Nr. ACC 180), and HeLa cells (DSMZ Nr. ACC 57) were maintained in DMEM complete medium (L-glutamine, 10% heat-inactivated foetal bovine serum (FBS), and 100 U/ml penicillin, and 100 μg/ml streptomycin) in addition, 1 mM sodium pyruvate and 1% non-essential amino acids were added to Caco-2 medium. Prof. Dr. Thomas Efferth (JGU, Mainz) provided human T cell leukaemia cells CCRF-CEM (DSMZ Nr. ACC 240) and CEM/ADR5000 cells. Both cell lines grew in a suspension. They were maintained in RPMI1640 complete medium (Efferth et al. 2002). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. All experiments were performed with cells in the logarithmic growth phase. The Caco-2 cells described were an ideal model for studying MDR because they highly express ABC-transporter proteins, including P-gp (=MDR1), MRP1, and BCRP. Multidrug-resistant subline CEM/ADR5000 is known to exclusively overexpress p-gp, thus these cells represent an ideal model for evaluating the modulatory effects of different compounds on P-gp (Efferth et al. 2003; Gillet et al. 2004).

Cytotoxicity assay

The MTT cytotoxicity assay is widely used, particularly in the field of drug development (Carmichael et al. 1987). Briefly, cells were seeded in 96-well plates with a density of 2×10^4 cells/well. The cells were treated with various concentrations of *C. majus* extract (up to 4 mg/ml) and chelidonine (up to 500 μM) for 24 h. Then 0.5 mg/ml MTT was added to each well and incubated for 4 h. The formed formazan crystals were dissolved in DMSO. Absorbance was detected at 570 nm using Tecan Safire II™ (Crailsheim, Germany). Suspended cells CCRF-CEM and CEM/ADR5000 (3×10^4 cells/well) were incubated with test samples for 48 h and MTT procedure was completed as outlined above.

Relative resistance was calculated as follows:

$$\text{Relative resistance} = \frac{IC_{50} \text{ value of the resistant cell line (CEM/ADR5000)}}{IC_{50} \text{ value of the sensitive parental cell line (CCRF-CEM)}}$$

MDR reversal assay

Fully differentiated cells were harvested and seeded at a density of 2×10^4 cells/well (for Caco-2 cells) and 3×10^4 cells/well (for CEM/ADR5000 cells) in flat-bottom 96-well plates; they were incubated for 24 h at 37 °C. Doxorubicin was added at various concentrations to the cells, with or without non-toxic concentrations of the reversal agent (mainly 20 μg/ml alkaloid extractor 20 μM chelidonine). The cytotoxicity was estimated by using MTT assay as mentioned above. The reversal ratio was calculated as follows:

$$\text{Reversal ratio} = \frac{IC_{50} \text{ value of doxorubicin}}{IC_{50} \text{ value of doxorubicin in combination with MDR inhibitor}}$$

ABC transporter activity

ABC transporter activities of the alkaloid extract and chelidonine were determined using rhodamine 123 (Rho123) and calcein-AM (CAM). Rho123 and calcein-AM are known substrates, not only for P-gp but also for MRP1 (Twentyman et al. 1994). Calcein-AM and Rho123 are readily effluxed in MDR-overexpressing cancer cells. Caco-2 cells: cells were seeded at 2×10^3 cells/well in 96-well plates and cultured under standard conditions until a confluent monolayer was formed (by day 6) as determined by light microscopy. After washing, cells were pre-incubated for 30 min at 37 °C with different concentrations of test samples in order to determine dose dependence. Rho123 (1 μg/ml) or calcein-AM (150 nM) were then added and the cells were further incubated