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ORIGINAL ARTICLE

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Inhibition of P-glycoprotein transport function and reversion of MDR1 multidrug resistance by cnidiadin

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Abstract *Purpose*: Overexpression of P-glycoprotein (Pgp) encoded by the MDR1 gene is one of the major obstacles to successful cancer chemotherapy. The goal of this study was to evaluate if, among other natural coumarins, cnidiadin, a furanocoumarin present in traditional Chinese medications and in a spice commonly used in Greek food, inhibits Pgp transport activity and has the potential to reverse MDR1 multidrug resistance. *Methods*: Using MDR1-transfected Madin-Darby canine kidney (MDCK-MDR1) cells as a model of cells expressing the human MDR1 phenotype, and verapamil or CsA or both as positive control, we tested the capacity of six natural coumarins (umbelliferone, esculin, esculetin, cnidiadin, angelicin and psoralen) to induce the accumulation of rhodamine-123 (R-123) and [^{3}H]-vinblastine ([^{3}H]-VBL) and to modulate the photolabeling of Pgp by SDZ 212-122, a diazirin cyclosporin A. The growth-inhibitory effect of cnidiadin and its capacity to enhance the cell toxicity of vinblastine (VBL) or vincristine (VCR) was then evaluated by the WST-1 assay in two cell lines overexpressing Pgp (MDCK-MDR1 and vincristine-resistant KB/VCR). *Results*: Cnidiadin was the only tested coumarin capable of significantly accumulating R-123 and [^{3}H]-VBL and inhibiting Pgp photolabeling in MDCK-MDR1 cells. The dose-dependent increase in [^{3}H]-VBL uptake (IC_{50} 26.5 μM) induced by cnidiadin in the dose range 1–100 μM correlated with inhibition of Pgp photolabeling. At 10 μM cnidiadin inhibited photolabeling by 59% and sensitized both MDCK-MDR1 and KB/VCR cells to vinca alkaloids. *Conclusion*: Cnidiadin is a cytotoxic agent capable in vitro of competitively inhibiting the binding and efflux of drug by Pgp and of enhancing the cell toxicity of vinca alkaloids in two cell lines (MDCK-MDR1 and mutant human carcinoma KB/VCR) overexpressing Pgp. This suggests that diet or traditional preparation containing cnidiadin may contribute to the reversal of MDR1 multidrug resistance and may affect the bioavailability of Pgp substrates orally administered. However, due to its cell toxicity, clinical interest in cnidiadin as a chemosensitizer appears to be limited.

Keywords Coumarin · P-glycoprotein (Pgp) · Multidrug resistance (MDR) · Cancer · Reversal activity · Drug interaction

Abbreviations CsA: Cyclosporin A · dz-CsA: The diazerine-cyclosporin A derivative SDZ 212-122 · KB/VCR cells: KB cell line resistant to vincristine · MDR: Multidrug resistance · MDR1: Multidrug resistance encoded by the MDR1 gene · MDCK-MDR1 cells: MDR1-transfected Madin-Darby canine kidney cells · MRP: Multidrug resistance-associated protein · Pgp: P-glycoprotein · R-123: Rhodamine-123 · TLC: Thin-layer chromatography · VBL: Vinblastine · VCR: Vincristine

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Introduction

P-glycoprotein (Pgp) is an inducible protein transporter belonging to the ATP-binding cassette (ABC) protein family that mediates the energy-dependent efflux of many drugs, including chemotherapeutics, out of cells.

In cancer cells, the overexpression of Pgp encoded by the MDR1 gene contributes to multidrug resistance (MDR) [1], and is already considered as one of the major obstacles to successful cancer chemotherapy [2, 3]. The MDR1 phenotype is commonly observed as the escape from chemotherapy of leukemic and breast cancer, and in kidney and colon cancer cells that overexpress Pgp at baseline, and are therefore intrinsically resistant to chemotherapy [4]. The discovery two decades ago that verapamil, trifluoroperazide, cyclosporin A (CsA) and certain CsA analogues can contribute to the sensitization of resistant cancer cells to chemotherapy by competitively inhibiting Pgp transport triggered research into chemosensitizers (also termed MDR-reversal agents) [4–7]. Although disappointing results have been obtained with the first generation of reversal agents due to dose-limiting toxicity and other side effects, three recent randomized trials have confirmed the clinical potential of this approach. Indeed, significant benefits have been observed in patients treated with combinations of Pgp inhibitors and chemotherapy (see reference 4 for a review). However, effective and clinically applicable reversal agents remain to be discovered. The observation that numerous plant-derived dietary compounds modulate Pgp transport has led to interest in the possible use of natural compounds, or related chemicals, in combination with chemotherapy [8–14].

The goal of the present study was to assess whether cnidiadin, a furanocoumarin present in certain traditional Chinese medications and in *Tordylium apulum* (Apiaceae), a plant commonly used as a spice in Greek cooking with the name "*kafkalithra*", can affect Pgp transport activity, and whether it can increase the uptake and cell toxicity of vinblastine (VBL) in a stable clone of MDR1-transfected Madin-Darby canine kidney (MDR1-MDCK) cells. In all experiments, cnidiadin (2′*S*,3′*R*-2′ hydroxyisopropyl-3′ hydroxyisopropyl-dihydroangelicin, Fig. 1) was tested versus verapamil or CsA

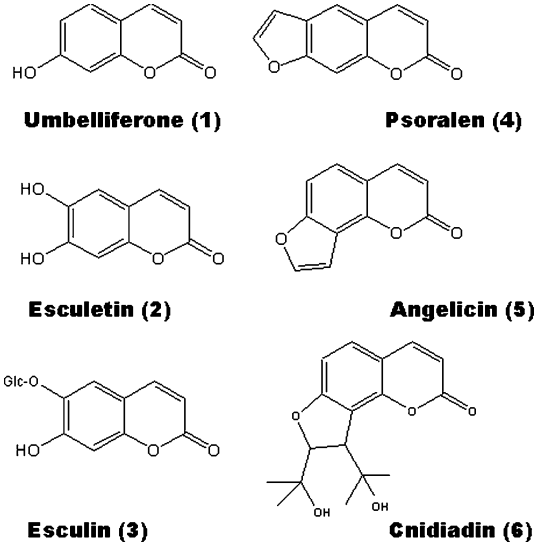


Fig. 1 Structures of the coumarins tested

that have both demonstrated clinical activity as reversal agents [4]. To provide information on structure-activity relationships, cnidiadin was compared to angelicin (7,8-furano-1,2-benzopyrone), psoralen (6,7-furano-1,2-benzopyrone), umbelliferone (7-hydroxycoumarin), esculetin (6,7-dihydroxycoumarin) and esculin (the 6-β-D-glucoside of esculetin). Esculin is present in large amounts in *Aesculum hippocastenum* seeds whose extracts are traditionally used in the EU to treat venous insufficiency. The results demonstrated that cnidiadin was the only tested coumarin that competitively inhibited Pgp transport activity in a significant manner. At 10 μM, cnidiadin increased both the uptake of ^{3}H-VBL and the cell toxicity of VBL on MDCK-MDR1 cells and decreased the IC_{50} value of vincristine (VCR) in a human mutant carcinoma cell line overexpressing resistance to this vinca alkaloid. These results suggest that cnidiadin has potential as a chemosensitizer.

Material and methods

Cells and materials

MDR1-transfected Madin-Darby canine kidney (MDCK-MDR1) cells were a gift from Amanda Yancy (AstraZeneca Pharmaceuticals). They were cultured in DMEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum. Stock cell cultures were maintained as monolayers in 75-cm^2 culture flasks in Glutamax Eagle's minimum essential medium (MEM) with Earle's salts supplemented with 10% fetal calf serum, 5 ml of 100 mM solution of vitamins, 5 ml 100 mM sodium pyruvate, 5 ml ×100 non-essential amino acids and 2 mg gentamicin base. Cells were grown at 37°C under a humidified atmosphere containing 5% CO_2.

Resistant KB/VCR cells resulted from long-term exposure of the parental sensitive KB human oral epidermoid carcinoma cell line to VCR. Wild-type KB (ECACC, Salisbury, UK) and mutant KB/VCR cells were grown on RPMI-1640 medium (GibcoBRL) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin. All cells were grown at 37°C under a humidified atmosphere containing 5% CO_2.

^{3}[H]-VBL (11.3 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Oakville, Ontario). CsA, SDZ 212-122, a diazirine-CsA (dz-CsA) derivative and the monoclonal antibody (mAb) directed against CsA were gifts from Novartis Pharma Canada. The mAb Ab-2 directed against human MDR1 was purchased from NeoMarkers (Fremont, Calif.). The antimouse IgG horseradish peroxidase-linked whole antibody was purchased from Jackson Immunoresearch Laboratories (West Grove, Pa.). All components of the medium were from Gibco (Gaithersburg, Md.) or Gibco-BRL (Paisley, UK). Umbelliferone, esculetin, esculin, angelicin and psoralen were purchased from Extrasynthèse (St Genay, France). R-123 and all other chemicals were from Sigma-Aldrich Canada (Oakville, Ontario).

Isolation of cnidiadin

Cnidiadin, was isolated with a yield of 0.035% from dried leaves of *Tordylium apulum* collected during June 2002 near Delphes (Greece). A voucher specimen of the collected leaves has been deposited in the Laboratory of Pharmacognosie et Biotechnologies (Université d'Auvergne) under the reference CBGTA-03. Cnidiadin was isolated, as described previously [15], by ether extraction ($n = 3$) of powdered leaves, followed by TLC of the dried residue on silica gel 60 with *n*-hexane-ethyl acetate (5:1, v/v), and preparative TLC on silica gel with *n*-hexane-ethyl acetate [3:1, v/v]. The purity of the sample (more than 97%) was checked by RP-HPLC on a Spherisorb RP-18 S5 ODS-2 (20×4.6 mm ID) column. Analysis was conducted in gradient mode at a flow rate of 1 ml/min with a mixture of double-distilled water containing 3% glacial acid acetic (solvent A) and acetonitrile containing 3% glacial acid acetic (solvent B) as components of the mobile phase. The separation started with a mixture containing 6% B. The percentage of B was increased by 0.6% per min for 20 min, then by 3% per min for 5 min and by 5% per min for another 5 min. Cnidiadin was detected by fluorescence at 340/425 nm. The structure of cnidiadin has been previously determined [15]. An authentic sample of cnidiadin generously provided by Prof. Lin Rui-Chao (National Institute for the Control of Pharmaceutical and Biological Products, Beijing) was used as a reference.

R-123 accumulation in MDCK-MDR1 cells

MDCK-MDR1 cells were seeded at 150,000 cells per well in 24-well plates and cultured for 4 days at 37°C in an atmosphere containing 5% CO_2. At confluence, cells were washed twice with HBSS (1.3 m*M* $CaCl_2$, 5.4 m*M* KCl, 0.44 m*M* KH_2PO_4, 0.5 m*M* $MgCl_2$, 0.83 m*M* $MgSO_4$, 137 m*M* NaCl, 4.2 m*M* $NaHCO_3$, 0.34 m*M* Na_2HPO_4 and 25 m*M* D-glucose, pH 6.5) at 37°C, then preincubated for 30 min at 37°C with HBSS in the presence or absence of coumarins or of positive controls (CsA or verapamil) at the tested concentrations in DMSO at 0.1% (v/v). Finally, 20 μ*M* R-123 was then added to each well, and the plates were incubated for 2 h at 37°C. At this step, the accumulation of R-123 was stopped by washing the cells five times with cold PBS (150 m*M* NaCl, 2.7 m*M* KCl, 1.3 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4, pH 7.4), and the cells were lysed with 0.1% Triton X-100 at room temperature. The fluorescence of cell lysates was measured with a SpectraMax Gemini spectrofluorometer (Molecular Devices) at a wavelength of 485 nm for excitation and 538 nm for emission.

Photoaffinity labeling with dz-CsA

Pgp photolabeling was assessed with the CsA analogue SDZ 212-122 which possesses a strong affinity for Pgp [6]. MDCK-MDR1 membranes (50 μg) isolated as previously described [6] were incubated for 1 h in the dark at 25°C in 10 m*M* Tris-HCl pH 7.5, containing SDZ 212-122 (0.1 μ*M*) and verapamil (10 μ*M*) or coumarins at the indicated concentrations, or DMSO at 0.1% (controls). The membranes were then crosslinked at 254 nm for 5 min at 4°C with a Stratalinker UV 2400 lamp (Stratagene, La Jolla, Calif.). Laemmli electrophoresis buffer (62.5 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.01% bromophenol blue) was added to the membrane samples, and the proteins were resolved by SDS-PAGE on 6.25% acrylamide-bisacrylamide (29.2:0.8) gels with a Mini-Protean II apparatus. The amount of dz-CsA linked to P-gp was immunodetected by Western blotting with a mAb against CsA. All experiments were performed at least three times. The intensities of the bands obtained from the photolabeling experiment were calculated with a personal densitometer SI (Molecular Dynamics, Sunnyvale, Calif.).

Detection of Pgp in KB/VCR cells

Detection of Pgp in KB/VCR cells was done by Western blotting with Ab-2 mAb as previously described [16]. Briefly, proteins (50 μg) from cell homogenates were resuspended in sample buffer and separated by SDS-PAGE on a 6.25% acrylamide-bisacrylamide (29.2:0.8) gel. Pgp was immunodetected by incubation using the mAb Ab-2 (1:1000, 2 h at room temperature) as primary antibody and horseradish peroxidase-conjugated rabbit anti-mouse IgG as secondary antibody. Detection was made with ECL reagents according to the manufacturer's instructions. To ascertain that similar amounts of protein were loaded in each gel, Western blots were always stained with Coomassie blue.

[^{3}H]-VBL accumulation in MDCK-MDR1 cells

MDCK-MDR1 cells were seeded at 150,000 cells per well in 24-well plates and cultured for 4 days at 37°C in an atmosphere containing 5% CO_2. At confluence, the cells were washed twice with HBSS (1.3 m*M* $CaCl_2$, 5.4 m*M* KCl, 0.44 m*M* KH_2PO_4, 0.5 m*M* $MgCl_2$, 0.83 m*M* $MgSO_4$, 137 m*M* NaCl, 4.2 m*M* $NaHCO_3$, 0.34 m*M* Na_2HPO_4 and 25 m*M* D-glucose, pH 6.5) at 37°C. Cells were then preincubated for 30 min at 37°C with HBSS containing DMSO (0.1% v/v) with or without cnidiadin in the range 0–100 μ*M*, or CsA in the range 0–10 μ*M*, or other coumarins at 10 or 100 μ*M*. In some experiments, cnidiadin at 0 or 100 μ*M* was tested in the presence or absence of indomethacin. ^{3}H-VBL (0.23 μCi) were then added at a concentration of 20 n*M* to each well, and the cells were further incubated for 2 h at 37°C. ^{3}H-VBL accumulation was stopped by washing the cells five times with cold PBS (150 m*M* NaCl, 2.7 m*M* KCl, 1.3 m*M* KH_2PO_4, 8.1 m*M* Na_2HPO_4,

pH 7.4). The cells were then lysed with 0.1% Triton X-100 at room temperature. The cell lysates were placed into scintillation vials and the radioactivity was counted.

Cell proliferation assay

MDCK-MDR1 cells were seeded at 5000 cells per well in 96-well plates. VBL was added at 0.6 μ*M*, with or without cnidiadin (10 μ*M*) and cells were grown for 3 days at 37°C under an atmosphere containing 5% CO_2. Cell survival was assayed with a standard WST-1 assay using a previously described procedure [17]. In brief, 10 μl WST-1 (a tetrazolium salt) were added to each well and the soluble formazan dye produced by metabolically active cells was monitored at 37°C by reading the absorbance at 450 nm with a ThermoMax spectrophotometer (Molecular Devices).

Results

Cnidiadin promotes accumulation of R-123 in MDCK-MDR1 cells

To explore their capacity to inhibit Pgp transport activity, we first examined whether cnidiadin and other tested coumarins affected the uptake of R-123, a fluorescent Pgp substrate commonly used to investigate the activity of Pgp inhibitors. In a first series of experiments, all coumarins were tested at concentrations of 10 and 100 μ*M* versus the vehicle (DMSO 0.1%). A strong increase in florescent dye was observed in MDCK-MDR1 cells treated with cnidiadin at 100 μ*M*, while no significant accumulation was detected when other coumarins were tested at this concentration or with cnidiadin at 10 μ*M* (Fig. 2a). The potency of cnidiadin was then measured in relation to positive controls. CsA (10 μ*M*) and verapamil (50 μ*M*) increased R-123 uptake by 7.0- and 3.2-fold, respectively. Exposure to 100 μ*M* cnidiadin induced a higher accumulation of R-123 (8.9 times the control level). However, R-123 uptake was not affected when cnidiadin was used at 10 μ*M* (Fig. 2b).

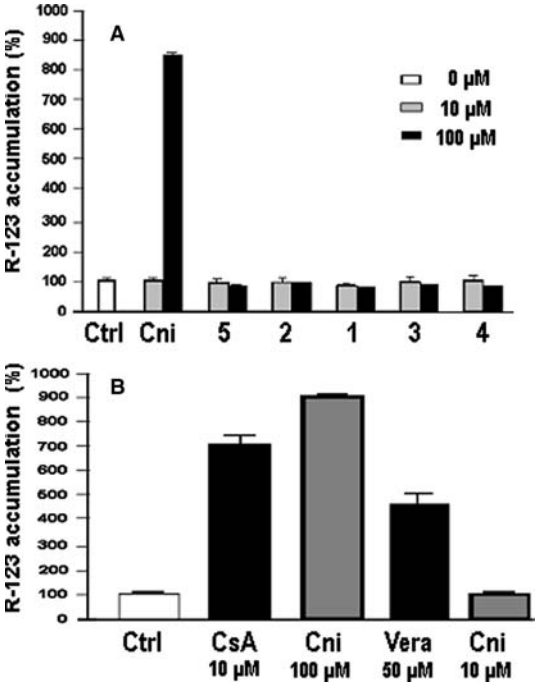


Fig. 2 Effect of cnidiadin and other coumarins on R-123 accumulation in MDCK-MDR1 cells. **a** The accumulation of R-123 in MDCK-MDR1 cells was measured over 2 h in the presence of 0, 10 and 100 μ*M* of cnidiadin (*Cni*), angelicin (*5*), psoralen (*4*), esculin (*3*), esculetin (*2*) or umbelliferone (*1*). **b** The R-123 accumulation promoted by cnidiadin at 100 μ*M* and 10 μ*M* was compared with that promoted by CsA (10 μ*M*) and by verapamil (*Vera*, 50 μ*M*). The results are presented as percentages and are the means from three independent experiments

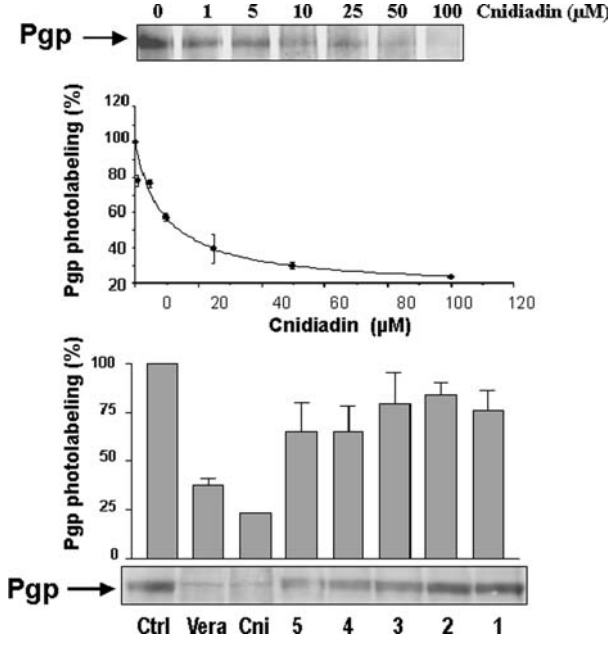


Fig. 3 Inhibition of P-gp photolabeling by cnidiadin and other coumarins. **a** Dose-dependent inhibition of Pgp photolabeling by cnidiadin. Membranes from MDCK-MDR1 cells (50 μg) were coincubated with dz-CsA (0.1 μ*M*) and cnidiadin in the range 0–100 μ*M*, then crosslinked with UV light. Proteins were resolved by SDS-PAGE using 6.25% polyacrylamide gels. The residual amount of Pgp photolabeled by dz-CsA was determined by blotting with a CsA mAb as described in Materials and methods. The corresponding bands were analyzed by laser densitometry. The results are presented as a dose-response curve. **b** Inhibition of Pgp photolabeling by cnidiadin, verapamil and other coumarins. The inhibition of dz-CsA photolabeling by 100 μ*M* cnidiadin (*6*) was compared to inhibition by 10 μ*M* verapamil (*Vera*) or by 100 μ*M* angelicin (*5*), psoralen (*4*), esculin (*3*), esculetin (*2*) or umbelliferone (*1*). DMSO 0.1% was used as the control 100% photolabeling. The levels of photolabeled P-gp were estimated by laser densitometry. The results are presented as the mean ± SD ($n = 3$) percentage of photolabeled Pgp measured in the presence of individual agents as compared to the control.

Cnidiadin inhibits Pgp photolabeling

To verify that cnidiadin competitively interacts with Pgp, we then examined whether cnidiadin inhibited the photolabeling of Pgp by SDZ 212-122, a photoactivable CsA analogue which possess a diazirine (dz) group at position 8. Membranes from MDCK-MDR1 cells were coincubated with the dz-CsA and cnidiadin, then the amount of Pgp linked to the CsA derivative was detected by Western blotting and the level of photolabeled Pgp was quantified by densitometry. In the range of concentrations 1–100 μ*M*, cnidiadin inhibited Pgp photolabeling in a strong and dose-dependent manner (Fig. 3a). At 1, 10 and 25 μ*M*, the inhibition reached 9%, 59% and 68.5%, respectively (Fig. 3a). At 25 μ*M*, the inhibition promoted by cnidiadin (68.5%) was equivalent to that promoted by verapamil at 10 μ*M* (66%) (Fig. 3b). Although at 100 μ*M*, all coumarins inhibited the photolabeling of Pgp by dz-CsA, cnidiadin appeared to be the only compound that efficiently competed with this CsA analogue for the binding of Pgp. Indeed, the highest degree of inhibition by a coumarins other than cnidiadin (34% for angelicin at 100 μ*M*) was markedly weaker than that promoted by cnidiadin at 10 μ*M*. Since at this latter concentration, cnidiadin did not induce significant accumulation of R-123, the inability of angelicin, psoralen, umbelliferone, esculin and esculetin to induce accumulation of R-123 was effectively related to their low capacity to bind Pgp.

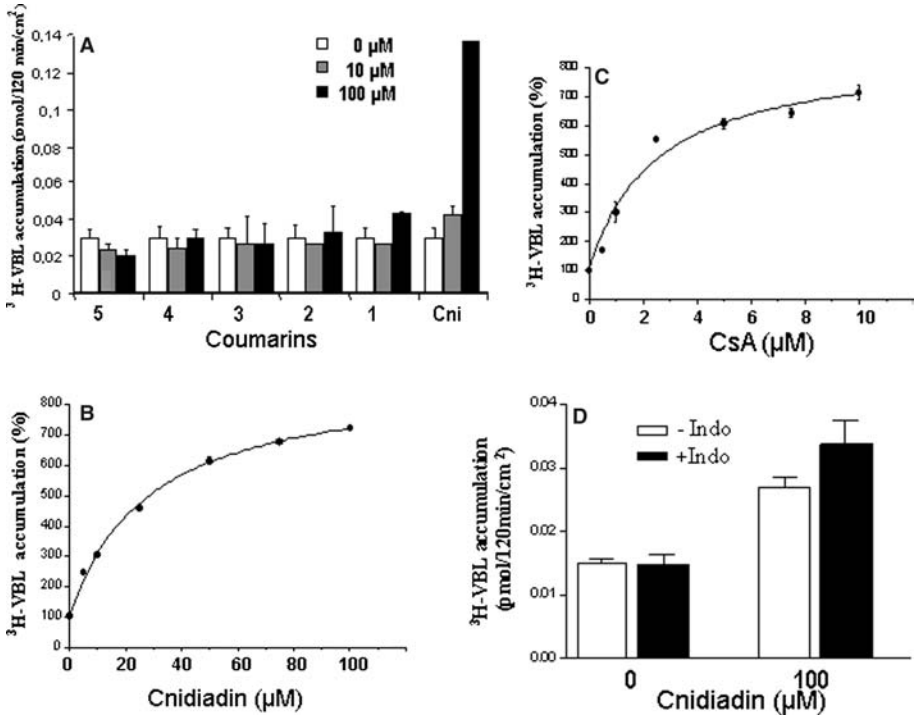
The order of potency for inhibition of Pgp photolabeling of the tested coumarins was: cnidiadin >>> angelicin ≅ psoralen > umbelliferone, esculin and esculetin. These results indicate that although the nuclear prenylation of the 1,2-benzopyrone nucleus favors the binding to Pgp, by itself, the additive nucleus does not confer a significant capacity to compete with SDZ 212-122. In this activity, the efficacy of cnidiadin appears due to the presence of the two isopropyl chains linked on the dihydroangelicin nucleus, rather than to the nucleus itself.

Fig. 4 Effect of treatment on [^{3}H]-VBL uptake in MDCK-MDR1 cells. **a** Modulation of [^{3}H]-VLB uptake by cnidiadin, angelicin (5), psoralen (4), esculin (3), esculetin (2) and umbelliferone (1). The uptake of VLB was measured after a 120-min exposure to each coumarin at 0, 10 and 100 μ*M* as described in Materials and methods. **b** Dose-response curve of the accumulation of [^{3}H]-VLB following treatment with cnidiadin in the dose-range 0–100 μ*M*. **c** Dose-response curve of the [^{3}H]-VLB accumulation following treatment with CsA in the dose-range 0–10 μ*M*. **d** Effect of cotreatment with indomethacin, an inhibitor of MRP, on the uptake of [^{3}H]-VBL by cnidiadin. [^{3}H]-VBL uptake was measured after a 120-min exposure of cells to solvent (DMSO 0.1%) or cnidiadin (100 μ*M*) in the presence (*white bars*) or absence (*black bars*) of indomethacin. The data presented are the means ± SD of the values obtained from three independent experiments performed in triplicate

Cnidiadin increases the uptake of [^{3}H]-VBL in MDCK-MDR1 cells

Although it is a potent Pgp substrate, the small and water-soluble R-123 could not be used as a model to explore the potency of cnidiadin as a reversal agent. This point was evaluated by measuring the uptake of the radiolabeled anticancer agent [^{3}H]-VBL into MDCK-MDR1 cells.

In a first series of experiments, [^{3}H]-VBL uptake was assessed after a 120-min exposure to each coumarin at 0, 10 and 100 μ*M*. At 10 μ*M* cnidiadin increased [^{3}H]-VBL



uptake, as did the other coumarins at 100 μ*M* (Fig. 4). These data confirmed that cnidiadin was the only tested coumarin exhibiting potency as a chemosensitizer.

In further studies, the potencies of cnidiadin and CsA were compared. In these experiments, cnidiadin was tested in the concentration range 0–100 μ*M*, and CsA in the concentration range 0–10 μ*M*. Both induced accumulation of [^{3}H]-VBL in a highly dose-dependent manner (Fig. 4b, c). The maximal uptakes were very close (7.2 times the control level for cnidiadin and 7.0 times for CsA) and the two dose-response curves showed similar profiles. However, 10 μ*M* of CsA were sufficient to cause the maximal accumulation while 100 μ*M* of cnidiadin were needed. Cnidiadin (IC_{50} 26.4 μ*M*) thus appears a less-potent reversal agent than CsA (IC_{50} 3.4 μ*M*),

However, cnidiadin at 10 μ*M* significantly interacted with Pgp and inhibited its transport activity (Fig. 4b).

In humans, other members of the ABC superfamily have been implicated in MDR. These include the multidrug resistance protein-1 (MRP1), its homologues MRP2–6 that transport glutathione, glucuronate and sulfate-conjugated drugs, and the breast cancer resistance protein (BCRP) [4]. Certain dietary polyphenolics, such as quercetin, modulate the transport of VBL by different multidrug transporters [18, 19]. To evaluate how, in MDCK-MDR1 cells, cnidiadin-mediated [^{3}H]-VBL accumulation was specific to Pgp, we examined the effect of indomethacin, an inhibitor of MRP, on the uptake of [^{3}H]-VBL by cnidiadin. In the absence of cnidiadin, indomethacin did not affect VBL accumulation (Fig. 4d). This suggests that MDCK-MDR1 cells do not express MRP pumps or that activity of MRP proteins is not detectable in these cells using this technique. Since the only slight variation of accumulation was observed in the presence cnidiadin, a lack of inhibitory activity of cnidiadin against MRP proteins can be suggested.

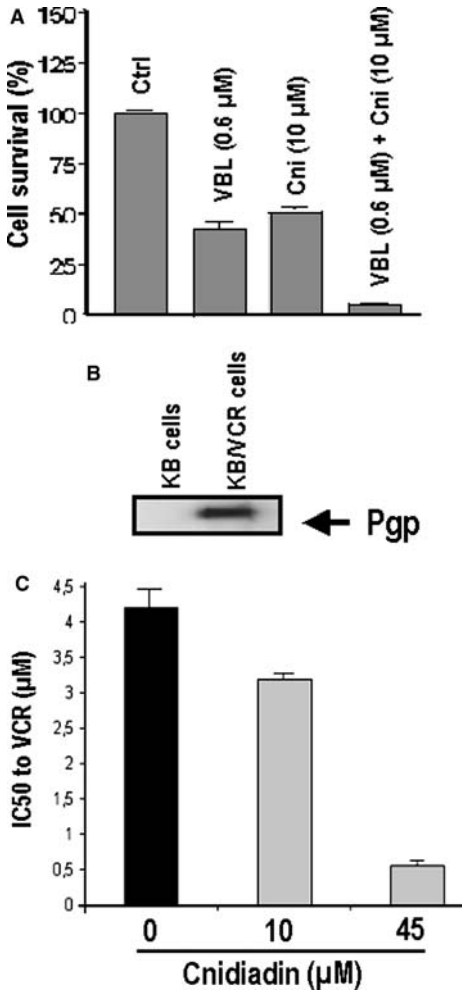


Fig. 5 Effect of a cotreatment by cnidiadin and vinca alkaloids on MDCK-MDR1 and KB/VCR cells. **a** Cnidiadin enhances the cell toxicity of VBL in MDCK-MDR1 cells. The survival of MDCK-MDR1 cells exposed continuously to VBL (0.6 μ*M*), cnidiadin (10 μ*M*) or a combination of the two drugs for 72 h was evaluated with the WST-1 assay as described in Material and methods. DMSO (0.1%) was used as a control. The results presented are the mean ± SD percentage survival in relation to control cells (100%). Values were obtained from three independent experiments. **b** Western blot analysis of Pgp expression in mutant human epidermoid carcinoma KB/VCR and wild-type KB cells. Proteins were separated on 6.25% polyacrylamide gels and transblotted onto a an Immobilon-P membrane. Pgp was detected with MDR Ab-2 mAb as described in Material and methods. **c** Cotreatment with cnidiadin sensitizes KB/VCR cells to VCR. IC_{50} values of VCR were determined after a 72-h contact with cnidiadin at 0, 10 and 45 μ*M*. Cell toxicity was measured with the WST-1 assay as described in Material and methods. Values are the means ± SD from three independent experiments

Cnidiadin is a cytotoxic compound enhancing the cell toxicity of vinca alkaloids in resistant cells overexpressing Pgp

Further experiments were done to verify whether cotreatment with cnidiadin may enhance the cell toxicity of vinca alkaloids on resistant cells overexpressing Pgp. In these experiments, the vinca alkaloid (VBL or VCR) was used at its IC_{50}, and cnidiadin at 10 μ*M* or its IC_{50}. Cell toxicity was evaluated after a 72-h continuous exposure of the cells to the different chemicals singly or combined.

Assays were first conducted on MDCK-MDR1 cells. The level of resistance of this cell line to VBL was approximately 14. This value was calculated as the IC_{50} of a drug in the resistant cell line divided by that of the drug in the wild-type parental cell line (0.04 μ*M*). When VBL was used alone at 0.6 μ*M*, it killed 57% of MDCK-MDR1 cells. The proportion of killed cells reached 93% when VBL was used in association with cnidiadin at 10 μ*M* (Fig. 5a). Because treatment with cnidiadin alone at 10 μ*M* killed about 50% of MDCK-MDR1 cells, it cannot be determined from these results whether the beneficial effect of cnidiadin was the result of an additive cell toxicity or a reversal effect or both. However, they unambiguously show that 10 μ*M* cnidiadin enhanced the toxicity of VBL in MDCK-MDR1cells by 163%.

The MDCK-MDR1 cell line is not a cancer line. To evaluate whether cnidiadin has the potential to sensitize

multiresistant human cancer cells to vinca alkaloids, we further tested the effect of cotreatment with cnidiadin/VCR on the human resistant cancer cell line (KB/VCR). This mutant epidermoid carcinoma line resulted from long-term exposure of sensitive wild-type KB cells to VCR. It is highly resistant to VCR. Western blots performed with an antibody recognizing only the human MDR1 isoform (MDR mAb Ab-2), showed that this resistance is associated with expression of the MDR1 gene (Fig 5b). The level of resistance to VCR of the mutant cell line (IC_{50} 4.21 ± 0.33 μM) was established as 105. Interestingly, KB/VCR cells were much more resistant to cnidiadin (IC_{50} 43.47 ± 0.27 μM) than MDCK-MDR1 cells (IC_{50} 10 μM). Notably, no cell toxicity was detected after exposure to 10 μM cnidiadin. However, at this concentration, cnidiadin sensitized KB/VCR cells to VCR. Indeed, when cells were treated with VCR in combination with 10 μM cnidiadin, the $IC_{50}$of VCR decreased from 4.21 ± 0.33 μM to 3.18 ± 0.17 μM, but was decreased to 0.56 ± 0.13 μM in combination with cnidiadin at 45 μM (about the IC_{50}) (Fig. 7c).

Discussion

Furanocoumarins exist in many plants from the Rutaceae, Apiaceae and Leguminoseae families. Some of these plants are sources of spices, herbal medicines, common vegetables (celery, parsley and parsnips), or beverages (grapefruit and other Citrus juices). The biological roles of furanocoumarins in their host plants are not fully understood. However, it is established that these chemicals are phytoallexins protecting plants from attack by insects, viruses and bacteria. Literature data show that certain of these dietary furanocoumarins may affect human health. Effects may be beneficial or deleterious. By blocking DNA adduct formation, the citrus coumarin, isopimpinellin, may exhibit chemopreventive activity [20]. Furanocoumarins from grapefruit mediate drug interactions and affect drug bioavailability and metabolism by modulating Pgp transport function and the activity of cytochrome P450 [12–14].

It has been reported that cnidiadin inhibits the proliferation of non-small-cell bronchial carcinoma cells by blocking the cell cycle in the G_1 phase [15]. In this study, using MDCK-MDR1 cells as a model of cells expressing the human MDR1 phenotype, we tested cnidiadin for its capacity to (1) induce the accumulation of two Pgp substrates (the lipophilic cation R-123 and the radiolabeled anticancer agent [^{3}H]-VBL), and (2) compete with a CsA analogue for binding to Pgp. In preliminary assays, cnidiadin was tested in comparison with three simple coumarins (umbelliferone, esculin and esculetin) and two furanocoumarins (angelicin and psoralen). CsA and verapamil were used as positive controls. Cnidiadin was the only tested coumarin capable of significantly inhibiting the extrusion of the lipophilic cation R-123 and the radiolabeled anticancer agent [^{3}H]-VBL out of MDCK-MDR1 cells. At high concentrations (100 μM), cnidiadin induced the accumulation of R-123 more efficiently than the two positive controls, and was slightly more potent than CsA in its ability to induce the accumulation of [^{3}H]-VBL. These results indicate that, in contrast to other tested coumarins, cnidiadin can inhibit Pgp transport and may act as a reversing agent. Interestingly, in the dose range 0–100 μM, cnidiadin substantially increased [^{3}H]-VBL uptake in a dose-dependent manner. The maximal accumulation (7.2 times the control level) was close to the accumulation (7.0 times) promoted by 10 μM CsA. At low concentrations, cnidiadin (IC_{50} 26.4 μM) is however a less potent reversal agent than CsA (IC_{50}: 3.2 μM). Nevertheless, 10 μM cnidiadin effectively induced the accumulation of [^{3}H]-VBL in this cell-line.

Suppressing the expression of P-gp at either the transcriptional or protein level is a key approach to reverse MDR. Understanding the mechanism by which cnidiadin increases [^{3}H]-VBL uptake was therefore important. The demonstration that this effect is due to a competition for the binding to Pgp was shown by a correlation between the dose-response curves of photolabeling inhibition and of [^{3}H]-VBL accumulation. Consistent with a specific inhibition of Pgp efflux, [^{3}H]-VBL uptake was not increased by an inhibitor of MRP in the absence of cnidiadin, and was only slightly increased when this inhibitor was used in combination with cnidiadin. These results support the hypothesis that, in MDCK-MDR1 cells, the accumulation of [^{3}H]-VBL following treatment with cnidiadin is almost completely specific to a competitive inhibition of Pgp transport activity. Comparison of Pgp photolabeling inhibition by cnidiadin and the other tested coumarins shows that this effect is in a large part due to the two hydroxyisopropyl chains linked on the dihydroangelicin nucleus.

To further evaluate the potency of cnidiadin as a reversal agent, we tested its capacity to sensitize MDCK-MDR1 and KB/VCR cells to vinca alkaloids. Our findings demonstrate that cnidiadin is a cytotoxic agent, and this is in agreement with previous findings in non-small-cell lung carcinoma (NSCLC) N6 cells, showing that cnidiadin is a cell-cycle inhibitor, blocking cells in the G_1 phase [15]. The IC_{50} in NSCLC-N6 cells was established as 13.1 μg/ml (39.8 μM) [15]. MDCK-MDR1 cells appear much more susceptible to cnidiadin than N6 cells. Indeed, a single treatment with 10 μM cnidiadin decreased the survival of this cell line by 50%. Used together with 0.6 μM VBL, 10 μM cnidiadin increased the cell toxicity of VBL by 163%. This finding indicates sensitization of this resistant cell line to VBL, but does not permit the part of additive cell toxicity in this effect to be determined.

To evaluate a possible benefit of cnidiadin in the treatment of human resistant tumors overexpressing Pgp, we then tested its capacity to enhance the cell toxicity of VCR in KB/VCR cells. This mutant human epidermoid carcinoma line that expresses the MDR1 gene exhibits high resistance (105-fold) to VCR and mild

susceptibility to cnidiadin (IC_{50} 43.5 μM). No cell toxicity was detected when cnidiadin was used at 10 μM as a single agent. However, at this non-toxic concentration, cnidiadin decreased by 24.6% the IC_{50} value of VCR in KB/VCR cells. The beneficial effect of the cotreatment supports a synergism between VCR and cnidiadin. The exact mechanisms by which cnidiadin potentiates VCR, the toxicity of this furanocoumarin in normal cells and its specificity for cancer cells remains to be evaluated. However, our results clearly establish that cotreatment of KB/VCR cells with VCR and cnidiadin at non-toxic and toxic concentrations sensitized this resistant cell line to VCR.

Considering the important role of Pgp in the oral bioavailability of drugs [21], it is possible that cnidiadin in food or folk medications may play a complementary role against MDR by reversing barriers to drug availability. Indeed, Pgp is present in the intestine in the brush border of mature enterocytes where it pumps Pgp substrates from the enterocytes back to the intestinal lumen, preventing their absorption into blood. It has been reported previously that ingestion of furanocoumarins from grapefruit juice increases the bioavailability of Pgp substrates by partially inhibiting intestinal Pgp transport activity [22]. Inhibiting Pgp-mediated drug efflux may have beneficial consequences, but may also contribute to drug interactions [22–25]. In vivo studies are thus needed to evaluate if ingestion of food or folk medicine containing cnidiadin has positive or deleterious effects.

In conclusion, this study demonstrated that cnidiadin is a cytotoxic compound and a Pgp substrate capable, in vitro, of competitively inhibiting the binding and efflux of drug by Pgp. The tumoricidal activity of this furanocoumarin, and its capacity to sensitize resistant cells overexpressing Pgp to vinca alkaloids suggest that diets and traditional preparations containing cnidiadin may contribute to (1) reverse multidrug resistance encoded by the MDR1 gene, and (2) increase the bioavailability of orally administered chemotherapeutic agents in humans. Due to its cell toxicity and weak reversal activity at low concentrations, clinical interest in cnidiadin as a reversal drug may, however, be limited.

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