
Title: Collateral sensitivity of resistant MRP1-overexpressing cells to flavonoids and derivatives through GSH efflux

Author: Doriane Lorendeau Lauriane Dury Estelle Genoux-Bastide Florine Lecerf-Schmidt Claudia Simõ es-Pires Pierre-Alain Carrupt Raphaël Terreux Sandrine Magnard Attilio Di Pietro Ahcène Boumendjel Hélène Baubichon-Cortay

PII: S0006-2952(14)00294-9
DOI: http://dx.doi.org/doi:10.1016/j.bcp.2014.05.017
Reference: BCP 11984

To appear in: *BCP*

Received date: 14-4-2014
Revised date: 16-5-2014
Accepted date: 19-5-2014

Please cite this article as: Lorendeau D, Dury L, Genoux-Bastide E, Lecerf-Schmidt F, Simõ es-Pires C, Terreux P-AC, <ce:inter-ref id=ïntr0005ẍlink:href=m̈ailto:pierre-alain.carrupt@unige.chz̈pierre-alain.carrupt@unige.ch</ce:inter-ref>, Raphaël, Magnard S, Di Pietro A, Boumendjel A, Baubichon-Cortay H, Collateral sensitivity of resistant MRP1-overexpressing cells to flavonoids and derivatives through GSH efflux, *Biochemical Pharmacology* (2014), http://dx.doi.org/10.1016/j.bcp.2014.05.017

# Collateral sensitivity of resistant MRP1-overexpressing cells to flavonoids and derivatives through GSH efflux

*Doriane Lorendeau[†], Lauriane Dury[†], Estelle Genoux-Bastide[‡], Florine Lecerf-Schmidt[‡], Claudia Simões-Pires[¥], Pierre-Alain Carrupt[¥], Raphaël Terreux[§], Sandrine Magnard[†], Attilio Di Pietro[†], Ahcène Boumendjel[‡£] and Hélène Baubichon-Cortay[†£]*

[†]Equipe Labellisée Ligue 2012, Université Lyon 1, Univ Lyon, France; CNRS, UMR 5086 ; BMSSI, IBCP, 7 passage du Vercors, F-69367, France, doriane.lorendeau@ibcp.fr, lauriane.dury@ibcp.fr, s.magnard@ibcp.fr, a.dipietro@ibcp.fr, h.cortay@ibcp.fr

[‡]Département de Pharmacochimie Moléculaire Université Grenoble-Alpes, CNRS UMR 5063, , Grenoble, France, ahcene.boumendjel@ujf-grenoble.fr, florine.lecerfschmidt@gmail.com

[§]Equipe "Bioinformatique : structures et interactions", Université Lyon 1, Univ Lyon, France; CNRS, UMR5086; Bases Moléculaires et Structurales des Systèmes Infectieux, IBCP 7 passage du Vercors, F-69367, France, r.terreux@ibcp.fr,

[¥] Pharmacochimie, Section des Sciences Pharmaceutiques , Université de Genève, Université de Lausanne , Genève, Suisse, pierre-alain.carrupt@unige.ch, claudia.avello@unige.ch,

[£] Both senior investigators equally contributed to the work

***Corresponding author information:***

Hélène Baubichon-Cortay

Phone: (+33) 47272 2691

Fax (+33) 47272 2604

E-mail: hcortay@ibcp.fr

IBCP, UMR 5086 , 7 passage du Vercors, F-69367, France

**Abbreviations**

ABC: ATP binding cassette, MDR: Multidrug resistance, MRP1 and MRP2: Multidrug resistance protein 1 and 2, P-gp: P-Glycoprotein, BCRP: Breast cancer resistance protein, BHK-21: Baby Hamster Kidney 21, GSH: reduced glutathione, GSSG: oxidized glutathione, SR-verapamil: racemic verapamil, MTT: 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide, NAC: N-AcetylCysteine, $IC_{50}$: concentration required to obtain 50% of the maximal effect, SR: Selective ratio, G418: Geneticin, PBS: Phosphate buffer salt, AcOEt: Ethyl acetate, cHex: Cyclohexane, 3D-QSAR: Three dimensional quantitative structure activity relationship, CoMSIA: Comparative molecular similarity indices, SD: standard deviation.

**Abstract**

The multidrug resistance protein 1 (MRP1) is involved in multidrug resistance of cancer cells by mediating drug efflux out of cells, often in co-transport with glutathione (GSH). GSH efflux mediated by MRP1 can be stimulated by verapamil. In cells overexpressing MRP1, we have previously shown that verapamil induced a huge intracellular GSH depletion which triggered apoptosis of the cells. That phenomenon takes place in the more global anticancer strategy called "collateral sensitivity" and could be exploited to eradicate some chemoresistant cancer cells. Seeking alternative compounds to verapamil, we screened a library of natural flavonoids and synthetic derivatives. A large number of these compounds stimulate MRP1-mediated GSH efflux and the most active ones have been evaluated for their cytotoxic effect on MRP1-overexpressing cells versus parental cells. Interestingly, some are highly and selectively cytotoxic for MRP1-cells, leading them to apoptosis. However, some others do not exhibit any cytotoxicity while promoting a strong GSH efflux, indicating that GSH efflux is necessary but not sufficient for MRP1-cells apoptosis. In support to this hypothesis, structure activity relationships show that the absence of a hydroxyl group at position 3 of the flavonoid C ring is an absolute requirement for induction of MRP1-cells death, but is not for GSH efflux stimulation. Chrysin (compound **8**) and its derivatives, compounds **11** and **22,** exhibit a high selectivity toward MRP1-cells with a $IC_{50}$ value of 4.1 µM for compound **11** and 4.9 µM for chrysin and compound **22,** making them among the best described selective killer compounds of multidrug ABC transporter-overexpressing cells.

**Keywords**

Multidrug resistance; Multidrug Resistance Protein 1; glutathione; flavonoids; collateral sensitivity.

Chemical compounds studied in this article: Chrysin (PubChem CID: 5281607); Galangin (PubChem CID: 5281616), Kaempferol (PubChem CID: 5280863)

## 1. Introduction

Resistance of tumours to multiple structurally-unrelated anticancer drugs is a common clinical problem that limits the curative potential of chemotherapy in clinical oncology. Eradication of resistant cells in tumours would then have a considerable impact for curing cancer by chemotherapy. The multidrug resistance (MDR) phenotype is in part due to the overexpression within plasma membranes of drug transporters able to bind and extrude a wide variety of compounds. Three human transporters belonging to the ATP-binding cassette (ABC) proteins superfamily have been described, which can actively extrude anticancer drugs from the cell at the expense of ATP hydrolysis: P-glycoprotein (P-gp or ABCB1), multidrug resistance protein 1 (MRP1 or ABCC1) and breast cancer resistance protein (BCRP, or ABCG2)[1]. MRP1 preferentially transports negatively-charged substrates, *i.e.* organic anions, generally present as either glutathione-, glucuronate- or sulfate- conjugates. Its main endogenous substrate is leukotriene $C_4$, but it also transports reduced glutathione (GSH) and its oxidized form (GSSG). In addition, some hydrophobic drugs, such as vinblastine and vincristine, are also transported by MRP1 by symport with GSH[2]. MRP1 modulation would be relevant since its expression is correlated with cancer incidence or prognostic impact[3]. Considering that MRP1 co-transports chemotherapeutic drugs with GSH, GSH depletion could become an experimental approach to sensitize tumor cells to therapy[4].

Previously, we discovered that verapamil stimulates MRP1-mediated glutathione transport out of the cell after binding to the transporter[5]. The fast and huge glutathione extrusion triggers a selective apoptosis of cells overexpressing MRP1[6]. Hence, it is conceivable to propose the

set-up of a novel and original strategy, *i.e.* a new therapeutic approach using ligands of MRP1 that act as stimulators of its GSH transport activity and that trigger apoptosis of cancer cells overexpressing MRP1 without affecting normal cells. This strategy takes place in a specific new therapeutic approach based on collateral sensitivity of cancer cells overexpressing multidrug ABC transporters, where the physio-pathological changes associated with their overexpression can be considered as an **Achilles' heel**[7], (Szakacs *et al.*, Chem. Rev., 2014; in press). In this research field, experiments have been first carried out on MDR cells overexpressing P-glycoprotein and a number of compounds have been shown to induce selective cytotoxicity towards MDR cells, such as NSC73306[8-12]. Recently, the orphan drug tiopronin demonstrated collateral sensitivity toward various MDR cell lines overexpressing P-glycoprotein or MRP1[13]. Successful targeting of multidrug-resistant cells would reduce the tumor burden, and indirect problems observed with the classical use of multidrug ABC transporters inhibitors.

When using verapamil as a sensitizer, we have showed that the *S*-isomer, but not the *R*-isomer, of verapamil is the causative agent of MRP1-mediated GSH efflux [5], indicating a sharp structure activity relationship. The major known drawback of verapamil is its cardiotoxicity, which excludes any clinical development in cancer treatment. In order to find new MRP1 ligands, less toxic and more potent than *S*-verapamil, we investigated flavonoids analogs with a special focus on derivatives that have been shown to bind to ABC transporters. On a series of flavonoids derivatives, we evaluated both the stimulation of GSH efflux and the induction of cell death. We found very active compounds on both activities either in transfected cells and selected cells and cell death was identified as apoptosis. Some other flavonoids are only active on GSH efflux stimulation, which demonstrates that GSH efflux is necessary but not sufficient to trigger cell death of MRP1-cells. Only those which do not have an hydroxyl group at position 3 of the C ring are active for the induction of MRP1-cells death.

The best one, 3-methoxy-chrysin (compound **11**) ($IC_{50}$ of 4.1 µM on MRP1-cells), exhibits a high selective ratio upper than 24.5.

## 2. Materials and Methods

### 2.1. Chemical compounds

Compounds **1-8**, **10 and 20-21** shown in table 1 were purchased from Sigma-Aldrich company (Saint Quentin Fallavier, France). Compounds **9**, **11-19** were prepared according to a previous report[14]. Flavones **22-38** were prepared by condensation of 4',6'-dimethoxy-2'-hydroxyacetophenone (Sigma-Aldrich, Saint Quentin Fallavier, France) with substituted benzaldehyde (Sigma-Aldrich, Saint Quentin Fallavier, France) ( in the presence of KOH in a mixture of EtOH:$H_2O$ which affords chalcones intermediates. The chalcones intermediates were treated in oxidative cyclization conditions with iodine in DMSO to afford 5,7-dimethoxyflavones (**24, 26 and 28-32**[15]. During the oxidation step and only in one case, we observed the incorporation of iodine into the A-aromatic ring at the C-6 carbon (compound **30**). 5-Hydroxyflavones and 5,7-dihydroxyflavones (**22, 23, 25 and 27**) were obtained by treating their methoxylated derivatives with boron tribromide (Sigma-Aldrich, Saint Quentin Fallavier, France) in $CH_2Cl_2$ to afford either totally demethylated or a mixture of mono and demethylated derivatives.

4'-Aminoflavone **32** was prepared by reduction of 4'-nitroflavone **31** with $SnCl_2$ (Sigma-Aldrich, Saint Quentin Fallavier, France) in HCl according to conventional procedures[16]. 4'-benzamide flavones (**33**, **38**) were obtained by treatment of **32** with substituted benzoyl chlorides.

NMR spectra were recorded on a Brüker AC-400 instrument (400 MHz). Electrospray ionization ESI mass spectra were acquired by the Analytical Department of Grenoble University on an Esquire 300 PlusBrukerDaltonis instrument with a nanospray inlet.

### 2.2. Antioxidant and radical scavenger activities measurements

All chemicals were purchased from Sigma-Aldrich company, Saint Quentin Fallavier, France.

#### 2.2.1. Oxygen radical absorbance capacity (ORAC) assay.

Antioxidant activities of the tested compounds were determined by their ability to preserve from oxygen radical species the fluorescence of fluorescein exposed to peroxyl radicals generated by 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH)[17]. Black polypropylene 96-well plates were filled with 60 nM fluorescein (in glycine buffer pH 8.3), together with test samples or vehicle (in 2% DMSO), and pre-incubated at 40°C for 15 min. The oxidative reaction was obtained by adding 5 mM AAPH (in glycine buffer pH 8.3) to wells containing samples, positive control (here quercetin, which does not induce GSH efflux) and oxidized fluorescein control. Non-oxidized fluorescein controls were added with the same volume of assay buffer. The plate was incubated at 40°C for 90 min with continuous shaking at 150 rpm, and cooled down at room temperature (5 min) prior to fluorescence reading at 485/528 nm. Pure compounds were first tested at 10 µM according to pre-established screening criteria[18]. For each compound with a percentage of fluorescein protection higher than 50%, the $EC_{50}$ (concentration required to obtain 50% of the maximal effect) value was determined by a dose-response curve of at least 6 concentrations.

#### 2.2.2. DPPH radical scavenging assay.

Capacities of samples to scavenge the stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) were determined spectrophotometrically by measuring the loss of absorbance of DPPH at 515 nm [19]. 96-well microplates were filled with test sample solution (in ethanol containing up to 2% DMSO) or vehicle for the DPPH control. The reaction was initiated by the addition of 80 µM DPPH (in ethanol). The decrease in absorbance at 515 nm was monitored at room temperature after 10 min in order to determine the percentage of scavenged radical. Samples

were tested at 40 µM for screening purposes to provide a concentration ratio of compound over radical of 0.5 (R=0.5), as previously defined [18].For each compound with a percentage of scavenged DPPH higher than 20%, the $ER_{50}$ (ratio between $EC_{50}$ and radical concentration) values were determined by on dose-response curves of at least 6 concentrations by calculating as ratios between compound concentration and radical concentration.

### 2.3. Molecular Modeling

Compounds were analysed by the Sybyl X 1.3 molecular modelling suite software[20], and minimized by the MMFF94 force field[21] with a dielectric constant of 80 and an electrostatic cut-off of 16Å. Minimized molecules were aligned on the central common core and put in a database; lateral chains of molecules were manually checked and aligned on a common position, and the modified conformation was minimized. The differences in internal energy between the two conformations must be lower than 20 $kcal.mol^{-1}$ in order to validate the aligned conformations. Using the aligned molecules, a 3DQSAR study was initiated with the CoMFA[22] program. Grids of electrostatic and steric potential fields were computed using the C3 atom with a charge of +2 as a probe. Grids were filtered with 4 $kcal.mol^{-1}$ as the minimal variation to select probes, and the validation by leave one out (LOO) method was chosen.[23]

### 2.4. Cell lines

All cell lines were grown at 37°C in 5 % $CO_2$. BHK-21 (Baby Hamster Kidney-21) cells stably transfected with wild-type *mrp1* or K1333L *mrp1* mutant have been previously described[24, 25]. They were grown in DMEM/F-12, GlutaMAX Supplement culture medium (Gibco-Life Technologies, Saint Aubin, France) supplemented with 15 mM HEPES, 1 % penicillin/streptomycin (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France) and 5

% of heat-inactivated fetal bovine serum (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France), in the presence of 200 µM methotrexate for transfected cells. The small cell lung cancer cell lines NCI-H69 and H69AR (from ATCC number HTB-119 and CRL-1135, respectively), which are human lung epithelial carcinoma, have been previously described[26]. The H69AR line is derived from the NCI-H69 line after 14 months selection with increased doxorubicin concentrations. They were grown in RPMI 1640 medium with HEPES and L-glutamine (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France) supplemented with 1 % penicillin/streptomycin, 10 % of heat-inactivated fetal bovine serum and 1mM sodium pyruvate (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France). H69AR were monthly selected with doxorubicine at 0.8 µM (Sigma-Aldrich, Saint Quentin Fallavier, France). The other cell lines were grown in DMEM High Glucose culture medium with L-Glutamine (PAA, GE Healthcare Life sciences, Velizy-Villacoublay, France). supplemented with 1 % penicillin/streptomycin and 10 % of heat-inactivated fetal bovine serum. The NIH3T3/P-gp (ATCC)[27] grown medium was also supplemented with 60 ng/mL of colchicine (Sigma-Aldrich company, Saint Quentin Fallavier, France). Human MDR1-G185 P-glycoprotein-transfected (Pgp-expressing) HEK293 cells, a gift from Dr S. Bates[28], and ABCG2-transfected HEK293 cells, obtained by transfection of cells with pcDNA3.1-ABCG2 using lipofectamine (Invitrogen, Life technologies, Saint Aubin, France), were respectively grown with 2 and 1 mg/mL of G418 (Invitrogen, Life technologies, France). MDCKII (Madin-Darby Canine kidney) transfected with *mrp2* (MCDKII-MOAT17)[29] and the MDCKII parental cell line were a gift from Dr E. Georges (McGill University, Montreal, Canada). MDCKII-MOAT17 was maintained with 800 µg/mL of G418.

**2.5. Cell proliferation analysis.**

The MTT colorimetric assay was used to assess the sensitivity of parental cells and MRP1-cells to the different compounds (by determination of $IC_{50}$: concentration of compound where 50 % of the growth inhibition are observed)[30]. Briefly, growth inhibition assays were performed by plating cells in 96-well plates at density $2.0 \times 10^4$ cells/well for NCI-H69 and H69AR, at $3.2 \times 10^3$ cells/well for BHK-21-wt and $4 \times 10^3$ cells/well for BHK-21-MRP1 cell lines. Cells were cultured for 17 h before addition of compounds diluted in complete culture medium, with a fixed final DMSO concentration of 0.5 %. The cells were then incubated for 24 or 72 h in a humidified tissue-culture chamber (37 °C, 5 % $CO_2$). Surviving cells were detected by the bromo-3(4,5-dimethyl-2-thiazoyl) 2,5-diphenyltetrazolium (MTT) assay. The MTT solution was added to a final concentration of 0.5 mg/ml and cells were then incubated for 4 h at 37 °C. For NCI-H69 and H69AR cell lines, the 96-well plates were thereafter centrifuged at 700 g for 10 min to pull down floating cells, the MTT dye was carefully aspirated and 200 µL of 50 % ethanol/50 % DMSO mixing solution was added to each well to solubilize the reduced formazan dye under shaking. The absorbance of formazan was determined by spectrophotometry at 570 nm. The effect of drugs on cell growth was determined from differences in absorbance between drug-treated cells *versus* untreated cells or solvent control. $IC_{50}$ values were calculated from dose response curves obtained from triplicate independent experiments. For cell rescue experiments, BHK-21-wt and BHK-21-MRP1 cells were incubating with flavonoids at 20 µM with increasing concentrations of N-acetylcysteine (NAC) (purchased from Sigma-Aldrich) for 72h and with increasing concentrations of Z-VAD-FMK, a generic pan-caspase inhibitor (purchased from Calbiochem, Merck Chemicals, UK) for 24h.

### 2.6. Total cellular glutathione determination.

All chemicals were purchased from Sigma-Aldrich, Saint Quentin Fallavier, France

Total cellular glutathione contents (GSH + GSSG) were measured using the enzymatic method described by Tiezte[31] and modified by Anderson[32] to the microtiter plate. Cells were seeded into 96-well plates at a density of $1.0 \times 10^5$ cells/well and cultured for 17 h before addition of compounds at 20 µM. DMSO concentration was fixed at 0.5 %. After a 3h-incubation, cells were washed twice with 100 µL cold PBS, and then lysed by 100 µL PBS supplemented with 0.1 % Triton X-100. After homogenization, lysis was performed by incubation 1 h at 4° C under strong shaking prior to freezing. Then 25 µL of lysate were transferred to a new plate for GSH titration, and 10 µL to another plate for protein quantitation. GSH determination was performed by adding 100 µL of reaction buffer containing 266.6 µM NADPH, 555 µM 5,5'-dithiobis-(2-nitrobenzoic acid), 10 U/mL GSH reductase and PBS-Triton 0.1 %. The plate was then placed in a microplate reader and the absorbance was read at 412 nm every 30 s for 2 min. The content of total cellular glutathione was quantified by comparison with known glutathione standards. The slope was determined for each sample and led to the determination of the GSH contents. Protein titration was performed on the remaining 10 µL by the Bicinchoninic Acid method assay. The measured total glutathione was expressed in nmol per mg protein, and the results were obtained from triplicates.

## 3. Results

### 3.1. Flavonoids and derivatives as inducers of a strong intracellular GSH depletion

Flavonoids are natural compounds extensively studied for their anti- and pro-oxidant properties. They have a broad range of biological activities by interacting with various proteins and their use in cancer chemoprevention and chemotherapy is of great interest[33, 34]. In order to circumvent the MDR phenotype of cancer cells due to multidrug ABC transporters, flavonoids and derivatives were shown to inhibit drug transport mediated by P-glycoprotein[35-38], MRP1[39, 40] and BCRP[41, 42]. A new type of activity has been observed for some flavonoids that are effective in depleting the glutathione content of tumor cells overexpressing MRP1, thanks to MRP1-mediated GSH efflux[33, 43-45].

In 2004, we reported for the first time collateral sensitivity of MRP1-cells. Indeed, we showed that verapamil triggered selective apoptosis of MRP1-overexpressing cells after a strong cellular GSH depletion[6], resulting from the stimulation of GSH efflux. This efflux stimulated by verapamil was demonstrated to be mediated by MRP1[46]. Afterwards, our results were confirmed by Laberge *et al*. who studied both verapamil and the flavonoid apigenin[47], this last one being only active on H69AR cells but not on transfected Hela$^{ABCC1}$ cells.

Here, we further prospect for compounds with the same desirable properties by searching the requested structural requirements. This could lead to the elaboration of novel anticancer agents that would act as collateral sensitivity agents. Natural and synthetic flavonoids (Table 1) were submitted to a first screen to assess their ability to modify the intracellular levels of GSH in two cell lines: Baby Hamster Kidney-21 wild-type parental cells (BHK-21-wt) (control cells) and BHK-21 cells transfected by the *MRP1* gene (BHK-21-MRP1), which only express MRP1[24], previously used to study collateral sensitivity to verapamil[6].

The total cellular glutathione levels were measured in both control cells and *MRP1*-transfected cells treated or not with each compound at 20 µM and expressed in percentage (untreated cells glutathione levels considered as 100 %). The 20 µM concentration was

selected from previous works describing the effects of flavonoids on cellular GSH depletion [44, 45]. Percentages of depletion caused by tested compounds were calculated for each cell line and the effect on the *MRP1*-transfected cells was then corrected from effects on control cells to get the “net GSH depletion". This "net depletion" can be assumed as a "net GSH efflux" since it is specifically induced by stimulated MRP1, as already described on membrane vesicles for verapamil and for bioflavonoids[45, 46]. In addition, an increase of extracellular GSH was measured in a concomitant way with the decrease of intracellular GSH in BHK-21-MRP1 cells treated with verapamil (unpublished results).

The values of resulting net efflux of cellular GSH are shown in Table 1. Our hit compound previously identified was the unsubstituted flavone (compound **1**) which induced a 42 % net efflux of GSH. From our previous investigations dealing with flavonoids interactions with ABC proteins[35-42, 48], we learned that the hydroxylation pattern of the A-ring was decisive. In the present study, the hydroxylation at C-5 or C-7 (compounds **2** and **6**) induces a significant increase in GSH efflux, whereas the hydroxylation at C-6 (compound **4**) is harmful for the GSH depletion.

The OH-methylation of 7-hydroxyflavone (compound **7**) leads to a decreased activity, which highlights the influence of the hydrogen bond and/or hydrophobic properties. The concomitant presence of hydroxyl groups at both C-5 and C-7 positions (compound **8,** compared to **2** and **6**) does not lead to a significant increase in GSH depletion. Interestingly, the replacement of the 5-OH or 6-OH (compounds **2** and **4**) by an amino group (compounds **3** and **5**) has no major impact on the activity. The presence of an additional hydroxyl group on the B-ring (apigenin, compound **20**) provides a less active derivative. Insertion of a hydroxyl group at the 3-position of the C-ring favors the activity, leading to one of the most active derivatives (galangin, compound **10**). The positive role of the 3-OH group is confirmed by comparing the activity of derivatives **20** and **21**.

The role of hydroxylation on the A-ring is confirmed by the screening of a series of derivatives with methoxy groups at the 5 and 7 positions and having different substituents at the 4'-position. As shown in table 1 (compounds **24, 26, 28-32**), all derivatives are less active, indicating once again the crucial role of the hydroxyl groups at 5 and 7 positions.

Finally, we set up 5 compounds (**33-38**) that have a flavonoidic skeleton and bear substituents at 4'-position that make them verapamil-like structures. Unfortunately, we observed only very low, if any, GSH efflux.

### 3.2. 3D-QSAR for GSH efflux mediated by MRP1

The 38 compounds that were screened for GSH efflux were modelled and aligned and a 3D-QSAR study with CoMSIA [20-23] was initiated. The PLS algorithm with an optimal number of component set at 4, gave a good correlation coefficient, $q^2 = 0.723$, with the LOO method and $r^2 = 0.952$ in calibration (Figure 1).

The analysis of volumes contribution (Figure 2) reveals a negative steric contribution (yellow) at both ends of the molecule, suggesting a narrow pocket for this type of compounds. The volume size is well suited for flavonoid compounds with molecular backbone deprived of heavy moieties

### 3.3. Induction of MRP1-cells death by selected flavonoids.

Compounds cytotoxicity at 20 µM was then investigated against BHK-21-wt cells and BHK-21-MRP1. Figure 3 shows that the compounds that are the most cytotoxic against BHK-21-MRP1 cells and the less against parental cells are chrysin (compound **8**) and its derivatives (compounds **11 to 15**) and the two compounds 5,7-dihydroxy-4'-fluoroflavone and 5,7-dihydroxy-4'-bromoflavone (**22** and **25** respectively). Some other compounds, 5-hydroxyflavone, 8-(1,1-DMA)-3-methoxy-chrysin and apigenin (compounds **2**, **17** and **20**

respectively) exhibit a good cytotoxicity against BHK-21-MRP1 cells but are also cytotoxic against BHK-21-wt cells to a lesser extent. Surprisingly, compounds **3,** compounds **10**, **16**-**18** (galangin and derivatives), compound **19**, compound **21** (kaempferol), compounds **23**, **24** and **32**, while inducing a significant GSH depletion, do not trigger cell death of BHK-21-MRP1 cells. Given these observations, it can be suggested that there is no strict correlation between both activities of GSH efflux and cell death. However, among compounds triggering selective cytotoxicity, GSH efflux stimulation is the pre-requisite for cell death induction. Indeed, the addition of N-acetyl-cysteine (Figure 4), a synthetic precursor of intracellular cysteine and GSH, or GSH (data not shown) in culture medium prevents cell death induced by chrysin (compound **8**) and flavonoids **11** and **22**, both strong GSH efflux inducers. This was previously shown with verapamil[6], indicating that these flavonoids would act in a similar way to verapamil regarding GSH depletion and cell death.

MRP1 has been demonstrated to be the causative agent of the selective cell death induced by verapamil[6]. Flavonoids (flavonoid **8**, **11** and **22** for the most active ones), like verapamil, are only effective on BHK-21 cells overexpressing wild-type MRP1 and not on those overexpressing MRP1 mutated in its hydrolytic site (K1333L) (Figure 5) suggesting that the functional transporter is required to induce selective cell death.

The addition of the pan-caspase inhibitor Z-VAD-FMK in culture medium prevents the death of MRP1-cells triggered by the natural compound chrysin and the flavonoid derivatives **11** and **22** after 24h of co-incubation (Figure 6). This result is in favor of an apoptotic cell death pathway induced by these flavonoids.

In order to establish valuable structure-activity relationships, the $IC_{50}$ of the compounds were determined. Only the 24 compounds that induced more than 38.5 % of net GSH depletion were tested. We included verapamil for comparison purposes and compound **38** as a negative control that does not induce GSH depletion (as shown in Table 1). Collateral sensitivity

(abbreviated as "SR" for selective ratio) was calculated for each compound as the ratio of $IC_{50}$ for parental cells divided by $IC_{50}$ for MRP1-cells. A SR greater than 2 is indicative of significant collateral sensitivity. In order to validate our proof of concept in a human chemoresistant cell line, we chose to determine SR on both human drug-sensitive cells NCI-H69 (carcinoma cells from small cell lung cancer) and drug-resistant MRP1-overexpressing cells H69AR (derived from NCI-H69 cells after 14 months of culture in the presence of increasing adriamycin concentrations), cell lines that are of better relevance in anticipation of *in vivo* studies. H69AR cells overexpress MRP1, and P-gycoprotein and BCRP were not detected by western-blot (data not shown). They are characterized by a lower basal GSH content compared to NCI-H69 (11 ± 5 nmol GSH/mg protein *versus* 39 ± 11 nmol GSH/mg protein), which results from the overexpression of MRP1, and they behave similarly to BHK-21-MRP1 cells toward GSH efflux in the presence of verapamil, which results in a near complete GSH depletion (1.5 ± 0.5 nmol GSH/mg after a 3h-exposure).

As shown in Table 2, galangin (compound **10**) and kaempferol (compound **21**) provide only weak activities on H69AR cells, as shown for BHK-21-MRP1, indicating that the absence of a hydroxyl group at position 3 seems to be an absolute requirement to obtain an active molecule. This is confirmed by results obtained with chrysin (compound **8**) which differs from galangin only by the absence of the 3-OH group and which exhibits one of the most potent activities with a high selective ratio (SR) upper than 20.5, even higher than verapamil's (Table 2). The later structural requirement is also supported by comparison of compound **16** (8-(1,1-DMA)-galangin) with compound **17 (**8-(1,1-DMA)-3-O-methylgalangin) where 3-OH is substituted by 3-OMe. Compound **16** has no cytotoxic effect on MRP1-overexpressing cells (SR = 1) while compound **17** exhibits a SR = 10. The presence of hydrophobic substituents like prenyl and geranyl does not seem to induce any additional effect on cytotoxicity (compound **8** *versus* compounds **12**, **13**, **14**, **15**). As observed for BHK-21-MRP1 cells,

galangin and derivatives (compounds **10**, **16**, **18**), kaempferol (compound **21**) and compounds **3, 19**, **23**, **24** and **32** do not trigger cell death of H69AR, while inducing a GSH depletion (Table 1 and 2 and Figure 3). In overall, when comparing both transfected cells and selected cells models, the effects of the different compounds are quite similar, even if BHK-21-wt cells are slightly more sensitive than the NCI-H69 cells.

Flavonoids are known to possess, for many of them, antioxidant capacities[49]. The absence of cytotoxicity observed for example with galangin (compound **10**) and kaempferol (compound **21**) could be explained by such properties, despite the strong GSH efflux triggered by these compounds. High reactivity of the hydroxyl groups of some flavonoids is responsible for their free radical scavenger properties [50], so they could scavenge the oxidative consequence of this GSH efflux. We checked this hypothesis by measuring the antioxidant properties of some flavonoids, as described in the Materials and Methods section. Among those exhibiting oxygen radical absorbance capacity, preventing fluorescein oxidation, some of them (compounds **8**, **11**, **25** and **27**) induce cell death and others (compounds **10**, **21** and **32**) do not (Table 3). We also determined, using the radical DPPH•, their peroxyl radical scavenger capacity. Among compounds triggering GSH depletion but no selective cell death (compounds **3**, **10**, **16**, **18**, **19**, **21**, **23**, **24**, **32**) , only galangin (compound **10**) and kaempferol (compound **21**) exhibit this capacity with an $ER_{50}$ (ratio between $EC_{50}$ and radical concentration) of 2.44 ± 0.05 and 0.36 ± 0.02 respectively, compared to 0.28 ± 0.01 determined for quercetin. Taking into account their strong scavenging capacities, we cannot exclude that the absence of cytotoxicity of galangin and kaempferol (compounds **10** and **21)** can be related to their antioxidant properties. However, some other flavonoids also show antioxidant capacities (table 3) but induce cell death.

Finally, the two flavonoid derivatives **11** and **22** and the natural compound chrysin (compound **8**) exhibit the highest SR (Table 2), despite antioxidant properties (Table 3).

Compound **11** gives the best $IC_{50}$ for cytotoxicity against H69AR (4.1 µM) (Table 2), even though it does not induce the highest GSH efflux (66%) (Table 1).

### 3.4. 3D-QSAR for cell death

The 25 initial compounds screened for cell death were modelled and aligned and a 3D-QSAR study with CoMSIA [20-23] was initiated. The establishment of a standard QSAR proving to be difficult, we chose an $IC_{50}$ of 70 µM as a cut off and compounds showing a higher $IC_{50}$ were considered to be inactive (and coded with a 100 value) while compounds showing a lower value $IC_{50}$ were considered to be active (and coded with a 0 value) (Table 4).

Analysis revealed that 3 molecules out of the 25 initial compounds were outliers. The PLS algorithm, with an optimal number of 6 components, gave a good correlation coefficient: $q^2$ = 0.803 with the LOO method and $r^2$ = 0.975 in calibration (Figure 7). All compounds were well predicted, positive or negative, with LOO methods.

The “yes or no” response of the cell death QSAR shows the difficulty to predict cellular cell death which is often multifactorial and involves different pathways. The 3D-QSAR of GSH efflux and cell death (Figures 2 and 8) reveal strong differences between the two maps of fields, particularly regarding electrostatic fields whose contributions are negative only for QSAR of cell death. This suggests that the target of offending flavonoids might not be unique. The lack of direct correlation between GSH efflux and selective cell death emphasizes this hypothesis.

### 3.5. Specificity of "cytotoxic" flavonoids for MRP1 as compared to other ABC drug transporters

We checked the specificity of the active flavonoid derivative **11** and chrysin (compound **8**) for MRP1. Thus, we tested their effect on resistant cell lines overexpressing either P-gp

(NIH3T3-P-gp and HEK-293-MDR1), MRP2 (MDCKII-MRP2) and BCRP (HEK-293-BCRP) in comparison with their respective parental cell lines (Table 5). None of these other MDR cell lines are hypersensitive to the two compounds, indicating that they selectively act on MRP1-cells.

**4. Discussion**

Cancer cells are usually treated with chemotherapeutic drugs, which unfortunately also induce cytotoxicity for non cancer cells. Many strategies are currently in development to act specifically against cellular targets only present in cancer cells. To overcome the MDR phenotype of cancer cells related to multidrug ABC transporters (P-glycoprotein, MRP1 and BCRP), different approaches are being studied. The classical one consists of using inhibitors in order to chemosensitize resistant cancer cells to chemotherapeutic drugs. A more promising trend is to exploit the phenomenon of collateral sensitivity of resistant cancer cells specifically due to the overexpression of the MDR transporters[51]. This overexpression can indeed be considered as an Achilles' heel that can be selectively targeted through this new therapeutic approach. This strategy, in addition to improving response to chemotherapy, would become a novel and specific anticancer therapy since the compounds would target resistant cancer cells that overexpress either P-glycoprotein, MRP1 or BCRP[12, 52] and (Szakacs *et al*., Chem. Rev., 2014; in press).

We describe in this work new findings about flavonoids which can be used as a new class of modulators able to selectively target chemoresistant cells overexpressing MRP1. We demonstrate for some of them a "killer" potency instead of the more traditional effects in cancer prevention. The flavonoids chrysin (compound **8**), 3-methoxy-chrysin (compound **11**) and 5,7-dihydroxy-4'-fluoroflavone (compound **22**) are more effective (SR > 20 to 24.5) than verapamil and show $IC_{50}$ of about 4 µM, which is 3-fold lower compared to that of verapamil.

Compounds **8** and **22** trigger apoptosis within MRP1-cells as shown by prevention of BHK21-MRP1 cells death using the pan-caspase inhibitor Z-VAD-FMK. These compounds would be expected to be less toxic than verapamil since polyphenolic compounds are common in the diet and have been suggested to have a number of beneficial health effects including prevention of cancer, cardiovascular disease, diabetes, and others.

Some studies showed that both VP-16 cell line, which overexpresses MRP1, and KB-V1 cell line, which overexpresses P-glycoprotein, develop a relative sensitivity to the sensitizer tiopronin of respectively 42.5-fold and 51-fold compared to the parental cell line. However, in both cases, collateral sensitivity exhibited by these MDR cell lines was not linked to interaction of tiopronin with MRP1 or P-gp, but seemed to require alterations associated with the development of MDR[13]. In another work using indomethacin, the doxorubicin-resistant MRP1-overexpressing human SCLC cell line GLC4-Adr was shown to be highly sensitive to the compound when compared to the parental line GLC4. Although GSH level was decreased by 31.5 % after 2 hours of incubation, the reduced cellular GSH level seemed not to be the primary cause of indomethacin-induced apoptosis[53]. On the contrary, we previously demonstrated that a strong GSH level diminution induced upon activation of MRP1 transport activity by verapamil[46], triggered apoptosis of MRP1-overexpressing cells[6]. When using cells overexpressing an inactive mutant of MRP1 defective for transport activity, neither GSH efflux (data known shown) nor concomitant cell death (figure 5) are observed with flavonoids, as already shown with verapamil[6], indicating an effective MRP1-dependent mechanism. This GSH efflux inducing cell death is directly dependent of MRP1-overexpression since *MRP1*-transfected cells (BHK21-MRP1 cells) show the same profile of selective sensitivity than selected MRP1-overexpressing cells (H69AR), although the two cell lines are strongly different. Furthermore, incubation with GSH or N-acetyl-cystein prevents

cell death (figure 4) showing a direct link between intracellular GSH level decrease and cell death.

Cancer cells are in a permanent oxidative stress state and one mechanism they use for hindering oxidative stress is to increase their intracellular GSH level and regulate the different components of the GSH synthesis system. Furthermore, beyond antioxidant properties of GSH, there is a strong relationship between apoptosis and GSH[54]. In this regard, several agents that target the GSH system have been developed in an attempt to improve cancer chemotherapy[55]. Our work lies with this approach. However, whereas flavonoids were used in addition to chemotherapeutic drugs to potentialize their effect[43], we describe for the first time their direct effect on resistant MRP1-cells as inducers of apoptosis. Cells overexpressing MRP1 (BHK21-MRP1 and H69AR) present a dramatically decreased intracellular GSH level in comparison to parental cells (BHK-21-wt and NCI-H69)[6]. Indeed, in the presence of verapamil, GSH content of H69AR is further reduced to a very low level, as shown in this work (results § 3.3) and as described[47], which probably induces a strong oxidative stress in the cell or acts as a critical regulator of apoptosis[54]. Modulation *via* MRP1 of cellular GSH levels would be therefore a powerful approach for cancer therapy[56]. Although depletion of intracellular GSH *via* stimulation of MRP1 transport activity by bioflavonoids was previously reported [45], we report here the first study about the structural requirements among flavonoid derivatives as selective cancer agents. Apigenin, already shown to be effective on H69AR[47], also leads to strong GSH efflux and cell death of BHK21-MRP1 cells (Table 1 and Figure 3) . Chrysin is the best natural compound with a SR higher than 20.5 and an $IC_{50}$ of 4.9 ± 3.9 µM. Chrysin has already been used for its strong stimulation of GSH efflux in MRP1-overexpressing cells, in order to potentiate the doxorubicin effect in human lung epithelial cancer[33]. From our results, this could be explained by the own cytotoxicity of chrysin against MRP1-cells. Potentiation of prooxidants toxicities towards cancer cells using

GSH depletion, induced for example by flavonoids, is already proposed as a strategy to sensitize cancer cells[43, 56] and we propose here that chrysin and derivatives could be directly used as selective anticancer agents against resistant cancer cells, their low range $IC_{50}$ being compatible with biologically achievable plasma level measured for a similar flavonoid, quercetin [57].

On the other hand, a hydroxyl group at position 3 of the flavonoid skeleton is incompatible with cytotoxicity against MRP1-overexpressing cells since galangin or kaempferol only induce GSH efflux but do not trigger cytotoxicity. This structural element is greatly valuable for further investigations of flavonoid derivatives. A strong GSH efflux, though necessary, is not sufficient for cell death induction, as also highlighted for indomethacin[53] and xanthones we previously studied[58], questioning the mechanism through which GSH depletion triggers the downstream events of apoptosis[59]. Free radical scavenger properties of galangin (compound **10**) and kaempferol (compound **21**) and probably those of galangine derivatives (compounds **16** and **18**) are strong. However, this does not totally explain their lack of selective cytotoxicity since other compounds exhibiting similar antioxidant capacities are able to induce MRP1-cells death. Furthermore, flavonoids **3**, **19**, **21**, **23**, **24** and **32** also are not cytotoxic for MRP1-cells whereas they induce GSH efflux. Therefore, it can be supposed that there would be different targets, the first one being MRP1 that leads to a huge cellular GSH depletion, while other targets, probably involving additional pathways, would lead to cell death solely when cells are enough depleted in their GSH content. The “yes or no” response of the QSAR of cell death (Figure 7) emphasizes this hypothesis. The implication of other cellular pathways in the induction of apoptosis is under investigation.

Among the flavonoid derivatives tested, compounds **11** and **22** are highly selective for cells overexpressing MRP1 as illustrated by their high SR (> 24.5 and > 20.5 respectively). The exact value of these SR could not be determined since the tested concentrations were

technically limited to 100 µM, concentration which is not toxic for control cells. They induce a collateral sensitivity towards cells overexpressing MRP1 at low concentrations ($IC_{50}$ of 4.1 and 4.9 µM respectively) and are much more powerful than tropionin ($IC_{50}$ of 290 µM)[13] or indomethacin ($IC_{50}$ 30 µM)[53], and may be considered among the best compounds described for their collateral sensitivity towards MDR cells that overexpress MRP1[13, 53, 58, 60].

In conclusion, our work brings a significant framework to consolidate the novel approach called collateral sensitivity as an emerging strategy in cancer treatment. The structural elements found here will allow to design new anticancer agents that target selectively MDR tumors overexpressing MRP1 through the induction of a strong GSH efflux, probably leading to oxidative stress. The *in vivo* efficacy of our best compounds is currently being assessed using H69AR xenografts in mice. Whereas safety is expected, as often described using dietary bioflavonoids, their cytotoxicity is being evaluated. Depending on the results, we could modify flavonoid structure while maintaining structural requirements needed for cell death induction described in this work. The relevance of this new strategy can be emphasized on the basis that the recurrent tumors often acquire MDR phenotype and that this novel anticancer therapy, which is selective for MDR tumors, can be applied upon failure of conventional chemotherapies, while being non toxic for non MRP1-overexpressing cells.

**Acknowledgments**

We thank la Région Rhône-Alpes for the doctoral fellowship accorded to E. G-B. F. L-S is thankful to Région Rhône-Alpes for the financial support during her joint PhD thesis between the University Joseph Fourier and the University of Geneva. We thank the French Ministère de l'Enseignement Supérieur et de la Recherche and the Ligue Nationale contre le Cancer for the doctoral fellowships accorded to Doriane Lorendeau and Lauriane Dury. A part of the financial support of this work was provided by grants from the French ANR and Hungarian

NIH (2010-INT-1101-01) and from the Ligue Nationale contre le Cancer (Equipe labellisée Ligue 2012). Authors are grateful to ANR (Agence Nationale pour la Recherche) for financial support (Labex Arcane (ANR-11-LABX-0003-01).

**Figure captions**

**Figure 1**. 3D-QSAR study based on GSH efflux at compound concentration of 20 µM.

**Figure 2**. CoMSIA volumes contribution of the 38 molecules submitted to the study of 3D-QSAR for GSH efflux mediated by MRP1. Volumes were plotted with 30 for negative contribution (yellow for steric and red for electrostatic fields) and 70 for positive contribution (green for steric and blue for electrostatic fields).

**Figure 3**. Cell survival of parental cells (BHK-21-wt) and MRP1-overexpressing cells (BHK-21-MRP1) in presence of 20 µM of flavonoid derivatives. Error bars represent standard deviation from three different experiments done in triplicate.

**Figure 4**. Prevention of BHK-21-MRP1 cell death induced by compounds **8**, **11** and **22** and racemic verapamil (SR-verapamil) by extracellular addition of N-acetyl-cystein. Error bars represent standard deviation from three different experiments done in triplicate.

**Figure 5**. Cell survival of parental cells (BHK-21-wt), MRP1-overexpressing cells (BHK-21-MRP1) and mutated MRP1-overexpressing cells (BHK-21-MRP1m) in presence of racemic verapamil (SR-verapamil) and compounds **8**, **11** and **22**.

**Figure 6**. Prevention of BHK-21-MRP1 cell death induced by compounds **8**, **11** and **22** and racemic verapamil (SR-verapamil) by extracellular addition of the pan-caspase inhibitor Z-VAD-FMK. Error bars represent standard deviation from three different experiments done in triplicate.

**Figure 7**. 3D-QSAR of cell death with attributed coded values (0 active/100 inactive).

**Figure 8**. CoMSIA volumes contribution of the 22 molecules submitted to the study of 3D-QSAR for cell death. Volumes were plotted with 30 for negative contribution (yellow for steric and red for electrostatic fields) and 70 for positive contribution (green for steric and blue for electrostatic fields).

**Figure captions for Figures 2 and 8 in black and white**

**Figure 2**. CoMSIA volumes contribution of the 38 molecules submitted to the study of 3D-QSAR for GSH efflux mediated by MRP1. Volumes were plotted with 30 for negative contribution (light grey for steric fields) and 70 for positive contribution (black for steric and dark grey for electrostatic fields).

**Figure 8**. CoMSIA volumes contribution of the 22 molecules submitted to the study of 3D-QSAR for cell death. Volumes were plotted with 30 for negative contribution (dark grey for electrostatic fields) and 70 for positive contribution (light grey for steric and black for electrostatic fields).

## References


[1] Glavinas H, Krajcsi P, Cserepes J, Sarkadi B. The role of ABC transporters in drug resistance, metabolism and toxicity. Curr Drug Deliv 2004;1:27-42.

[2] Deeley RG, Cole SP. Substrate recognition and transport by multidrug resistance protein 1 (ABCC1). FEBS Lett 2006;580:1103-11.

[3] Munoz M, Henderson M, Haber M, Norris M. Role of the MRP1/ABCC1 multidrug transporter protein in cancer. IUBMB Life 2007;59:752-7.

[4] Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, et al. Role of Glutathione in Cancer Progression and Chemoresistance. Oxid Med Cell Longev 2013;2013:10.

[5] Perrotton T, Trompier D, Chang XB, Di Pietro A, Baubichon-Cortay H. (R)- and (S)-verapamil differentially modulate the multidrug-resistant protein MRP1. J Biol Chem 2007;282:31542-8.

[6] Trompier D, Chang XB, Barattin R, du Moulinet D'Hardemare A, Di Pietro A, Baubichon-Cortay H. Verapamil and its derivative trigger apoptosis through glutathione extrusion by multidrug resistance protein MRP1. Cancer Res 2004;64:4950-6.

[7] Turk D, Szakacs G. Relevance of multidrug resistance in the age of targeted therapy. Curr Opin Drug Discov Devel 2009;12:246-52.

[8] Hall MD, Brimacombe KR, Varonka MS, Pluchino KM, Monda JK, Li J, et al. Synthesis and structure-activity evaluation of isatin-beta-thiosemicarbazones with improved selective activity toward multidrug-resistant cells expressing P-glycoprotein. J Med Chem 2011;54:5878-89.

[9] Hall MD, Salam NK, Hellawell JL, Fales HM, Kensler CB, Ludwig JA, et al. Synthesis, activity, and pharmacophore development for isatin-beta-thiosemicarbazones with selective activity toward multidrug-resistant cells. J Med Chem 2009;52:3191-204.

[10] Ludwig JA, Szakacs G, Martin SE, Chu BF, Cardarelli C, Sauna ZE, et al. Selective toxicity of NSC73306 in MDR1-positive cells as a new strategy to circumvent multidrug resistance in cancer. Cancer Res 2006;66:4808-15.

[11] Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219-34.

[12] Turk D, Hall MD, Chu BF, Ludwig JA, Fales HM, Gottesman MM, et al. Identification of compounds selectively killing multidrug-resistant cancer cells. Cancer Res 2009;69:8293-301.

[13] Goldsborough AS, Handley MD, Dulcey AE, Pluchino KM, Kannan P, Brimacombe KR, et al. Collateral sensitivity of multidrug-resistant cells to the orphan drug tiopronin. J Med Chem 2011;54:4987-97.

[14] Boumendjel A, Bois F, Beney C, Mariotte AM, Conseil G, Di Pietro A. B-ring substituted 5,7-dihydroxyflavonols with high-affinity binding to P-glycoprotein responsible for cell multidrug resistance. Bioorg Med Chem Lett 2001;11:75-7.

[15] Yao N, Song A, Wang X, Dixon S, Lam KS. Synthesis of flavonoid analogues as scaffolds for natural product-based combinatorial libraries. J Comb Chem 2007;9:668-76.

[16] Cholody WM, Martelli S, Konopa J. 8-Substituted 5-[(aminoalkyl)amino]-6H-v-triazolo[4,5,1-de]acridin-6-ones as potential antineoplastic agents. Synthesis and biological activity. J Med Chem 1990;33:2852-6.

[17] Huang D, Ou B, Hampsch-Woodill M, Flanagan JA, Prior RL. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J Agric Food Chem 2002;50:4437-44.

[18] Nurisso A, Simoes-Pires C, Martel S, Cressend D, Guillot A, Carrupt PA. How to increase the safety and efficacy of compounds against neurodegeneration? A multifunctional approach. Chimia (Aarau) 2012;66:286-90.

[19] Ancerewicz J, Migliavacca E, Carrupt P-A, Testa B, Brée F, Zini R, et al. Structure–Property Relationships of Trimetazidine Derivatives and Model Compounds as Potential Antioxidants. Free Radic Biol Med 1998;25:113-20.

[20] Allen MS, Tan YC, Trudell ML, Narayanan K, Schindler LR, Martin MJ, et al. Synthetic and computer-assisted analyses of the pharmacophore for the benzodiazepine receptor inverse agonist site. J Med Chem 1990;33:2343-57.

[21] Cramer RD, Patterson DE, Bunce JD. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. J Am Chem Soc 1988;110:5959-67.

[22] Cramer RD, 3rd, Patterson DE, Bunce JD. Recent advances in comparative molecular field analysis (CoMFA). Prog Clin Biol Res 1989;291:161-5.

[23] Marshall GR, Cramer RD, 3rd. Three-dimensional structure-activity relationships. Trends Pharmacol Sci 1988;9:285-9.

[24] Chang XB, Hou YX, Riordan JR. ATPase activity of purified multidrug resistance-associated protein. J Biol Chem 1997;272:30962-8.

[25] Hou Y, Cui L, Riordan JR, Chang X. Allosteric interactions between the two non-equivalent nucleotide binding domains of multidrug resistance protein MRP1. J Biol Chem 2000;275:20280-7.

[26] Mirski SE, Gerlach JH, Cole SP. Multidrug resistance in a human small cell lung cancer cell line selected in adriamycin. Cancer Res 1987;47:2594-8.

[27] Cardarelli CO, Aksentijevich I, Pastan I, Gottesman MM. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. Cancer res 1995;55:1086-91.

[28] Robey RW, Honjo Y, Morisaki K, Nadjem TA, Runge S, Risbood M, et al. Mutations at amino-acid 482 in the ABCG2 gene affect substrate and antagonist specificity. Br J Cancer 2003;89:1971-8.

[29] Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LC, et al. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. J Clin Invest 1998;101:1310-9.

[30] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.

[31] Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. Anal Biochem 1969;27:502-22.

[32] Anderson ME. Determination of glutathione and glutathione disulfide in biological samples. Methods Enzymol 1985;113:548-55.

[33] Brechbuhl HM, Kachadourian R, Min E, Chan D, Day BJ. Chrysin enhances doxorubicin-induced cytotoxicity in human lung epithelial cancer cell lines: The role of glutathione. Toxicol Appl Pharm 2012;258:1-9.

[34] Chahar MK, Sharma N, Dobhal MP, Joshi YC. Flavonoids: A versatile source of anticancer drugs. Pharmacogn Rev 2011;5:1-12.

[35] Boumendjel A, Di Pietro A, Dumontet C, Barron D. Recent advances in the discovery of flavonoids and analogs with high-affinity binding to P-glycoprotein responsible for cancer cell multidrug resistance. Med Res Rev 2002;22:512-29.

[36] Conseil G, Baubichon-Cortay H, Dayan G, Jault JM, Barron D, Di Pietro A. Flavonoids: a class of modulators with bifunctional interactions at vicinal ATP- and steroid-binding sites on mouse P-glycoprotein. Proc Natl Acad Sci U S A 1998;95:9831-6.

[37] Conseil G, Decottignies A, Jault JM, Comte G, Barron D, Goffeau A, et al. Prenyl-flavonoids as potent inhibitors of the Pdr5p multidrug ABC transporter from Saccharomyces cerevisiae. Biochemistry 2000;39:6910-7.

[38] Di Pietro A, Conseil G, Perez-Victoria JM, Dayan G, Baubichon-Cortay H, Trompier D, et al. Modulation by flavonoids of cell multidrug resistance mediated by P-glycoprotein and related ABC transporters. Cell Mol Life Sci 2002;59:307-22.

[39] Boumendjel A, Baubichon-Cortay H, Trompier D, Perrotton T, Di Pietro A. Anticancer multidrug resistance mediated by MRP1: recent advances in the discovery of reversal agents. Med Res Rev 2005;25:453-72.

[40] Trompier D, Baubichon-Cortay H, Chang XB, Maitrejean M, Barron D, Riordon JR, et al. Multiple flavonoid-binding sites within multidrug resistance protein MRP1. Cell Mol Life Sci 2003;60:2164-77.

[41] Ahmed-Belkacem A, Pozza A, Munoz-Martinez F, Bates SE, Castanys S, Gamarro F, et al. Flavonoid structure-activity studies identify 6-prenylchrysin and tectochrysin as potent and specific inhibitors of breast cancer resistance protein ABCG2. Cancer Res 2005;65:4852-60.

[42] Arnaud O, Boumendjel A, Geze A, Honorat M, Matera EL, Guitton J, et al. The acridone derivative MBLI-87 sensitizes breast cancer resistance protein-expressing xenografts to irinotecan. Eur J cancer 2011;47:640-8.

[43] Kachadourian R, Day BJ. Flavonoid-induced glutathione depletion: potential implications for cancer treatment. Free Radic Biol Med 2006;41:65-76.

[44] Kachadourian R, Leitner HM, Day BJ. Selected flavonoids potentiate the toxicity of cisplatin in human lung adenocarcinoma cells: a role for glutathione depletion. Int J Oncol 2007;31:161-8.

[45] Leslie EM, Deeley RG, Cole SP. Bioflavonoid stimulation of glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). Drug Metab Dispos 2003;31:11-5.

[46] Loe DW, Deeley RG, Cole SP. Verapamil stimulates glutathione transport by the 190-kDa multidrug resistance protein 1 (MRP1). J Pharmacol Exp Ther 2000;293:530-8.

[47] Laberge RM, Karwatsky J, Lincoln MC, Leimanis ML, Georges E. Modulation of GSH levels in ABCC1 expressing tumor cells triggers apoptosis through oxidative stress. Biochem Pharmacol 2007;73:1727-37.

[48] Boumendjel A, Macalou S, Valdameri G, Pozza A, Gauthier C, Arnaud O, et al. Targeting the multidrug ABCG2 transporter with flavonoidic inhibitors: in vitro optimization and in vivo validation. Curr Med Chem 2011;18:3387-401.

[49] Nijveldt RJ, van Nood E, van Hoorn DE, Boelens PG, van Norren K, van Leeuwen PA. Flavonoids: a review of probable mechanisms of action and potential applications. Am J Clin Nutr 2001;74:418-25.

[50] van Acker SA, van den Berg DJ, Tromp MN, Griffioen DH, van Bennekom WP, van der Vijgh WJ, et al. Structural aspects of antioxidant activity of flavonoids. Free Radic Biol Med 1996;20:331-42.

[51] Szakacs G, Annereau JP, Lababidi S, Shankavaram U, Arciello A, Bussey KJ, et al. Predicting drug sensitivity and resistance: profiling ABC transporter genes in cancer cells. Cancer Cell 2004;6:129-37.

[52] Hall MD, Handley MD, Gottesman MM. Is resistance useless? Multidrug resistance and collateral sensitivity. Trends Pharmacol Sci 2009;30:546-56.

[53] de Groot DJ, van der Deen M, Le TK, Regeling A, de Jong S, de Vries EG. Indomethacin induces apoptosis via a MRP1-dependent mechanism in doxorubicin-resistant small-cell lung cancer cells overexpressing MRP1. Br J Cancer 2007;97:1077-83.

[54] Franco R, Cidlowski JA. Apoptosis and glutathione: beyond an antioxidant. Cell Death Differ 2009;16:1303-14.

[55] Singh S, Khan AR, Gupta AK. Role of glutathione in cancer pathophysiology and therapeutic interventions. J Exp Ther Oncol 2012;9:303-16.

[56] Leitner HM, Kachadourian R, Day BJ. Harnessing drug resistance: Using ABC transporter proteins to target cancer cells. Biochem Pharmacol 2007;74:1677-85.

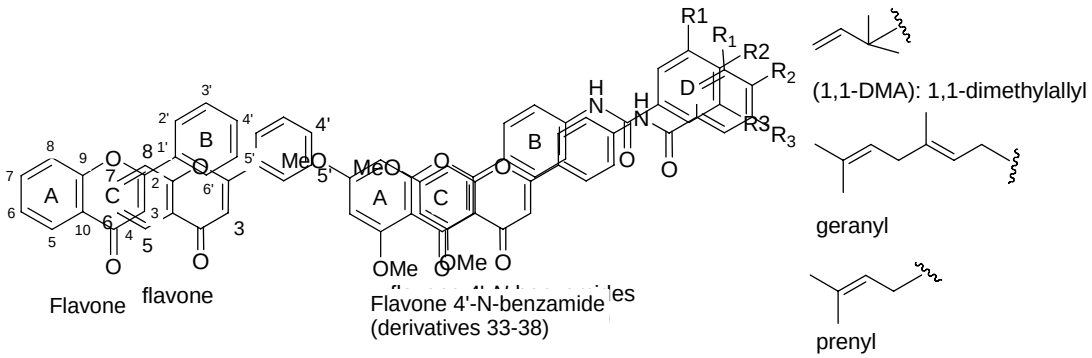
[57] Manach C, Morand C, Crespy V, Demigne C, Texier O, Regerat F, et al. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. FEBS Lett 1998;426:331-6.

[58] Genoux-Bastide E, Lorendeau D, Nicolle E, Yahiaoui S, Magnard S, Di Pietro A, et al. Identification of Xanthones as Selective Killers of Cancer Cells Overexpressing the ABC Transporter MRP1. ChemMedChem 2011;6:1478-84.

[59] Coppola S, Ghibelli L. GSH extrusion and and the mitochondrial pathway of apoptotic signalling. Biochem Soc trans 2000;28:56-61.

[60] Barattin R, Perrotton T, Trompier D, Lorendeau D, Di Pietro A, d'Hardemare Adu M, et al. Iodination of verapamil for a stronger induction of death, through GSH efflux, of cancer cells overexpressing MRP1. Bioorg Med Chem 2010;18:6265-74.

**Table 1**. Structures of studied compounds and effect on cellular GSH efflux in BHK-21 cells.




| Compound | Compound name | CLogP | Net GSH efflux at 20 µM (%) |
|---|---|---|---|
| 1 | flavone | 3.74 | 42.4 |
| 2 | 5-hydroxyflavone | 3.47 | 63.8 |
| 3 | 5-aminoflavone | 3.17 | 61.3 |
| 4 | 6-hydroxyflavone | 3.23 | 16.7 |
| 5 | 6-aminoflavone | 2.79 | 11.7 |
| 6 | 7-hydroxyflavone | 3.23 | 76.3 |
| 7 | 7-methoxyflavone | 3.77 | 42.2 |
| 8 | chrysin (5,7-dihydroxyflavone) | 2.94 | 76.5 |
| 9 | 5-hydroxy-7-ethoxyflavone | 3.85 | 39.3 |
| 10 | galangin (3,5,7-trihydroxyflavone) | 2.65 | 84.6 |
| 11 | 3-methoxy-chrysin | 2.93 | 65.6 |
| 12 | 6-prenyl-chrysin | 5.19 | 46.1 |
| 13 | 6-geranyl-chrysin | 7.04 | 58.0 |
| 14 | 8-prenyl-chrysin | 5.19 | 46.2 |
| 15 | 8-geranyl-chrysin | 7.04 | 52.1 |
| 16 | 8-(1,1-DMA)-galangin | 4.84 | 59.2 |
| 17 | 8-(1,1-DMA)-3-methoxy-chrysin | 5.12 | 43.2 |
| 18 | 8-prenyl-galangin | 4.9 | 39.9 |
| 19 | 8-prenyl-3-methoxy-chrysin | 5.18 | 57.6 |
| 20 | apigenin (5,7,4’-trihydroxyflavone) | 2.46 | 43 |
| 21 | kaempferol (3,5,7,4’-tetrahydroxyflavone) | 2.17 | 72.5 |
| 22 | 5,7-dihydroxy-4'-fluoroflavone | 3.11 | 74.5 |
| 23 | 5-hydroxy-7-methoxy-4'-fluoroflavone | 3.64 | 42.8 |
| 24 | 5,7-dimethoxy-4’-fluoroflavone | 3.92 | 43.8 |
| 25 | 5,7-dihydroxy-4’-bromoflavone | 3.75 | 47.4 |
| 26 | 5,7-dimethoxy-4’-ethoxyflavone | 3.65 | 26.7 |
| 27 | 5,7-dihydroxy-4’-tertiobutylflavone | 4.65 | 38.5 |
| 28 | 5,7-dimethoxy-4’-tertiobutylflavone | 5.46 | 0 |
| 29 | 5,7-dimethoxy-4’-isopropylflavone | 5.27 | 12.4 |
| 30 | 5,7-dimethoxy-6-iodo-4’-isopropylflavone | 6.28 | 15 |
| 31 | 5,7-dimethoxy-4’-nitroflavone | 3.71 | 0 |

| | | | |
|---|---|---|---|
| 32 | 5,7-dimethoxy-4'-aminoflavone | 2.83 | 49.3 |
| 33 | $R_1$ = F, $R_2$ = H, $R_3$ = F | 5.56 | 0,9 |
| 34 | $R_1$ = OMe, $R_2$ = H, $R_3$ = H | 5.73 | 15.1 |
| 35 | $R_1$ = H, $R_2$ = Et, $R_3$ = H | 4.68 | 0 |
| 36 | $R_1$ = F, $R_2$ = F, $R_3$ = H | 4.29 | 1.9 |
| 37 | $R_1$ = H, $R_2$ = I, $R_3$ = H | 4.9 | 15.1 |
| 38 | $R_1$ = OMe, $R_2$ = OMe, $R_3$ = H | 4.9 | 5.4 |

[a]Intracellular GSH level was measured in parental cells **(**BHK-21-wt) and *mrp1*-transfected cells (BHK-21-MRP1), after a 3h-incubation in absence or presence of compounds at 20 µM. The percentage of GSH efflux induced by the compounds was first determined in each cell line and the specific effect of each compound on the *mrp1*-transfected cells (net GSH efflux) was obtained after correction from the effect observed on parental cells.

**Table 2.** Cytotoxicity of flavonoid derivatives against MRP1-overexpressing cells (H69AR) and parental cells (NCI-H69).

| **Compound** | **$IC_{50}$ (µM)[a] H69AR** | **$IC_{50}$ (µM)[a] NCI H69** | **SR[c]** |
|---|---|---|---|
| **1** | 29.9 ± 12.7[b] | > 100 | > 3.3 |
| **2** | 23.2 ± 8.3 | > 100 | > 4.3 |
| **3** | > 100 | > 100 | 1 |
| **6** | 17,9 ± 8.9 | > 100 | > 5.6 |
| **7** | 16.8 ± 9.7 | > 100 | > 5.9 |
| **8** | 4.9 ± 3.9 | > 100 | > 20.4 |
| **10** | > 100 | > 100 | 1 |
| **11** | 4.1 ± 2.7 | > 100 | > 24.5 |
| **12** | 8.3 ± 7.8 | > 100 | > 12 |
| **13** | 14.1 ± 8.1 | > 100 | > 7.1 |
| **14** | 9.2 ± 6.1 | 99.9 | 10.8 |
| **15** | 8.3 ± 5.8 | > 100 | > 12.1 |
| **16** | > 100 | > 100 | 1 |
| **17** | 5.7 ± 4 | 59.2 ± 2.3 | 10.3 |
| **18** | > 100 | > 100 | 1 |
| **19** | > 100 | > 100 | 1 |
| **20** | 9.9 ± 4.7 | > 100 | > 10 |
| **21** | > 100 | > 100 | 1 |
| **22** | 4.9 ± 2.6 | > 100 | > 20.5 |

| | | | |
|---|---|---|---|
| **23** | > 100 | > 100 | 1 |
| **24** | 71 ± 22.6 | 81.9 ± 12 | 1.1 |
| **25** | 7.1 ± 0.1 | 68.6 ± 0.9 | 9.5 |
| **27** | 19.6 ± 2.6 | 43.3 ± 3.2 | 2.2 |
| **32** | > 100 | > 100 | 1 |
| **38** | > 100 | > 100 | 1 |
| **SR-verapamil** | 6.7 ± 4 | > 100 | > 14.8 |

[a]$IC_{50}$ values (concentration required to obtain 50% of the maximal effect) were calculated from dose-response curves. [b]Data are the mean ± SD of at least three independent experiments. [c]The hypersensitivity of MRP1-overexpressing cells was designed by the selective ratio (SR) calculated as the ratio between $IC_{50}$ against parental cells and $IC_{50}$ against MRP1-overexpressing cells for each compound.

**Table 3**. Anti-oxidant activities of flavonoids compared to SR.

| **Compound** | **SR** | **Activity (%)[a,b] at 10 µM** | **$EC_{50}$ (µM)[c]** |
|---|---|---|---|
| **Quercetin** | - | 98.4 ± 8.5 | 0.34 ± 0.04 |
| **1** | > 3.3 | 3 ± 3 | - |
| **6** | > 5.6 | 43 ± 1 | - |
| **7** | > 5.9 | 0 ± 5 | - |
| **8** | > 20.4 | 75 ± 26 | 3.03 ± 0.21 |
| **10** | 1 | 97.3 ± 8 | 1.43 ± 0.04 |
| **11** | > 24.5 | 91 ± 4 | 3.49 ± 0.38 |
| **21** | 1 | 98.1 ± 9.6 | 0.58 ± 0.06 |
| **25** | 9.5 | 86 ± 1 | 3.55 ± 0.25 |
| **27** | 2.2 | 69 ± 6 | 4.89 ± 0.12 |
| **32** | 1 | 88 ± 1 | 2.26 ± 0.32 |
| **SR-verapamil** | > 14.8 | 16 ± 5 | - |

[a]Percentage of non-oxidized fluorescein after expoxure to the free radical generator 2,2'-azobis (2-amidinopropane chloride) (AAPH). [b]For each compound with a percentage of fluorescein protection activity higher than 50%, the $EC_{50}$ value was determined by a dose-response curve of at least 6 concentrations. [c]$EC_{50}$ value obtained from three independent experiments in triplicate.

**Table 4.** Cut-off attribution for 3D-QSAR analysis for cell death

| **Compound** | Active (0)[a] Inactive (100) | **Compound** | Active (0) Inactive (100) |
|---|---|---|---|
| **1** | 0 | **21** | 100 |
| **2** | 0 | **24** | 100 |
| **6** | 0 | **25** | 0 |
| **8** | 0 | **27** | 0 |
| **10** | 100 | **32** | 100 |
| **14** | 0 | **38** | 100 |
| **15** | 0 | **12** | 0 |
| **16** | 100 | **13** | 0 |
| **17** | 0 | **11** | 0 |
| **18** | 100 | **22** | 0 |
| **20** | 0 | **23** | 100 |

[a]An $IC_{50}$ of 70 µM was chosen as the cut off below which compounds were considered as active and above which compounds were considered as inactive.

**Table 5**. Cytotoxicity of compound **11** and chrysin (compound **8**) against P-gp-, BCRP- and MRP2- overexpressing cell lines.

| **Cell lines** | Compound **11** **$IC_{50}$ (µM)** | Chrysin **$IC_{50}$ (µM)** |
|---|---|---|
| NIH3T3 | 60 | 70 |
| NIH3T3-P-gp | 60 | 70 |
| HEK-293 | 100 | > 100 |
| HEK293-MDR1 | 100 | > 100 |
| HEK-293 | 100 | 105 |
| HEK293-BCRP | 100 | 100 |
| MDCKII | > 100 | > 100 |
| MDCII-MRP2 | > 100 | > 100 |

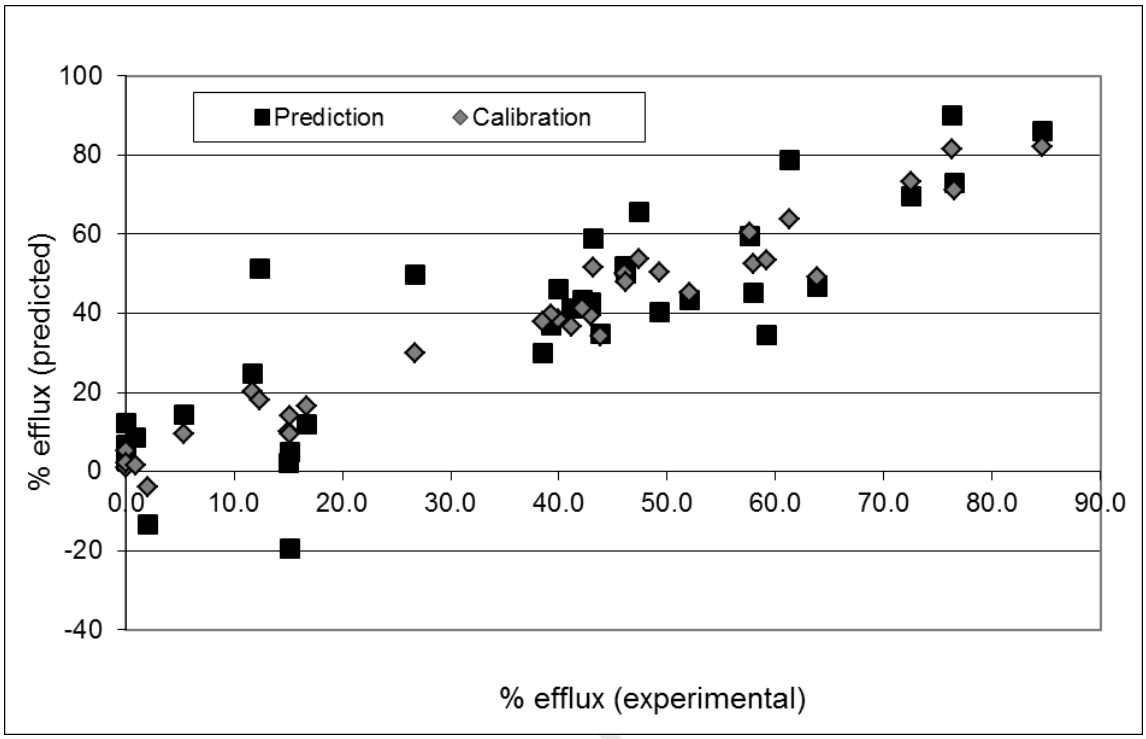



**Figure 1**

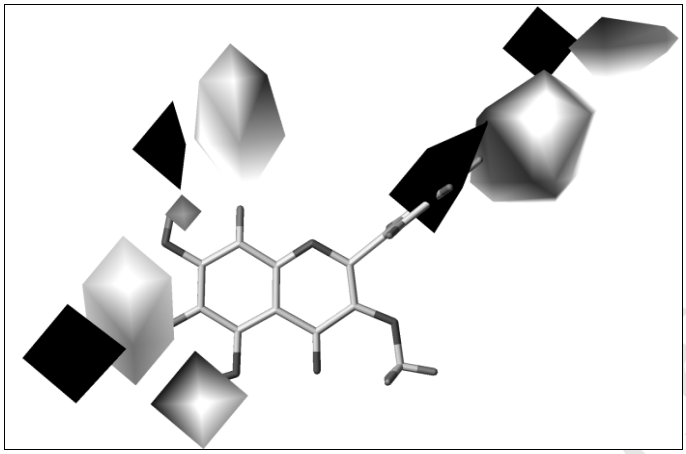


**Figure 2**

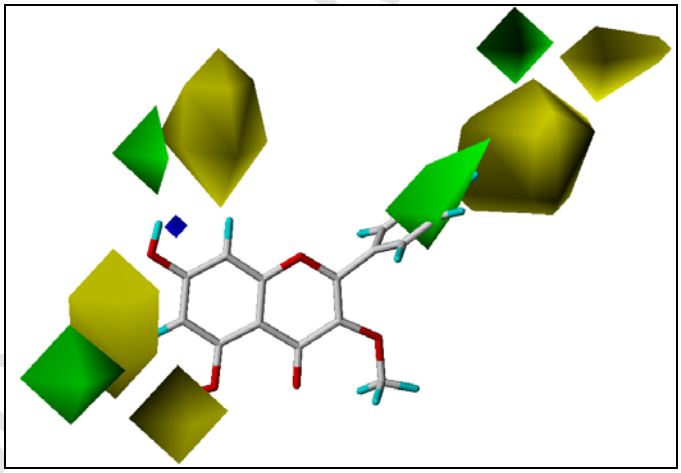


**Figure 2**

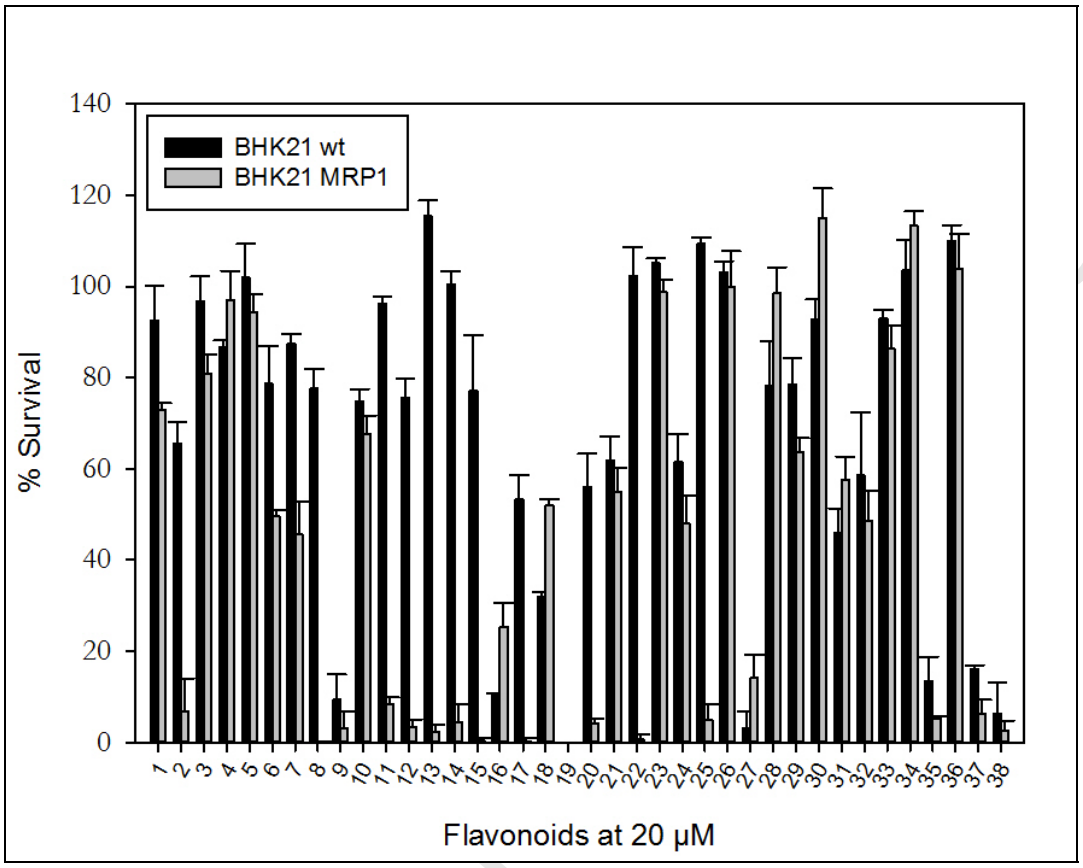



**Figure 3**

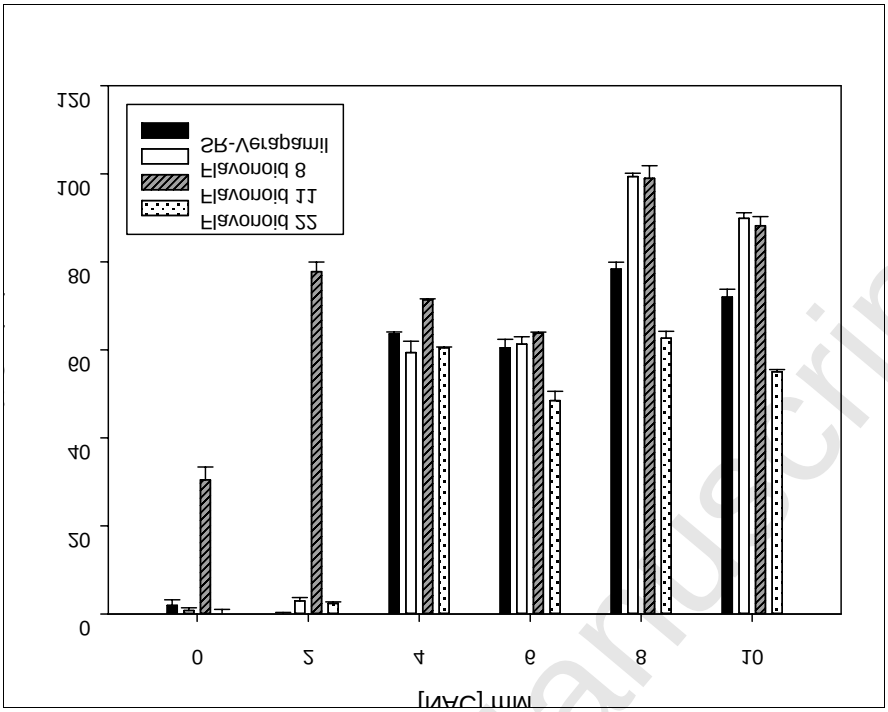



**Figure 4**

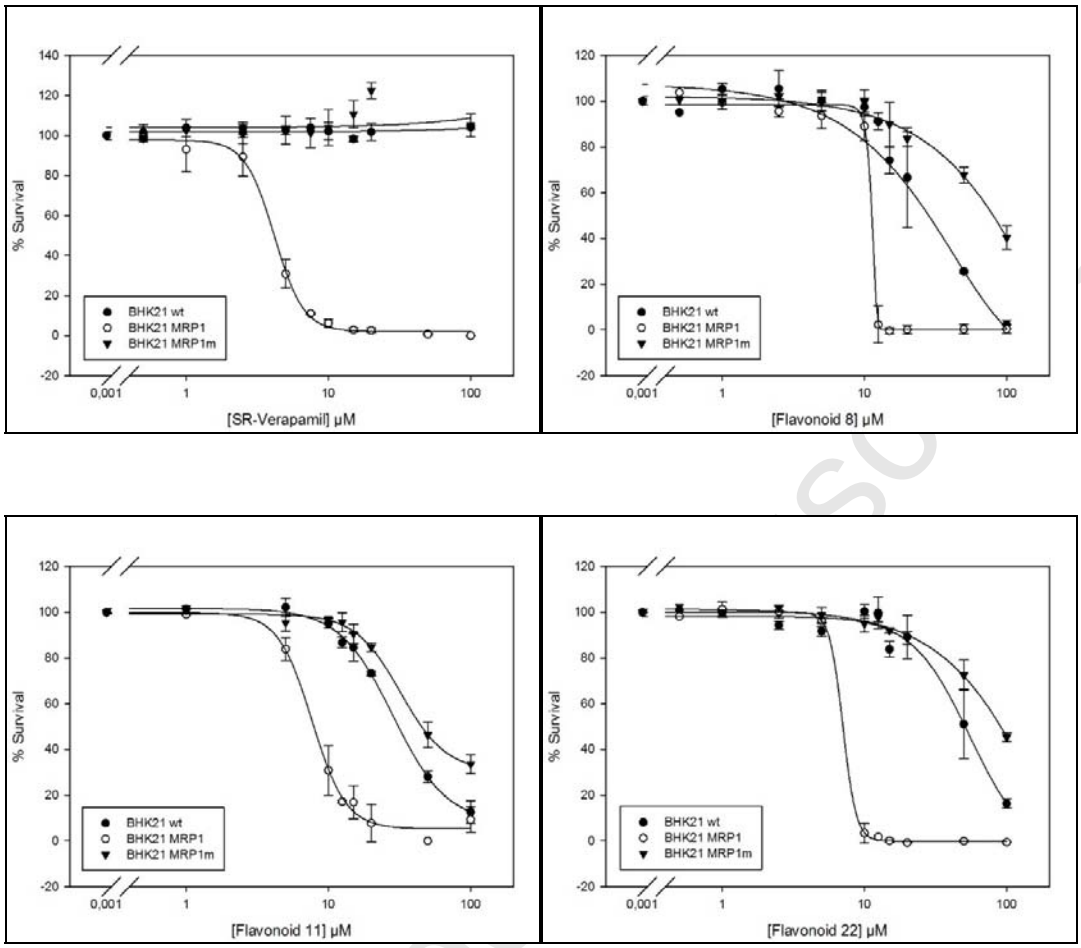



**Figure 5**

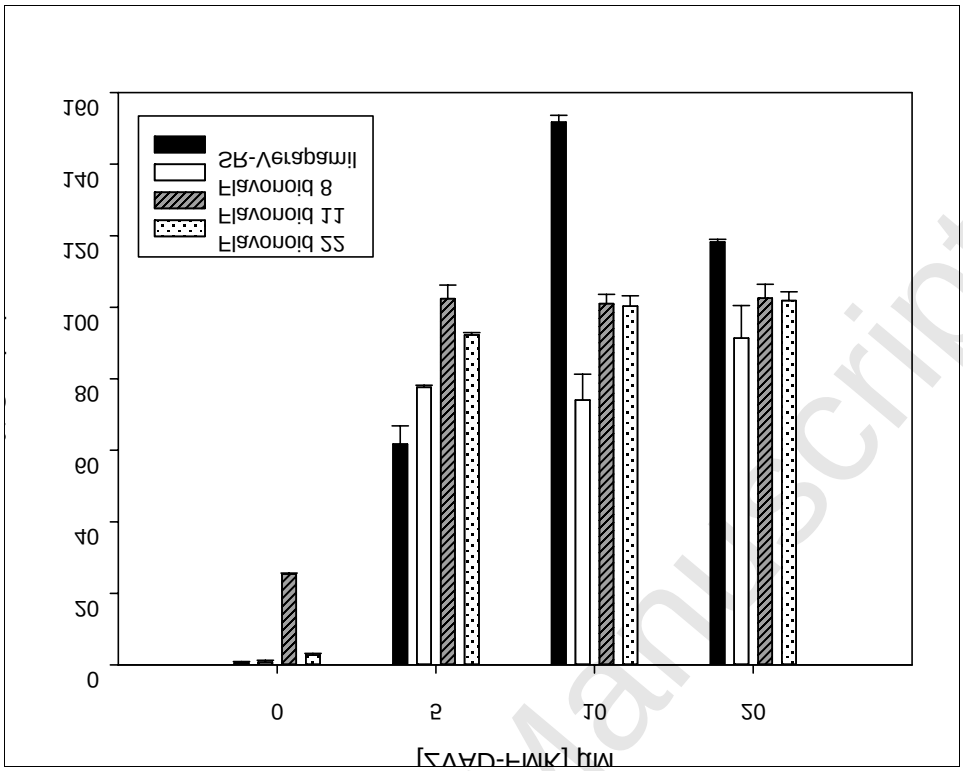



**Figure 6**

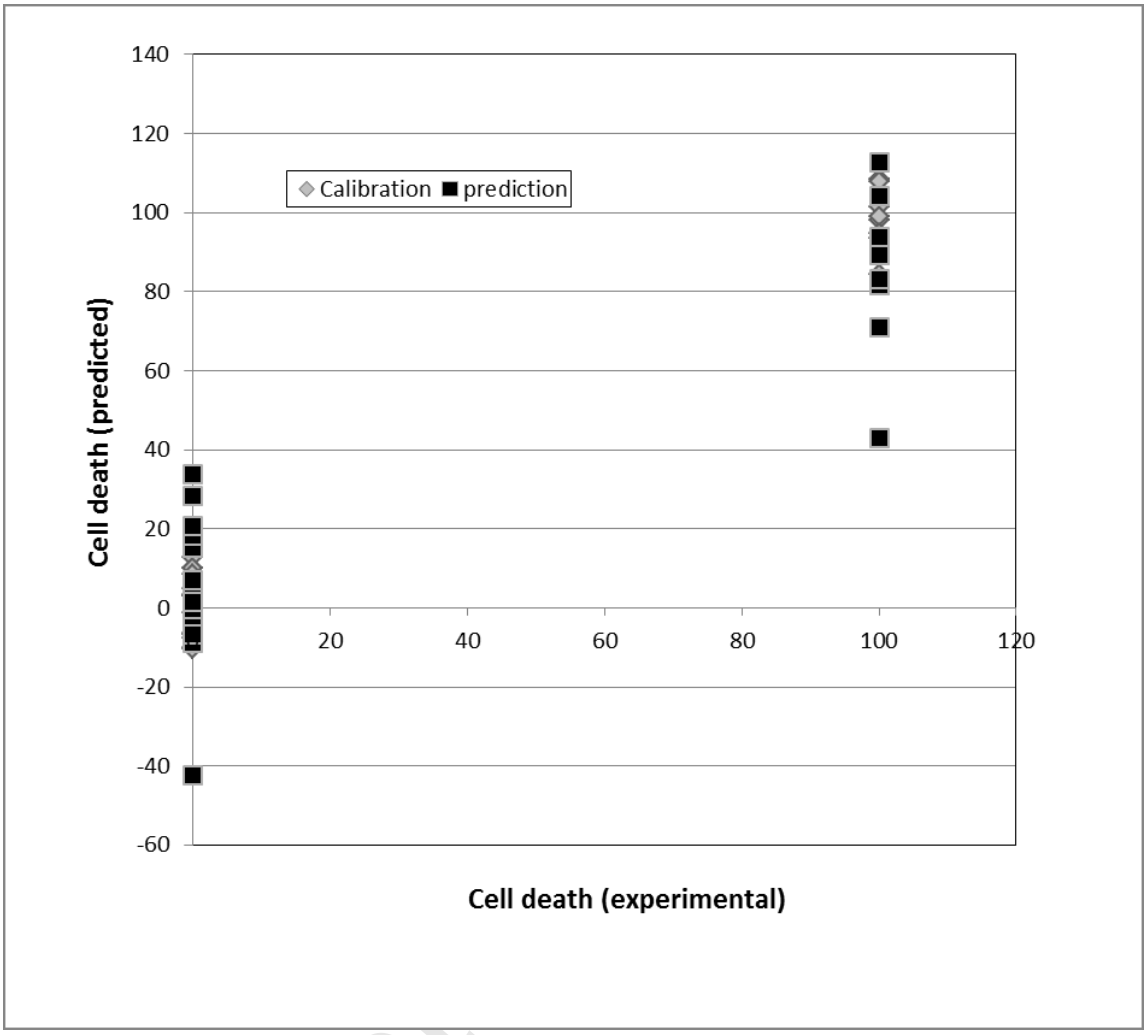



**Figure 7**

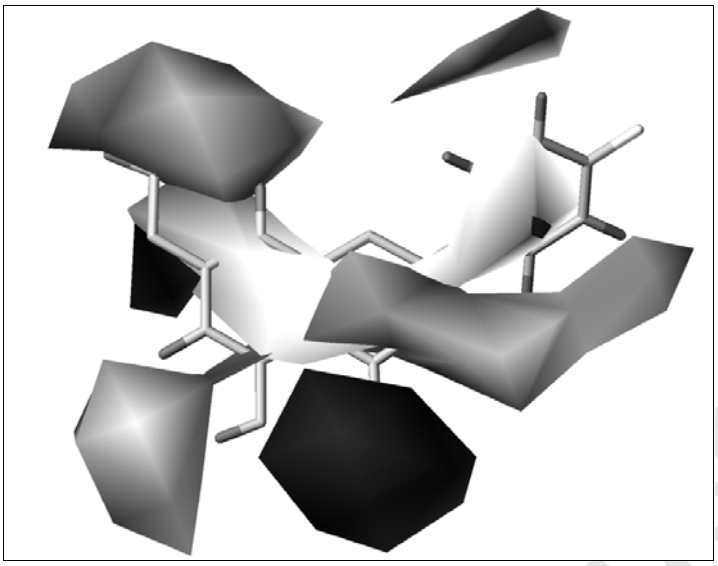


**Figure 8**

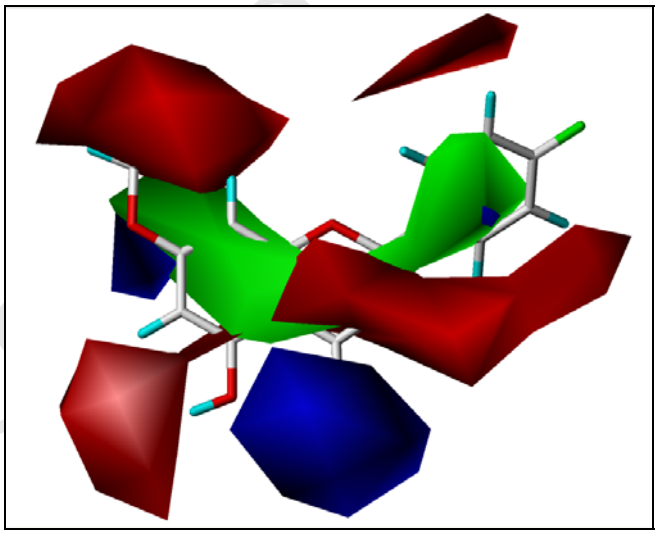


**Figure 8**

*Graphical Abstract (for review)