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# Synthesis and SAR Study of Novel Anticancer Protoflavone Derivatives – Investigation of Cytotoxicity and Interaction with the ABCB1 and ABCG2 Multidrug Efflux Transporters

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## Abstract

There is a constant need for new therapies against multidrug resistant (MDR) cancer. Natural compounds represent a promising class of novel anticancer agents. Recently, we have shown that protoflavones display activity in multidrug resistant cancer cell lines overexpressing the drug efflux pump P-glycoprotein. In the present study, 52 protoflavones, including 22 new derivatives were synthesized and tested against a panel of sensitive parental cells and their MDR derivatives obtained by transfection with the human *ABCB1* or *ABCG2* genes, or by adaptation to chemotherapeutics. With the exception of protoapigenone, identified as a weak ABCG2 substrate, all protoflavones bypass resistance conferred by these two transporters. The majority of the compounds exhibited mild to strong (up to 13 fold) selectivity against the MCF-$7_{Dox}$ and KB-V1 cell lines, but not to transfected MDR cells engineered to overexpress the MDR transporters. Our results suggest that protoflavones can overcome cancer multidrug resistance by evading efflux by P-glycoprotein.

Keywords: protoflavone, MDR cancer, collateral sensitivity / cross-resistance, ABCB1 / P-glycoprotein, ABCG2 / BCRP

## Introduction

Cancer is among the leading causes of morbidity and mortality worldwide. According to the World Cancer Report, 8.2 million cancer-related deaths were registered in 2012, and it is expected that annual cancer cases will rise from 14 million in 2012 to around 22 million within the next two decades.[1] Resistance to chemotherapy and molecularly targeted therapies is a major problem facing current cancer research.[2] Despite the availability of a broad range of diverse anticancer compounds with new mechanisms and molecular targets, cancer is often incurable due to the development of drug resistance.[3] Resistance can rapidly develop even in cases when the tumor initially responds to chemotherapy. Multi-drug resistance (MDR) can emerge as a result of reduced uptake or increased efflux of cytostatic agents – the latter is mediated by ATP-binding-cassette (ABC) proteins, primarily by P-glycoprotein (P-gp; ABCB1) and ABCG2, which confer resistance to a wide variety of compounds.[4],[5] There is a constant need for novel chemotherapeutics with marked and selective antitumor activity that can overcome resistance mediated by these transporters. The unfavorable prognostic impact of P-glycoprotein expression in several cancers has prompted overwhelming research efforts aimed at the clinical development of high affinity efflux inhibitors that were shown to overcome MDR in in vitro models. Unfortunately, even after decades of intensive research, a clinically effective inhibitor has not been identified. Recently, the discussion has shifted to alternative strategies, either to bypass the transporters or to exploit the collateral sensitivity (CS) of MDR cells.[6] Recent discoveries have shown that it is possible to invert the selective advantage of resistant cells to reverse the evolution of resistance.[7] For example, MDR-selective compounds were shown to specifically target ABC transporter over-expressing MDR cancer cells by exploiting the Achilles' heel conferred by the overexpression of the transporters.[8],[9],[10]

Our review of the literature identified several natural compounds that were reported to elicit preferential toxicity against MDR cells.[6] For example, the 4′-hydroxyflavone apigenin was identified in a screen as a specific killer of drug-selected H69AR cells and MRP1-transfected HeLa cells.[11] Flavonoids are naturally derived compounds that display both anti- and prooxidant properties. Flavonoids have been used in cancer chemoprevention and chemotherapy. A particularly interesting, rare group of natural flavonoids with a high antitumor potential contains protoflavones. Typically derived from ferns, protoflavones contain a non-aromatic, usually *p*-quinol B-ring or its di- or tetrahydro derivative. Based on the most frequently occurring chemical nomenclature, herein we refer to the flavone skeleton containing a 1′-OH group and a 2′,5′-dien-4′-one moiety in its B-ring as the "protoflavone" skeleton. Protoflavones can formally be derived from 4′-hydroxyflavones, like apigenin (**1**), and some, e.g. protoapigenone (**2**), the protoflavone analog of **1**, have been described as potent anticancer agents *in vitro* and *in vivo*. We have recently reviewed the chemistry and bioactivity of protoflavones.[12] The proapoptotic activity of protoflavones is mediated by oxidative stress[13] and the inhibition of ATR-dependent signaling.[14] We have previously shown that 6-methylated protoflavone derivatives exert mild selective cytotoxicity against a murine lymphoma cell line transfected with the human ABCB1 transporter, while other protoflavones, derived from apigenin, genkwanin or β-naphthoflavone, did not exhibit such a selectivity.[15] Furthermore, protoapigenone and its 1′-*O*-butyl- and propargylether, the β-naphthoflavone analog WYC0209, 6-methylprotoflavone and 6-bromoprotoflavone showed selective

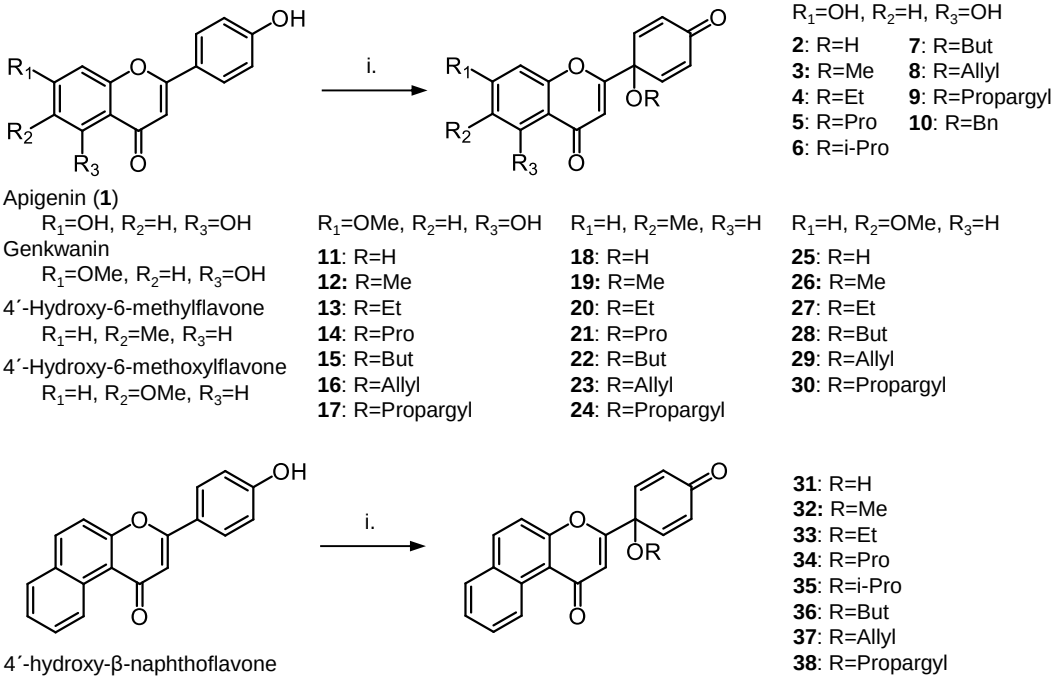
cytotoxicity against certain MDR cancer cell lines, such as NCI-H460 human non-small cell lung carcinoma cells adapted to doxorubicin, U87 human glioma and DLD1 human colorectal cells, both adapted to paclitaxel.[16] On the other hand, cross-resistance (CR) to protoflavones was observed in C6 rat glioma cells adapted to carmustine, and CS/CR pattern appeared to be in line with altered antioxidative capacity of the MDR cells as compared to their parental cell lines.[16]

Based on these results our aim was to systematically explore the cytotoxicity and antitumor potential of further protoflavone derivatives. In particular, we characterized the anticancer activity of a total of 52 compounds in a diverse panel of cancer cell lines including MDR derivatives expressing ABCB1 or ABCG2.

## Results

Thirty-seven protoflavones and protoflavone 1′-*O*-alkyl ethers were synthesized from apigenin (**1**), genkwanin, 4′-hydroxy-6-methylflavone, 4′-hydroxy-6-methoxyflavone and 4′-hydroxy-β-naphthoflavone, based on the synthetic route we have previously published for compounds **2-9**,[17] **11-24**[15] and **31-38**.[17] Briefly, an oxidative de-aromatization was performed by a common hypervalent iodine reagent, [*bis*(trifluoroacetoxy)iodo]benzene (PIFA) in acetonitrile in the presence of water or the alcohol to be coupled at position C-1′. Among these compounds, protoapigenone 1′-*O*-benzylether (**10**) and the 6-methoxylated derivatives (**25-30**) were obtained as new protoflavones; synthesis and structures of the compounds are presented in Scheme 1.

**Scheme 1.** Synthesis of protoflavones from commercially available 4′-hydroxyflavones[a]

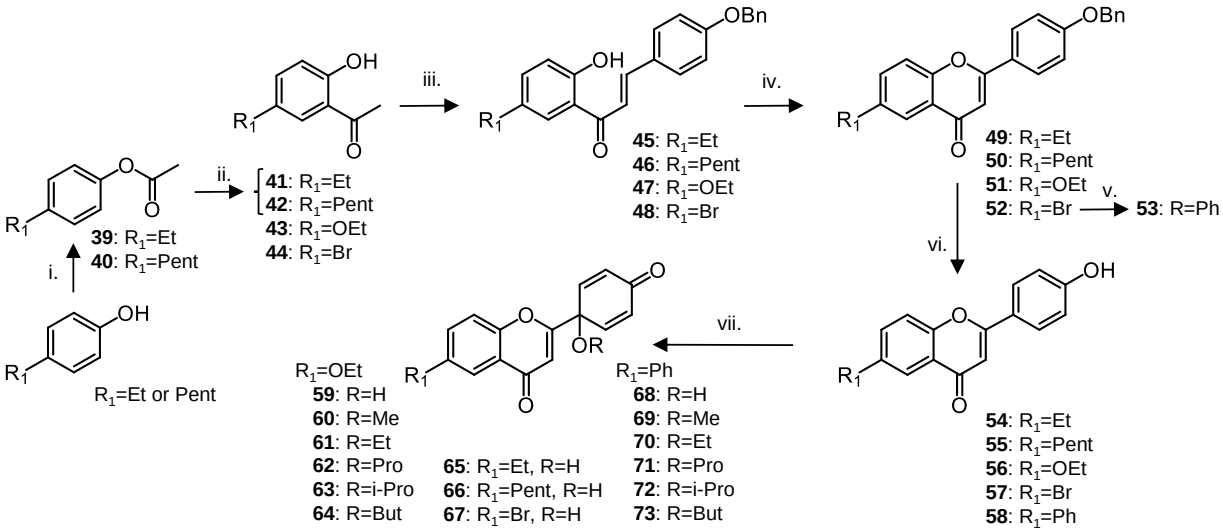



[a] Reagents: (i.) $CH_3CN$/ROH 9/1, PIFA (2 eq).

Total synthesis of a set of various 6-substituted protoflavones was achieved in 4-6 steps. In order to obtain starting materials (*i.e.* 5′-ethyl-2′-hydroxyacetophenone, **41**; and 5′-pentyl-2′-hydroxyacetophenone; **42**) for our 6-ethyl and 6-pentyl substituted target compounds, the

appropriate *p*-substituted phenols were acetylated and subjected to Fries-rearrangement reaction under the condition of dry $AlCl_3$ in dichloromethane.[18] The resulting 2′-hydroxyacetophenones and those commercially available with a 5′-ethoxy or -bromo substituent (**43** and **44**, respectively) were utilized in Claisen-Schmidt condensation reactions with p-benzyloxybenzaldehyde to yield chalchones (**45-48**), which, after performing ring closure with iodine in DMSO, yielded the corresponding 6-substituted 4′-benzyloxyflavones (**49-52**). The 6-bromo substituted compound (**52**) was subjected to Suzuki coupling in order to obtain the corresponding 6-phenylflavone (**53**). Debenzylation of the flavonoids obtained this way and subsequent oxidative de-aromatization of the flavones **54-58** with PIFA, as described above, allowed us to obtain the protoflavones with various substituents at positions C-6 and C-1′ (**59-73**). Scheme 2 summarizes the total synthetic procedure.

**Scheme 2.** Total synthesis of 6-substituted protoflavone derivatives[a]




[a] Reagents: (i.) $(CH_3CO)_2O$, *cc* $H_2SO_4$; (ii.) $AlCl_3$; (iii.) EtOH, 4-benzyloxybenzaldehyde, 50% $KOH/H_2O$; (iv.) $I_2$, DMSO; (v.) Phenylboronic acid, $K_2CO_3$, Tetrakis(triphenylphosphine)palladium(0); (vi.) 10% Pd-C/$H_2$; (vii.) $CH_3CN$/ROH 9/1, PIFA (2 eq).

In the first set of experiments, the cytotoxicity of compounds **2-38** and **59-73** were tested in two MDR/sensitive cancer cell line pairs (parental L5178 mouse lymphoma cells and $L5178_{B1}$ cells engineered to overexpress the human ABCB1 protein; parental MCF-7 breast cancer cells and the doxorubicin resistant derivative $MCF\text{-}7_{Dox}$, overexpressing P-gp). The fraction of $IC_{50}$ values obtained in P-gp negative vs. positive cells served as a quantification of the MDR selective effect (selectivity ratio, SR). Accordingly, SR $\leq$ 0.5 indicated that the compound is subject to P-gp-mediated resistance, whereas SR $\geq$ 2 suggested that the P-gp expressing cells demonstrate collateral sensitivity against the tested protoflavone derivative. Results of the cytotoxicity testing on the L5178 and MCF-7 models are summarized in Figure 1; detailed data are presented in Supplementary Table S1.

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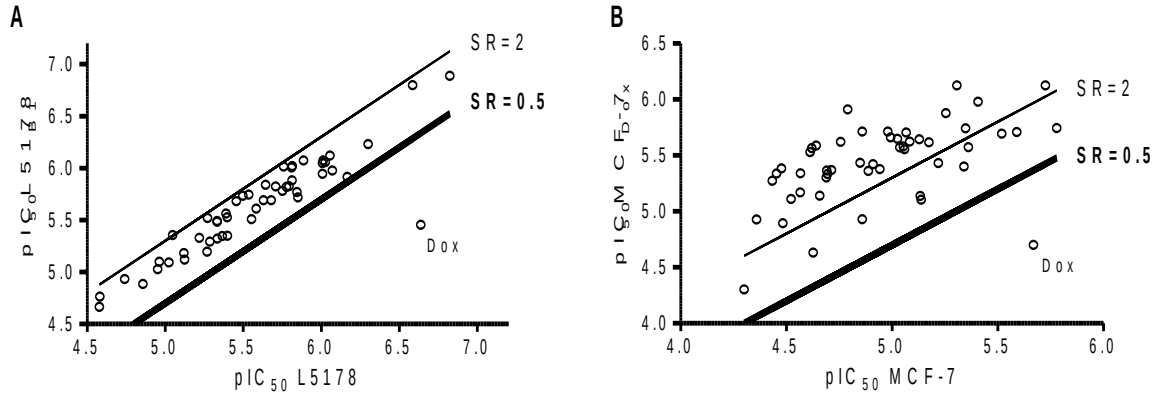


**Figure 1.** Cytotoxicity of protoflavones **2**-**38** and **59**-**73**. $pIC_{50}$ values were derived from dose response curves obtained from cell viability experiments on L5178/$L5178_{B1}$ mouse lymphoma cells (**A**) and MCF-7/$MCF\text{-}7_{Dox}$ cells (**B**). SR=Selectivity Ratio, calculated as $IC_{50}^{non\text{-}MDR}/IC_{50}^{MDR}$; n=3-4; Dox: doxorubicin.

The above results indicate that the synthesized protoflavone derivatives possess significant toxicity in the two cell line pairs. Interestingly, MCF7 cells were in general more resistant. Whereas the expression of ABCB1 did not modify the sensitivity of $L5178_B$ cells in comparison with the parental L5178 cell line, $MCF\text{-}7_{Dox}$ cells showed collateral sensitivity to several derivatives, with SR values exceeding 5 in the case of compounds **16**, **18**-**22**, **37**, **68**-**71** and **73**.

To substantiate the role of ABC transporters in the MDR-selective toxicity of the compounds, additional MDR models were included in the study. The cytotoxic activity of compounds **2**-**11**, **18**, **31**-**38**, **66** and **68**-**73** were tested in four additional MDR/sensitive cell line pairs, including A431, $A431_{B1}$, $A431_{G2}$, MES-SA, MES-SA/Dx5, KB-3-1 and KB-V1. These compounds represent a diverse sub-set of derivatives of the naturally occurring protoapigenone (**2**) and protogenkwanone (**11**), analogs of the synthetic WYC0209 (**31**) identified as a potential lead in previous studies,[12] as well as 6-methyl- 6-pentyl- and 6-phenyl derivatives (**18**, **66** and **68**-**73**) aiming to further explore SAR at C-6. The results are shown in Figures 2-3; detailed data are available as supplementary information (Tables S2-S3).

The tested protoflavone derivatives were equally toxic to A431, $A431_{G2}$ or $A431_{B1}$ cells ($IC_{50}$ values ranged from 0.60 μM to 7.27 μM), with the exception of protoapigenone (**2**), suggesting that the compounds tested herein are able to bypass ABCB1 or ABCG2. Resistance of $A431_{G2}$ cells to compound **2** was abolished in the presence of tariquidar, confirming that protoapigenone is an ABCG2 substrate (Figure 2).

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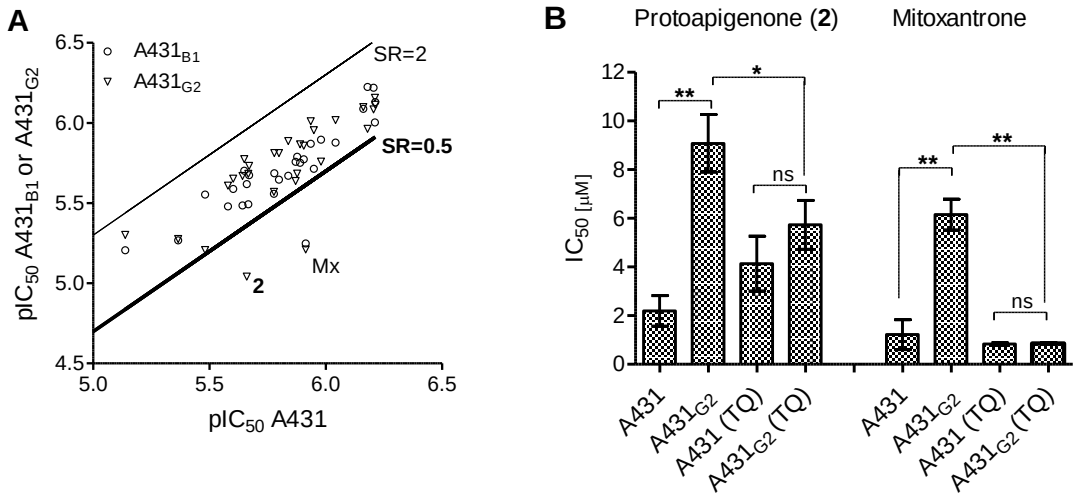


**Figure 2. A.** Cytotoxic activity of selected protoflavones against A431 (parental) cell line and two MDR derivatives engineered to overexpress ABCB1 ($A431_{B1}$) or ABCG2 ($A431_{G2}$). SR=Selectivity Ratio, calculated as $IC_{50}^{sensitive}/IC_{50}^{MDR}$; n=3-4; Mx: mitoxantrone. **B.** $IC_{50}$ values of **2** (protoapigenone) and mitoxantrone in A431 and $A431_{G2}$ cell lines in the presence and absence of 1 μM tariquidar, an ABCG2 efflux inhibitor.

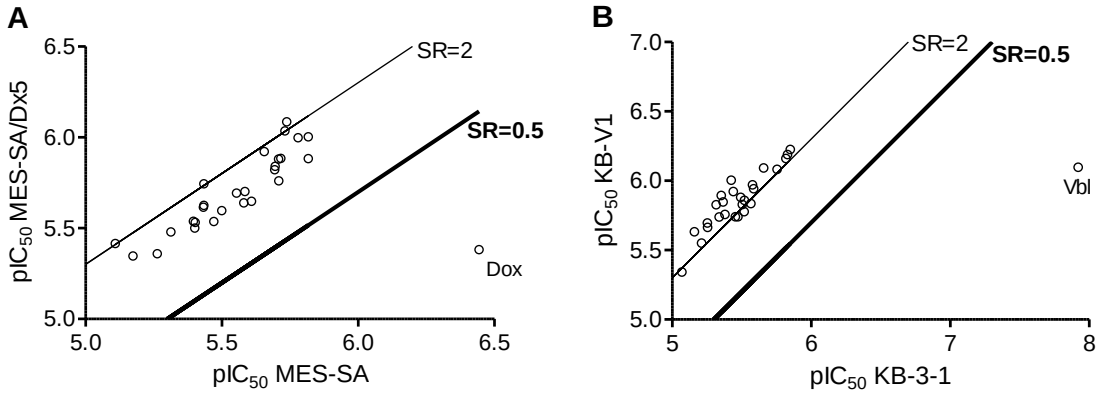


**Figure 3.** $pIC_{50}$ values measured in doxorubicin-selected MES-SA/Dx5[19] and vinblastine-selected KB-V1[20] cells compared to the $pIC_{50}$ values of MES-SA and KB-3-1 cells (**A** and **B**, respectively). Two compounds were selectively toxic against both MES-SA/Dx5 and KB-V1 cells (**2** and **66**), and none of the protoflavones showed substrate-like characteristics; Dox: doxorubicin, Vbl: vinblastine.

Finally, we tested the interaction of the compounds with Pgp to reveal if any of the compounds inhibit drug efflux. ABCB1 function was characterized using the calcein accumulation assay.[21] Each derivative was assayed at two concentrations in the presence of the fluorescent indicator. Except for the 6-phenylprotoflavone series (**68-73**), which showed moderate inhibition at 20 μM (14-46 %; see supplementary Table 4), none of the compounds inhibited the efflux of calcein AM by P-gp (Figure 4).

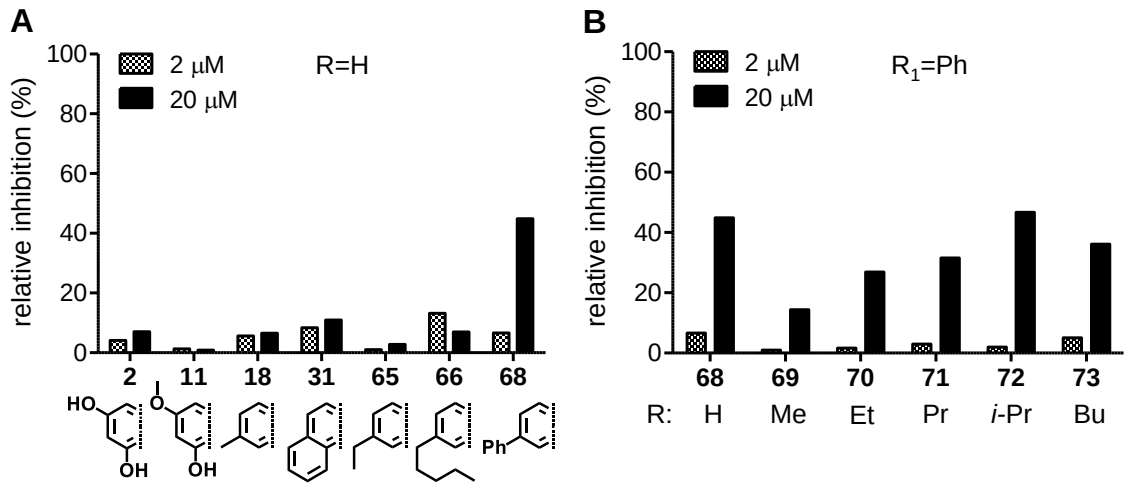


**Figure 4.** Relative inhibition of calcein AM efflux by **A** compounds **2**, **11**, **18**, **31**, **65**, **66** and **68** (where R=H refers to Scheme 1 and Scheme 2), and **B** derivatives of compound **68** (6-phenylprotoflavones). Relative inhibition was calculated from mean calcein intensities as [100*(sample - negative control)/(positive control - negative control)]. 20 μM verapamil was used as positive control, corresponding to full inhibition (100%).

## Discussion

This work was initiated with the aim to explore relevant structure-activity relationships of protoflavones with various substituents at the A-ring and particularly at C-6. Following the preparation of acetophenones **41** and **42**, a straightforward total-synthetic strategy[25] was applied to obtain 6-substituted protoflavones. It is worth mentioning that, even though related publications typically describe the use of a catalytic amount of iodine for the ring closure to obtain the flavone skeleton, utilizing a larger, 1 equivalent amount is far more efficient. Structure elucidation of the protoflavones was straightforward based on the mass and $^1$H NMR spectra. The expected change in the molecular mass, and, in case of the 1′-*O*-alkyl derivatives, the appearance of the characteristic $^1$H NMR signals and coupling pattern of the side chain proved the successful linking of water or alcohol. The build-up of the protoflavone type B-ring was evidenced by the change in the coupling constant of the two dublets of H-3′/H-5′ and H-2′/H-6′ from ca. 8.8 Hz to ca. 10.0 Hz, together with the remaining H-3 singlet and practically unchanged A-ring signals in the $^1$H NMR spectrum.

According to their B-ring substitution, cytotoxicity of the protoflavones on the utilized cell lines typically followed the previously observed structure-activity relationship: in most of the cases, 1′-OH substituted compounds were more toxic than those with 1′-alkoxy moieties and the isopropyl-ethers were the least cytotoxic derivatives. This, however, did not apply for protogenkwanone and its analogs (**11**-**17**): protogenkwanone 1′-*O*-methylether (**12**) exerted a stronger activity on the mouse lymphoma cells than **11**. Moreover, an at least two carbons long side-chain was necessary for this series of compounds to be slightly toxic on MCF-7 cells. Presence of a non-branching propyl or butyl ether side chain at the C-1′ of protoapigenone (as in compounds **5** and **7**) was previously found to be preferable for a strong cytotoxic activity.[17] This was also observed in the present study in most cell lines with the exceptions of the L5178

/ L5178$_{B1}$ and the MCF-7 / MCF-7$_{Dox}$ cell line pairs. Moreover, the introduction of a 1′-benzyloxy moiety (as in compound **10**) to protoapigenone also resulted in an increased toxicity in the A431 cell line and its MDR sub-cell lines, as well as in the KB-3-1 and KB-V1 cells. This provides further evidence for the importance of the size and/or lipophilicity of the substituent at C-1′ and suggests that a larger branching and/or unsaturated alkyl side-chain might also lead to an increased cytotoxicity, despite the generally lower activity of the 1′-*O*-isopropyl substituted derivatives as compared to those with linear alkyl chains.

Our attempt to increase the mild selective toxicity of 6-methylprotoflavones (**18**-**24**) previously observed in ABCB1 transfected L5178 cells[15] by introducing various C-6 substituents revealed one single compound reaching our chosen threshold of relevance against the L5178$_{B1}$ cells, 6-methoxyprotoflavone 1′-*O*-allylether (**29**). On the other hand, while all 6-methoxy compounds also showed tendency for such selectivity, other new derivatives showed decreased selective cytotoxicity against this cell line (Table S1). Similarly, none of the tested compounds, including 6-methylprotoflavone (**18**), showed selective toxicity against ABCB1 transfected cell lines, including A431$_{B1}$ (Table S2) and MDCK-II$_{B1}$ (not shown). Despite the equal toxicity of the studied compounds on parental and Pgp-transfected cell lines, several derivatives proved selectively toxic against MDR cell lines overexpressing P-gp as a result of long-term drug selection. In particular, MCF-7$_{Dox}$ cells (adapted to doxorubicin) showed collateral sensitivity to most compounds except for **4**, **31**, **35** and **59**-**65**, with structural differences of the A-ring clearly influencing activity (Table S1). Compound **68**, 6-phenylprotoflavone, for example, showed a remarkable, 13.2 fold selective cytotoxicity, while its β-naphthoflavone analog (**31**), where a fused aromatic ring is connected to the A-ring at the C-5/C-6 position, was non-selective. By comparing the selectivity ratios of compounds with different C-6 substituents, a clear SAR of the following order was observed: Ph > Me > OMe ≈ Pentyl, while the ethoxy substituted protoflavones (**59**-**64**) and the ethyl substituted compound **65** were non-selective. No such general SAR could be concluded for the C-1′ substituents, except for the lower selectivity observed for the isopropyl ether derivatives **35** and **72**. Interestingly, in case of the MES-SA / MES-SA/Dx5 cell line pair where the MDR sub-cell line was also obtained by adaptation to doxorubicin[19], collateral sensitivity was observed only for the classical, 1′-OH containing protoflavones (**2**, **11 18** and **66**, but SR was below threshold for **31** and **68**) and not for any of the 1′-*O*-alkylprotoflavones. Furthermore, the KB-V1 cell line, obtained from KB-3-1 by adaptation to vinblastine[20], also presented marginal CS towards most of the protoflavones, although SR values for several compounds fell just below the 2-fold threshold (Figure 3B, Table S3).

Statistical significance of the SAR was tested from two angles. Compounds were grouped either according to their A-rings or their substituents at C-1′, and the SR values of these groups were compared by one-way ANOVA* followed by Bonferroni's post hoc test. No differences were observed for the C-1′ substituents on any of the cell lines, not even when the data were normalized to the average of their corresponding series (i.e. analogs with the same A-ring).

* Each group containing at least 7 data points passed the Shapiro-Wilk normality test, suggesting the normal distribution of SR values under the influence of the presented chemical variations. Therefore, ANOVA is suitable for the statistical evaluation of these datasets.

However, the different A-ring containing protoflavone derivatives showed significant differences in their SR values on the MCF-7 / MCF-$7_{Dox}$ cell line pair; results are presented in Figure 5.

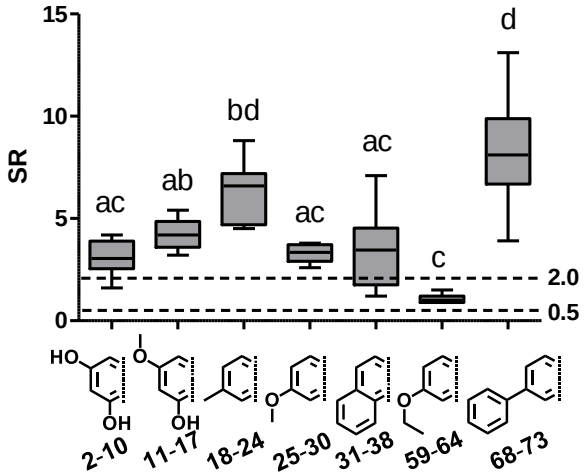


**Figure 5.** Selectivity ratio (SR) values for protoflavone analogs containing the same A-rings on the MCF-7 / MCF-$7_{Dox}$ cell line pair. Box-and-whisker plots represent medians, first and third quartiles and ranges; SR≥2.0 and SR≤0.5 represent CS and CR, respectively; different lower case letters represent datasets with statistically significant differences (i.e. groups with overlap in their marking are not significantly different) at $p \leq 0.05$ by one-way ANOVA followed by Bonferroni's post-hoc test.

All tested compounds were found similarly cytotoxic on the L5178 and the L$5178_{B1}$ cell lines, with a strong correlation between the two (Spearman r=0.9544). Interestingly, $IC_{50}$ values on the MCF-$7_{Dox}$ cell line also showed a good correlation to those on the parental mouse lymphoma cell line (Spearman r=0.7691), while the same correlation for MCF-7 was much weaker with several outliers (Spearman r=0.5812; without outliers, i.e. compounds **8**, **11**-**17**, **19**-**24**, **37**, **68**-**71** and **73**: r=0.7420). For a graphical interpretation of these correlations, see supplementary Figure S1.

These results suggest that the SR associated with the compounds on the MCF-7/MCF-$7_{Dox}$ is more a result of the resistance of MCF-7 to protoflavones, than the sensitivity of MCF-$7_{Dox}$. MCF-7 cells appear to be particularly resistant to the 7-methoxy group containing protogenkwanone derivatives (**11**-**17**), 6-methylprotoflavones (**19**-**24**), 6-phenylprotoflavones (**68**-**71** and **73**) and 1′-allyl group containing analogs (**8** and **37**), in line with the results presented in Figure S1. As such, adaptation of MCF-7 cells to doxorubicin has apparently resulted in the loss of initial resistance to protoflavones as an evolutionary cost of acquiring the MDR phenotype, and this manifested as collateral sensitivity.

Collateral sensitivity is causally linked to the adaption of MDR cells to a chemotherapeutic and may involve metabolic modifications, the upregulation of receptors[22] or the modulation of the redox homeostasis[23]. One limitation of studies relying on MDR cell lines is that the contribution of MDR pumps, versus other acquired cellular alterations, cannot be

delineated.[8],[9],[24] Collateral sensitivity of the MDR cell lines analyzed in this study indicates that resistance to doxorubicin or vinblastine may result in cellular alterations that render the cells susceptible to the protoflavone derivatives. However, in contrast to MDR-selective compounds,[10] protoflavone derivatives do not selectively target cells engineered to overexpress P-glycoprotein, suggesting that the increased toxicity observed in the MDR cells is not conferred by the efflux pumps. This was also supported by our observation that selectivity ratios did not decrease significantly when compounds **2**, **11**, and **18** were tested on the MES-SA / MES-SA/Dx5 cell line pair in the presence of tariquidar (data not presented).

It is important to point out that the lack of cross-resistance to most protoflavones in all MDR cell lines studied here indicates that these compounds can overcome MDR through bypassing efflux that is mediated by ABCB1 or ABCG2. SAR of the cytotoxic activity concerning the 6-substituents appears to differ from cell line to cell line, for example an order of Me > Et ≈ Pent ≈ Ph > Br > OEt > OMe can be recognized on L5178 and $L5178_{B1}$, Pent > Ph ≈ Me on KB-3-1 and KB-V1, while similar activities are exerted by 6-pentyl (**66**), 6-phenyl (**68**) and 6-methyl (**18**) compounds on MES-SA and MES-SA/Dx5. From a general overview, 6-alkyl substituted protoflavones appear to be somewhat more favorable anticancer agents over 6-alkoxy ones, even though nearly all compounds presented here can be considered as valuable leads against resistant cancers. As an interesting exception to this, however, resistance to protoapigenone (**2**) was observed in the ABCG2 transfected $A431_{G2}$ cell line, and a tendency for marginal resistance appeared also to its 1′-*O*-alkyl ethers. Resistance of this cell line to protoapigenone (**2**) markedly decreased in the presence of tariquidar, strongly suggesting that compound **2** is an ABCG2 substrate. ABCG2 did not confer resistance towards any of the other compounds including protogenkwanone (**11**), which differs from **2** only in its 7-methoxy group. This suggests that a non-substituted phenolic OH group at C-7 is necessary for protoflavones to be recognized by this transporter.

## Conclusions

Our *in vitro* studies on various A-ring and 1′-substituted protoflavones revealed 6-methoxyprotoflavone 1′-*O*-allyl ether (**29**) as an antitumor agent with a mild MDR selectivity (SR=2.0) in a murine lymphoma cell line transfected with the human ABCB1 efflux transporter.

The ability of protoflavones to evade efflux-mediated MDR was confirmed both in ABCB1 and ABCG2 expressing cell lines, with the exceptions of protoapigenone (**2**) which was identified as an ABCG2 substrate. MDR selective cytotoxicity was observed for most of the tested protoflavones in a breast cancer cell line adapted to doxorubicin ($MCF\text{-}7_{Dox}$) and SAR revealed importance of the A-ring substitution, while in the uterine sarcoma MES-SA/Dx5, another doxorubicin-selected cell line, only the 1′-OH containing compounds showed relevant selectivity. Since overexpression of ABCB1 did not sensitize cells, we conclude that the MDR-selective cytotoxicity of protoflavones is connected to other changes accompanying acquired drug resistance.

## Experimental Section

Structure elucidation was carried out by means of nuclear magnetic resonance (NMR) spectroscopy and mass spectroscopy (MS). NMR spectra were obtained on a Varian Gemini-

2000 200 MHz or Bruker Avance DRX-500 NMR spectrometer in chloroform-$d_1$, methanol-$d_4$, acetone-*d6*, or dimethyl sulfoxide-*d6*. Mass spectra were taken on an API 2000 triple-quadrupole (Ab Sciex, USA) or LCMS-IT-TOF (Shimadzu, Japan) with an ESI interface.

Compounds were purified by rotation planar chromatography on a Chromatothron equipment (Harrison Research, USA) with adequately chosen eluents of *n*-hexane-ethyl acetate on silica GF 254 (Merc, Germany) or with Flash Chromatograpy on a Combiflash Rf+ equipment (TELEDYNE Isco, USA) with eluents of *n*-hexane – ethyl acetate or methanol – dichloromethane on RediSep normal-phase silica flash columns (TELEDYNE Isco, USA). All compounds possessed a purity of ≥95.0% by means of HPLC-DAD, except for compounds **51** and **55** (92.20% and 93.41%, respectively), which served as intermediates for further synthesis and whose bioactivity was not tested. All chemicals were obtained from Aldrich, Inc. (USA).

**Synthesis of 5′-ethyl- and 5′-pentyl-2′-hydroxyacetophenone (41 and 42).** In the first step, 4-ethyl- and 4-pentylphenol acetate (**39** and **40**, respectively) were synthesized by adding 0.1 mol (10.2 g) of acetic anhydride and one drop of cc$H_2SO_4$ to 0.1 mol of 4-ethyl or 4-pentylphenol and stirring at room temperature for 20 min. The mixture was then poured into water and extracted with 3 x 50 ml EtOAc. The organic layer was evaporated under reduced pressure, re-dissolved in $CH_2Cl_2$ and crystallized anhydrous $AlCl_3$ was added little by little under ice bed cooling. The mixture was refluxed for 10 h, then the reaction was stopped by adding crushed ice. After filtration, the precipitate was purified on silica to obtain **41** or **42**.

**General Procedure for chalchone synthesis.** The 5′-substituted 2′-hydroxyacetophenone (**41-44**) and 4-benzyloxybenzaldehyde (3.0 g, 14.3 mmol) were dissolved in 50% EtOH, KOH/$H_2O$ solution (20 mL). The reaction was stirred at r.t. for 30 h, and then the solvent was evaporated under reduced pressure. The mixture was purified on silica gel (isocratic elution, n-hexane/EtOAc, 6:1) to afford **45-48**, respectively.

(E)-3-(4-benzyloxyphenyl)-1-(5-ethoxy-2-hydroxyphenyl)prop-2-en-1-one (**47**). Orange solid; yield: 82.2%; NP-HPLC purity: 99.15%; $^1$H NMR (500 MHz, chloroform-*d1*) δ 12.47 (1H, s, OH), 7.89 (1H, d, *J* = 15.35 Hz), 7.62 (2H, d, *J* = 8.60 Hz), 7.47 (1H, d, *J* = 15.55 Hz), 7.34-7.45 (6H, m), 7.12 (1H, dd, *J* = 9.00 Hz, 2.80 Hz), 7.02 (2H, d, *J* = 8.55 Hz), 6.96 (1H, d, *J* = 9.00 Hz), 5.13 (2H, s), 4.05 (2H, q, *J* = 6.95 Hz), 1.44 (3H, t, *J* = 6.95 Hz) 7.91 (2H, d, *J* = 8.45 Hz), 7.86 (1H, d, *J* = Hz), 7.67 (1H, d, *J* = 2.65 Hz), 7.4-7.38 (5H, br), 7.35 (1H, d, *J* = 7.25 Hz), 7.19 (1H, dd, *J* = 2.70 Hz, 8.50 Hz), 7.11 (2H, d, *J* = 8.50 Hz), 6.92 (1H, d, *J* = 9.00 Hz), 5.19 (2H, s), 4.07 (2H, q, *J* = 6.85 Hz), 1.34 (3H, t, *J* = 6.90 Hz) ; $^{13}$C NMR (125 MHz, chloroform-*d1*) 193.45, 161.32, 157.97, 151.11, 145.47, 136.46, 130.69, 128.83, 128.36, 127.72, 127.61, 124.29, 119.97, 119.31, 117.93, 115.53, 114.22, 70.29, 64,72, 15.06; ESI-MS (m/z): 375.3 [$M^+$+H].

**General Procedure for Flavone synthesis.** 3 mmol of chalchone (**45-48**) was dissolved in DMSO (5 mL), and 1 eq. iodine (76 mg, 0.3 mmol) was added. The solution mixture was stirred and heated to 110 °C. After 2 h, 10% $Na_2S_2O_3$ (50 mL) was added to remove iodine. The mixture was extracted with EtOAc (3 X 50 mL) and then purified by column chromatography on silica gel to afford compounds **49-52**, respectively.

6-Ethoxy-4′-benzyloxyflavone (**51**). Yield: 85.2%; NP-HPLC purity: 92.20%, pale yellow solid; $^{1}$H NMR (500 MHz, chloroform-*d1*) δ 7.86 (2H, d, *J* = 8.80 Hz), 7.56 (1H, d, *J* = 2.70 Hz), 7.48-7.38 (5H, benzyl), 7.35 (1H, d, *J* = 7.1 Hz), 7.25 (1H, dd, *J* = 2.85 Hz, 8.75 Hz), 7.08 (2H, d, *J* = 8.70 Hz), 6.72 (1H, s), 5.14 (2H, s), 4.13 (2H, q, *J* = 6.95 Hz), 1.44 (3H, t, *J* = 6.95 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 178.44, 163.26, 161.61, 156.43, 151.06, 136.34, 131.02, 128.95, 128.87, 128.41, 128.11, 127.62, 124.68, 124.61, 124.05, 119.46, 115.48, 105.72, 70.36, 64.36, 14.86; ESI-MS (m/z): 372.9 [$M^{+}$+H].

**Synthesis of compound 53 from 52 via Suzuki-coupling.** Compound **52** (407.26 mg, 1.0 mmol), 1.2 eq. phenylboronic acid (146.3 mg, 1.2 mmol) and 2 eq. (276.42mg, 2.0 mmol) $K_2CO_3$ were dissolved in 7 mL of water, and 25 mL of propanol and 5 mmol of Tetrakis(triphenylphosphine)palladium(0) were subsequently added. The reaction was performed under $N_2$ gas at 40ºC, to afford **53** as a yellow solid.

6-Phenyl-4′-benzyloxyflavone (**53**). Yield: 30 %; yellow solid; $^{1}$H NMR (200 MHz, chloroform-*d1*) δ 8.45 (1H, s), 7.89 (3H, d, *J* = 7.80 Hz), 7.67 (1H, d, *J* = 7.20 Hz), 7.50-7.30 (11H, m), 7.25 (1H, s,), 7.10 (2H, d, *J* = 8.40 Hz), 6.77 (1H, s), 5.15 (2H, s); $^{13}$C NMR (50 MHz, chloroform-*d1*) 177.35, 162.34, 160.55, 154.56, 138.32, 137.20, 135.16, 131.35, 127.91, 127.68, 127.35, 127.21, 127.00, 126.74, 126.41, 126.12, 125.66, 123.19, 122.48, 117.39, 114.33, 113.90, 105.18, 97.23, 69.17.

**General Procedure for Benzyl Group Removal**. A mixture of 0.5 mmol of the 4′-benzyloxyflavone (**49**-**53**), dry Pd/C (10%, 106 mg), and 20 mL of EtOAc was stirred at 25 °C under an atmosphere of hydrogen for 10 h. The mixture was filtered, washed with EtOAc, concentrated under vacuum and purified by column chromatography on silica gel to afford compounds **54**-**58**, respectively.

6-Pentyl-4′-hydroxyflavone (**55**). Yield: 85.4%; NP-HPLC purity: 93.41%, pale yellow solid; $^{1}$H NMR (500 MHz, DMSO-*d6*) δ 10.31 (1H, brs, OH), 7.95 (2H, d, *J* = 8.65 Hz), 7.81 (1H, s), 7.66 (1H, d, *J* = 8.45 Hz), 7.64 (1H, dd, J = 8.85 Hz, 1.85 Hz), 6.90 (2H, d, *J* = 9.75 Hz), 6.94 (2H, d, *J* = 8.60 Hz), 6.84 (1H, s), 2.70 (2H, t, *J* = 7.45 Hz), 1.61 (2H, quin, *J* = 7.00 Hz), 1.38-1.21 (4H, m), 0.85 (2H, t, *J* = 6.85 Hz); $^{13}$C NMR (125 MHz, DMSO-*d6*) 176.97, 162.97, 160.96, 154.05, 139.67, 134.43, 128.34, 123.43, 123.07, 121.7, 118.26, 115.97, 104.72, 34.42, 30.79, 30.55, 21.96, 13.94; ESI-MS (m/z): 309.5 [$M^{+}$+H].

**General procedure for protoflavone synthesis from 4′-hydroxyflavones**. Apigenin, genkwanin, 4′-hydroxy-6-methylflavone, 4′-hydroxy-6-methoxyflavone, 4′-hydroxy-β-naphthoflavone (Indofine, Hillsborough, NJ, USA), or the synthesized 4′-hydroxyflavone (**54**-**58**) was dissolved at 1 mg/mL concentration in a 9:1 v/v ratio mixture of acetonitrile and water or the alcohol to be coupled at position C-1′. Two equivalents of [*bis*(trifluoroacetoxy)iodo]benzene were added to the mixture. After stirring at 80 °C for 1 hour, the mixture was cooled down, evaporated under reduced pressure and purified by flash chromatography to obtain compounds **2**-**9**,[17] **10**, **11**-**24**,[15] **25**-**30**, **31**-**38**,[17] or **59**-**73**, respectively. Compounds **10**, **25**-**30** and **59**-**73** are reported here as new protoflavones.

Protoapigenone 1′-*O*-benzyl ether (**10**). Light brown solid; yield: 39,6%; RP-HPLC purity: 99.21%, $^{1}$H NMR (500 MHz, DMSO-*d6*) δ 12.47 (1H, s, OH), 7.36-7.43 (4H, m), 7.32 (1H, t,

*J* = 6.90 Hz), 7.18 (2H, d, *J* = 9.95 Hz), 6.57 (2H, d, *J* = 9.95 Hz), 6.50 (1H, s), 6.18 (2H, d, *J* = 9.15 Hz), 4.57 (2H, s); $^{13}$C NMR (125 MHz, DMSO-*d6*) 184.29, 181.32, 165.44, 164.37, 161.41, 157.41, 145.78, 137.53, 132.22, 128.35, 127.84, 127.65, 107.37, 103.68, 99.43, 94.01, 74.22, 66.52; ESI-MS (m/z): 377.2 [$M^+$+H].

6-Methoxyprotoflavone (**25**). Pale yellow solid; yield: 32.3%; NP-HPLC purity: 98.95%, $^1$H NMR (500 MHz, acetone-*d6*) δ 7.46 (1H, d, *J* = 2.95 Hz), 7.44 (1H, d, *J* = 9.35 Hz), 7.33 (1H, dd, *J* = 9.07 Hz, 2.90 Hz), 7.05 (2H, d, *J* = 10.00 Hz), 6.66 (1H, s), 6.33 (2H, d, *J* = 10.00 Hz), 6.11 (1H, s), 3.90 (3H, s); $^{13}$C NMR (125 MHz, chloroform-*d1*) 185.54, 179.13, 166.64, 157.4, 151.23, 147.00, 129.71, 124.57, 119.68, 107.97, 104.84, 69.85, 56.09; ESI-MS (m/z): 285.4 [$M^+$+H].

6-Methoxyprotoflavone 1′-*O*-methyl ether (**26**). Yellow solid; yield: 41.3%; NP-HPLC purity: 98.93%, $^1$H NMR (500 MHz, acetone-*d6*) δ 7.45 (1H, d, *J* = 3.20 Hz), 7.43 (1H, d, *J* = 8.7 Hz), 7.33 (1H, dd, *J* = 9.15 Hz, 3.00 Hz), 7.01 (2H, d, *J* = 10.05 Hz), 6.58 (1H, s), 6.54 (2H, d, *J* = 10.15 Hz), 3.90 (3H, s), 3.44 (3H, s); $^{13}$C NMR (125 MHz, DMSO-*d6*) 184.27, 176.53, 163.86, 156.74, 150.26, 145.91, 132.42, 123.96, 123.53, 119.89, 108, 104.77, 74.3, 55.74, 52.34; ESI-MS (m/z): 299.1 [$M^+$+H].

6-Methoxyprotoflavone 1′-*O*-ethyl ether (**27**). Yellow solid; yield: 39.3%; NP-HPLC purity: 98.39%, $^1$H NMR (500 MHz, acetone-*d6*) δ 7.45 (1H, d, *J* = 2.90 Hz), 7.43 (1H, d, *J* = 8.85 Hz), 7.33 (1H, dd, *J* = 10.00, 2.80 Hz), 7.03 (2H, d, *J* = 10.00 Hz), 6.63 (1H, s), 6.51 (2H, d, *J* = 9.95 Hz), 3.90 (3H, s), 3.64 (2H, q, *J* = 6.80 Hz) 1.28 (1H, t, *J* = 6.90 Hz); $^{13}$C NMR (125 MHz, DMSO-*d6*) 184.34, 176.55, 164.04, 156.73, 150.24, 146.39, 131.91, 123.96, 123.5, 119.88, 107.96, 104.78, 74.01, 60.3, 55.73, 15.56; ESI-MS (m/z): 313.0 [$M^+$+H].

6-Methoxyprotoflavone 1′-*O*-butyl ether (**28**). Yield: 46.3%; NP-HPLC purity: 98.67%, light brown solid; $^1$H NMR (500 MHz, acetone-*d6*) δ 7.45 (1H, d, *J* = 2.90 Hz), 7.43 (1H, d, *J* = 8.80 Hz), 7.32 (1H, dd, *J* = 9.15 Hz, 1.80 Hz), 7.02 (2H, d, *J* = 10.25 Hz), 6.63 (1H, s), 6.51 (2H, d, *J* = 9.85 Hz), 3.90 (3H, s), 3.59 (2H, t, *J* = 5.35 Hz) 1.65 (2H, q, *J* = 6.20 Hz), 1.51 – 1.35 (2H, m), 0.94 (3H, t, *J* = 7.20 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 184.93, 178.42, 164.14, 153.28, 149.20, 146.45, 132.77, 124.66, 124.25, 119.64, 108.89, 104.99, 74.65, 65.09, 56.11, 32.23, 19.43, 13.99; ESI-MS (m/z): 341.1 [$M^+$+H].

6-Methoxyprotoflavone 1′-*O*-allyl ether (**29**). Light brown solid; yield: 37.2 %; NP-HPLC purity: 95.17%, $^1$H NMR (500 MHz, acetone-*d6*) δ 7.44 (1H, d, *J* = 2.85 Hz), 7.43 (1H, d, *J* = 8.85 Hz), 7.30 (1H, dd, *J* = 9.15 Hz, 2.95 Hz), 7.02 (2H, d, *J* = 10.10 Hz), 6.61 (1H, s), 6.49 (2H, d, *J* = 9.85 Hz), 5.24 (1H, octet, *J* = 6.55 Hz), 4.61 (1H, d, *J* = 17.2 Hz), 4.43 (1H, d, *J* = 10.57 Hz), 3.37 (2H, d, *J* = 5.05 Hz), 3.86 (3H, s); $^{13}$C NMR (125 MHz, chloroform-*d1*) 184.69, 178.25, 163.8, 157.37, 151.09, 145.81, 145.78, 133.75, 132.87, 124.24, 123.68, 119.6, 117.69, 108.98, 105.01, 74.83, 66.23, 56.09; ESI-MS (m/z): 325.2 [$M^+$+H].

6-Methoxyprotoflavone 1′-*O*-proargyl ether (**30**). Light brown solid; yield: 37.7 %; NP-HPLC purity: 95.32%, $^1$H NMR (500 MHz, acetone-*d6*) δ 7.46 (1H, d, *J* = 2.90 Hz), 7.44 (1H, d, *J* = 8.85 Hz), 7.33 (1H, dd, *J* = 9.07 Hz, 2.80 Hz), 7.10 (2H, d, *J* = 9.90 Hz), 6.61 (1H, s), 6.50 (2H, d, *J* = 9.90 Hz), 4.44 (2H, d, *J* = 1.95 Hz), 3.90 (3H, s), 3.10 (1H, t, *J* = 2.20 Hz); $^{13}$C NMR

(125 MHz, DMSO-*d6*) 184.17, 176.5, 163.24, 156.76, 150.27, 144.90, 132.26, 123.95, 123.57, 119.92, 108.17, 104.76, 80.17, 78.41, 74.40, 55.74, 53.27; ESI-MS (m/z): 322.9 [$M^+$+H].

6-Ethoxyprotoflavone (**59**). Pale yellow solid; yield: 35.3%; NP-HPLC purity: 99.14%, $^1$H NMR (500 MHz, methanol-*d4*) δ 7.51 (1H, d, *J* = 2.90 Hz), 7.43 (1H, d, *J* = 9.20 Hz), 7.36 (1H, dd, *J* = 9.25 Hz, 3.00 Hz), 7,00 (2H, d, *J* = 9.95 Hz), 6.79 (1H, s), 6.39 (2H, d, *J* = 10.10 Hz), 4.14 (2H, q, *J* = 6.95 Hz), 1.44 (3H, t, *J* = 7.00 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 185.37, 178.99, 166.3, 156.73, 151.08, 146.77, 129.75, 124.79, 124.23, 119.58, 107.97, 105.45, 69.78, 64.40, 14.72; ESI-MS (m/z): 299.3 [$M^+$+H].

6-Ethoxyprotoflavone 1′-*O*-methyl ether (**60**). Yellow solid; yield: 42.0%; NP-HPLC purity: 99.21%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.48 (1H, d, *J* = 2.90 Hz), 7.26 (1H, d, *J* = 9.15 Hz), 7.20 (1H, dd, *J* = 9.15 Hz, 2.90 Hz), 6.79 (2H, d, *J* = 10.00 Hz), 6.71 (1H, s), 6.54 (2H, d, *J* = 9.95 Hz), 4.09 (2H, q, *J* = 7.00 Hz), 3.37 (3H, s), 1.41 (3H, t, *J* = 7.15 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 184.64, 178.16, 163.66, 156.66, 150.94, 145.68, 133.18, 124.65, 124.47, 119.51, 108.83, 105.65, 75.01, 64.37, 52.88, 14.76; ESI-MS (m/z, %): 313.5 [$M^+$+H].

6-Ethoxyprotoflavone 1′-*O*-ethyl ether (**61**). Yellow solid; yield: 41.2%; NP-HPLC purity: 98.12%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.49 (1H, d, *J* = 2.85 Hz), 7.26 (1H, d, *J* = 9.25 Hz), 7.20 (1H, dd, *J* = 9.20 Hz, 2.90 Hz), 6.80 (2H, d, *J* = 10.00 Hz), 6.78 (1H, s, H-3), 6.50 (2H, d, *J* = 10.00 Hz), 4.09 (2H, q, *J* = 7.00 Hz), 3.57 (2H, q, *J* = 6.85 Hz), 1.41 (3H, t, *J* = 6.85 Hz), 1.27 (3H, t, *J* = 6.95 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 184.83, 178.54, 164.14, 156.71, 151.02, 146.25, 132.68, 124.64, 124.53, 119.55, 108.77, 105.59, 74.71, 64.39, 61.03, 15.78, 14.75; ESI-MS (m/z, %): 327.6 [$M^+$+H].

6-Ethoxyprotoflavone 1′-*O*-propyl ether (**62**). Yellow solid; yield: 45.8%; NP-HPLC purity: 99.20%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.49 (1H, d, *J* = 2.85 Hz), 7.25 (1H, d, *J* = 9.25 Hz), 7.20 (1H, dd, *J* = 9.20 Hz, 2.90 Hz), 6.79 (2H, d, *J* = 9.80 Hz), 6.78 (1H, s, H-3), 6.51 (2H, d, *J* = 10.10 Hz), 4.09 (2H, q, *J* = 7.00 Hz), 3.46 (2H, t, *J* = 6.45 Hz), 1.65 (2H, sex, *J* = 7.15 Hz), 1.41 (3H, t, *J* = 6.90 Hz), 0.96 (3H, t, *J* = 7.40 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 184.85, 178.31, 163.97, 156.65, 150.95, 146.37, 132.68, 124.65, 124.45, 119.51, 108.84, 105.63, 74.59, 66.92, 64.37, 23.47, 14.77, 10.7; ESI-MS (m/z, %): 341.5 [$M^+$+H].

6-Ethoxyprotoflavone 1′-*O*-isopropyl ether (**63**). Yellow solid; yield: 43.1%; NP-HPLC purity: 98.20%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.48 (1H, d, *J* = 2.75 Hz), 7.25 (1H, d, *J* = 9.00 Hz), 7.20 (1H, dd, *J* = 9.15 Hz, 2.85 Hz), 6.81 (2H, d, *J* = 10.00 Hz), 6.80 (1H, s), 6.48 (2H, d, *J* = 9.95 Hz), 4.09 (2H, q, *J* = 7.00 Hz), 3.84 (2H, quin, *J* = 6.15 Hz), 1.42 (3H, t, *J* = 7.30 Hz), 1.21 (6H, d, *J* = 6.35 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 185.05, 178.35, 164.16, 156.64, 150.93, 146.72, 131.97, 124.64, 124.43, 119.51, 108.96, 105.62, 74.91, 68.93, 64.36, 24.82, 14.77; ESI-MS (m/z): 341.5 [$M^+$+H].

6-Ethoxyprotoflavone 1′-*O*-buthyl ether (**64**). Light brown solid; yield: 49.9%; NP-HPLC purity: 98.30%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.49 (1H, brs), 7.26 (1H, d, *J* = 9.10 Hz), 7.22 (1H, dd, *J* = 8.55 Hz, 2.85 Hz), 6.83 (1H, s), 6.79 (2H, d, *J* = 9.55 Hz), 6.51 (2H, d, *J* = 9.60 Hz), 4.09 (2H, q, *J* = 6.85 Hz), 3.84 (2H, t, *J* = 6.05 Hz), 1.61 (2H, q, *J* = 6.65 Hz), 1.45-1.32 (2H, m), 1.41 (3H, t, *J* = 6.40 Hz), 0.92 (3H, t, *J* = 7.25 Hz); $^{13}$C NMR (125 MHz,

methanol-*d4*) 186.46, 166.74, 158.31, 152.45, 148.05, 133.64, 125.79, 125.36, 120.82, 109.11, 106.55, 75.99, 66.07, 65.45, 33.38, 20.46, 15.07, 14.32; ESI-MS (m/z): 355.2 [$M^+$+H].

6-Ethylprotoflavone (**65**) Light brown solid; yield: 41.0 %; RP-HPLC purity: 95.0%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.92 (1H, s), 7.48 (1H, d, *J* = 8,55 Hz), 7.27 (1H, d, *J* = 8,65 Hz), 6.95 (2H, d, *J* = 9,30 Hz), 6.86 (1H, s), 6.38 (2H, d, *J* = 9,30 Hz), 2.72 (2H, q, *J* = 7.50 Hz), 1.25 (3H, t, *J* = 7.00 Hz); $^{13}$C NMR (125 MHz, chloroform-*d1*) 185.33, 179.31, 166.56, 154.82, 146.63, 142.31, 134.77, 129.89, 123.88, 123.37, 118.11, 108.68, 69.94, 28.44, 15.58; ESI-MS (m/z): 283.3 [$M^+$+H].

6-Pentylprotoflavone (**66**). Light brown solid; yield: 42.3%; NP-HPLC purity: 96.10%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 7.92 (1H, s), 7.46 (1H, dd, *J* = 8.45 Hz, 1.25 Hz), 7.28 (1H, d, *J* = 8.95 Hz), 6.90 (2H, d, *J* = 9.75 Hz), 6.79 (1H, s), 6.38 (2H, d, *J* = 9.80 Hz), 2,66 (2H, t, *J* = 7.60 Hz), 1.61 (2H, quin, *J* = 6.90 Hz), 1.38-1.21 (4H, m), 0.85 (3H, t, *J* = 7.05 Hz); $^{13}$C NMR (125.7 MHz, chloroform-*d1*) 176.91, 162.93, 160.90, 154.00, 139.61, 134.37, 123.38, 118.20, 104.68, 34.37, 30.48, 20.73, 21.90, 13.88; ESI-MS (m/z): 325.4 [$M^+$+H].

6-Phenylprotoflavone (**68**). Yellow solid; yield: 38.1%; NP-HPLC purity: 95.19%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 8.37 (1H, d, *J* = 1.40 Hz), 7.88 (1H, dd, *J* = 9.00 Hz, 1.85 Hz), 7.62 (2H, d, *J* = 7.55 Hz), 7.45 (2H, t, *J* = 6.75 Hz), 7.41 (1H, d, *J* = 8.95 Hz), 7.37 (1H, t, *J* = 7.65 Hz), 6.91 (2H, d, *J* = 10.10 Hz), 6.82 (1H, s), 6.57 (2H, d, *J* = 9.90 Hz); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 145.07, 131.64, 128.27, 127.94, 127.42, 126.95, 126.10, 122.51, 122.51, 117.47, 108.50, 65.77; HRESIMS $C_{21}H_{15}O_4$, calcd. 331.0970, found: 331.0973.

6-Phenylprotoflavone 1′-*O*-methyl ether (**69**). Yellow solid; yield: 50.2%; NP-HPLC purity: 97.74%, $^1$H NMR (500 MHz, chloroform-*d1*) *δ* 8.37 (1H, d, *J* = 1.50 Hz), 7.88 (1H, dd, *J* = 8.35 Hz, 1.50 Hz), 7.66 (2H, d, *J* = 7.85 Hz), 7.45 (2H, t, *J* = 7,10 Hz), 7.41 (1H, d, *J* = 8.85 Hz), 7.37 (1H, t, *J* = 7.00 Hz), 6.82 (2H, d, *J* = 9.95 Hz), 6.77 (1H, s), 6.57 (2H, d, *J* = 9.90 Hz), 3.41 (3H, s); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 144.39, 132.14, 131.81, 128.26, 127.94, 127.42, 127.26, 126.92, 126.10, 122.51, 121.72, 117.47, 108.49, 108.11, 51.71, 28.63; HRESIMS $C_{22}H_{17}O_4$, calcd. 345.1127, found: 345.1125.

6-Phenylprotoflavone 1′-*O*-ethyl ether (**70**). Yellow solid; yield: 48.2%; NP-HPLC purity: 98.10%, 1H NMR (500 MHz, chloroform-*d1*) *δ* 8.37 (1H, d, *J* = 1.50 Hz), 7.87 (1H, d, *J* = 8.60 Hz), 7.62 (2H, d, *J* = 7.50 Hz), 7.44 (2H, t, *J* = 7.20 Hz), 7.40 (1H, d, *J* = 8.85 Hz), 7.36 (1H, brt, *J* = 6.75 Hz), 6.84 (1H, s), 6.83 (2H, d, *J* = 10.10 Hz), 6.53 (2H, d, *J* = 9.80 Hz), 3.58 (2H, q, *J* = 7.00 Hz), 1.28 (3H, t, *J* = 6.80 Hz); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 144.84, 132.63, 131.78, 128.29, 127.96, 127.42, 127.24, 126.91, 126.10, 122.51, 121.70, 117.49, 108.52, 108.15, 59.85, 28.63, 14.60; HRESIMS $C_{23}H_{19}O_4$, calcd. 359.1283, found: 359.1280.

6-Phenylprotoflavone 1′-*O*-propyl ether (**71**). Light brown solid; yield: 46.4%; NP-HPLC purity: 99.39%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 8.37 (1H, d, *J* = 1.40 Hz), 7.88 (1H, dd, *J* = 8.85 Hz, 1.60 Hz), 7.62 (2H, d, *J* = 7.50 Hz), 7.44 (2H, brt, *J* = 6.45 Hz), 7.40 (1H, d, *J* = 8.70 Hz), 7.36 (1H, t, *J* = 7.20 Hz), 6.84 (1H, s), 6.83 (2H, d, *J* = 9.95 Hz), 6.54 (2H, d, *J* = 9.90 Hz), 3.48 (2H, t, *J* = 6.25 Hz), 1.67 (2H, sex, *J* = 6.95 Hz), 0.98 (3H, t, *J* = 7.35 Hz); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 145.07, 131.64, 128.27, 127.94, 127.42, 126.95, 126.10,

122.51, 117.47, 108.50, 65.77, 30.89, 28.63, 22.27, 9.51; HRESIMS $C_{24}H_{21}O_4$, calcd. 373.1440, found: 373.1438.

6-Phenylprotoflavone 1′-*O*-isopropyl ether (**72**). Light brown solid; yield: 42.2%; NP-HPLC purity: 99.30%, $^1$H NMR (500 MHz, chloroform-*d1*) δ 8.37 (1H, brs), 7.88 (1H, dd, *J* = 8.10 Hz, 1.25 Hz), 7.62 (2H, d, *J* = 7.85 Hz), 7.45 (2H, brt, *J* = 6.65 Hz), 7.41 (1H, d, *J* = 8.85 Hz), 7.37 (1H, t, *J* = 7.00 Hz), 6.82 (2H, d, *J* = 9.95 Hz), 6.77 (1H, s), 6.57 (2H, d, *J* = 9.90 Hz),3,89 (1H, sext, *J* = 6.25 Hz); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 145.44, 138.05, 132.61, 128.30, 127.96, 127.44, 127.24, 126.91, 126.10, 122.51, 121.70, 117.47, 108.62, 108.26, 97.24, 67.81, 28.63, 23.62; HRESIMS $C_{24}H_{21}O_4$, calcd. 373.1440, found: 373.1439.

6-Phenylprotoflavone 1′-*O*-butyl ether (**73**). Light brown solid; yield: 50.1%; NP-HPLC purity: 95.4%; $^1$H NMR (200 MHz, chloroform-*d1*) δ 8.40 (1H, d, *J* = 1.50 Hz), 7.89 (1H, dd, *J* = 9.02 Hz, 2.00 Hz), 7.64 (2H, d, *J* = 8.00 Hz), 7.47 (2H, brt, *J* = 6.60 Hz), 7.41 (1H, d, *J* = 8.20 Hz), 7.39 (1H, t, *J* = 7.00 Hz), 6.81 (1H, s), 6.56 (2H, d, *J* = 10.00 Hz), 6.39 (2H, d, *J* = 9.80 Hz), 3.54 (2H, t, *J* = 5.80 Hz), 1.68-1.36 (4H, m), 0.95 (3H, t, *J* = 7.20 Hz); $^{13}$C NMR (50 MHz, chloroform-*d1*) *δ* 145.01, 138.03, 132.63, 128.27, 127.96, 127.42, 127.24, 126.91, 126.10, 122.52, 121.72, 117.47, 108.52, 108.15, 63.89, 31.01, 28.63, 18.20; HRESIMS $C_{25}H_{23}O_4$, calcd. 387.1596, found: 387.1593.

**Cell lines.** L5178 mouse T-cell lymphoma cell line (ECACC catalog no. 87111908, U.S. FDA, Silver Spring, MD, U.S.), and its sub-cell line $L5178_{B1}$, derived from L5178 by transfection with pHa MDR1/A retrovirus,[26] were cultured in McCoy's 5A media supplemented inactivated horse serum. $L5178_{B1}$ cell line was selected by culturing the infected cells with 60 μg/L colchicine (Sigma). Breast cancer cell lines MCF7 and its sub-cell line obtained by adaptation to doxorubicin, $MCF7_{Dox}$[27] were cultured in EMEM media supplemented with non-essential amino acids, 1mM Na-pyruvate and 10% inactivated fetal bovine serum ($MCF7_{Dox}$ was cultured in presence of 1 μM of doxorubicin each third passage). All above cell lines were cultured at 37°C and 5% $CO_2$; all media contained Nystatin, 2 mM of L-glutamine, 100U of penicillin and 0.1mg of streptomycin, purchased from Sigma.

MES-SA human uterine sarcoma cell line and the doxorubicin selected MES-SA/Dx5 were obtained from ATCC. The human cervix carcinoma cell line KB-3-1 and its vinblastine selected derivative KB-V1 were a kind gift from Dr. Michael M. Gottesman (National Institutes of Health). A431 and the retrovirally transduced $A431_{B1}$ and $A431_{G2}$ are human skin-derived, epidermoid carcinoma cells were kind gifts from Dr. K. Német. MES-SA, KB-3-1, A431 and their derivative cell lines were maintained in DMEM completed with 10% FBS, 5 mM glutamine and 50 units/mL penicillin and streptomycin (Life Technologies).

**Cytotoxicity assay.** In case of mouse lymphoma cell lines L5178 and $L5178_{B1}$, $2x10^4$ cells per well were cultured in 96-wells microplates with different concentrations of the tested compound, in McCoy's 5A media, at 37ºC and 5% $CO_2$, for 24h.

With respect to MCF7 and $MCF7_{Dox}$, $1x10^4$ cells per well were seeded overnight and serial dilutions of the compounds were added the following day and incubated for 48h. In all cases, after the incubation time, 10% MTT was added to each well and incubated for 4h, when 100 μM of 10% sodium dodecyl-sulfate (SDS) dissolved in 0.01M HCl was added to each well. Results were read after o/n incubation. Fifty per cent inhibitory concentrations ($IC_{50}$) were calculated

using nonlinear regression curve fitting of log (inhibitor) versus normalized response with a variable slope and least squares (ordinary) fit of GraphPad Prism 5 software, for three independent samples.

In the case of A431 cell lines, MES-SA cell lines, KB-3-1 and KB-V1 cell lines, $5x10^3$ cells per well were cultured and incubated overnight in 96-well microplates. Serially diluted drugs were then added, and plates were incubated for additional 72 hours. Cytotoxicity was measured by Presto Blue cell viability reagent (Invitrogen) in a final dilution of 5%.

**FACS measurements.** Calcein accumulation assay was performed as described earlier[21]. Briefly, 250 000 cells per tube were pre-incubated for 5 minutes in the presence of 20 μM verapamil or the test protoflavone compounds. Calcein AM was added at a final concentration of 250 nM and incubated for an additional 10 minutes. Samples were then washed with ice-cold PBS and were kept on ice until measured by an Attune® Acoustic Focusing Cytometer.

**Supporting information available:** Cytotoxicity data on the studied cancer cell line pairs and corresponding selectivity ratios, as well as data of the calcein accumulation assay are presented as supplementary Tables S1-S4, and correlation of $IC_{50}$ values on the $L5178_{B1}$, MCF-7 and $MCF\text{-}7_{Dox}$ cell lines with those on the L5178 cells as Figure S1.

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