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# Phenylsulfonylfuroxans as Modulators of Multidrug-Resistance-Associated Protein-1 and P-Glycoprotein

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A series of furoxan derivatives were studied for their ability to interact with P-gp and MRP1 transporters in MDCK cells overexpressing these proteins. 3-Phenylsulfonyl substituted furoxans emerged as the most interesting compounds. All of them were capable of inhibiting P-gp, and a few also were capable of inhibiting MRP1. Substituents at the 4-position of 3-phenylsulfonylfuroxan scaffold were able to modulate the selectivity and the intensity of inhibition. In some cases, they reverted MRP1 inhibitor activity, namely, they were capable of potentiating MRP1 dependent efflux. When compounds **16** and **17** were coadministered with doxorubicin, they restored a high degree of the activity of the antibiotic. Preliminary immunoblotting studies carried out on these two compounds indicate that they are capable of nitrating P-gp, which in this form is likely unable to efflux the antibiotic.

## Introduction

Multidrug resistance (MDR[a]) generates cancer cells unresponsive to antineoplastic drugs treatment through several mechanisms.[1] Among them, the most extensively studied is the increased efflux of chemotherapeutic agents from cells due to a number of ATP binding cassette (ABC) transporters.[2]

Human ABC transporters belong to a family of 49 genes classified into seven subfamilies: ABC-**A**, ABC-**B**, ABC-**C**, ABC-**D**, ABC-**E**, ABC-**F**, ABC-**G**.[3,4] They use the energy of ATP hydrolysis to extrude compounds by a complex translocation process.[5] Some of these transporters play physiological functions in several barriers and are also involved in CNS disorders such as Alzheimer's disease, Parkinson's disease, and epilepsy.[6] They are localized in the luminal membrane of the endothelial cells constituting barriers such as the blood−brain barrier, blood−cerebrospinal fluid barrier, and blood−testis barrier.[7] This strategic localization permits modulation of the absorption and excretion of xenobiotics across these barriers.[8]

Moreover, because they efflux drugs,[9,10] reducing their concentration in tumor cells, overexpression of transporters in several tumor cell lines and tumor tissues results in MDR. Among these transporters, ABC-**B1**, better known as P-glycoprotein (P-gp), and ABC-**C1**−**6**, multidrug resistance associated proteins (MRP1−6) are the most representative pumps involved in MDR. P-gp contains 12 transmembrane helices organized in two membrane spanning domains (MSDs), each containing six transmembrane helices and two nucleotide binding domains (NBDs) responsible for ATP binding. MRP1−6 transporters differ from P-gp because they present three MSDs and the additional domain contains five transmembrane domains.[11]

Several strategies[5,10] have been employed to reverse MDR including coadministration of the antineoplastic agents with a MDR inhibitor such as elacridar, tariquidar and laniquidar (Chart 1). These complex molecules, belonging to third generation P-gp inhibitors, have been studied in clinical trials.[12−17] Preliminary results show that they have significant pharmacokinetic and pharmacodynamic limitations. In particular, they inhibited the CYP3A4 enzyme affecting chemotherapeutic detoxification and they showed poor selectivity toward other ABC transporters not involved in MDR.[17]

Recently, it has been reported that reduced endogenous NO production could be a possible mechanism responsible for MDR in HT29-dx doxorubicin-resistant human colon cancer cells and consequently that its restoration reverses resistance.[18] Moreover, exogenous NO influences the development of MDR. Indeed, it was found that *S*-nitroso-*N*-acetyl-D,L-penicillamine, *S*-nitrosoglutathione, and sodium nitroprusside, three well-known NO-donors, when used at 100 $\mu$M, markedly reduced the efflux of doxorubicin in HT29-dx and induced tyrosine nitration in MRPs transporters.[18] In addition, other studies reported that an inadequate supply of oxygen could induce MDR in solid cancers because several conventional anticancer drugs require oxygen for their maximal activity.[19] It has been demonstrated that hypoxia-induced MDR could be reverted by low concentrations (from 0.1 nM to 1 $\mu$M) of NO mimetics.[20,21]

Furoxan (1,2,5-oxadiazole 2-oxide) (**1**) (Figure 1) is an old heterocyclic system well-known to chemists because of its

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[a]Abbreviations: P-gp, P-glycoprotein; MDCK, Madin−Darby canine kidney; MRP, multidrug resistance associated proteins; MDR, multidrug resistance; ABC, ATP binding cassette; MSDs, membrane spanning domains; NBDs, nucleotide binding domains; NO, nitric oxide; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide.



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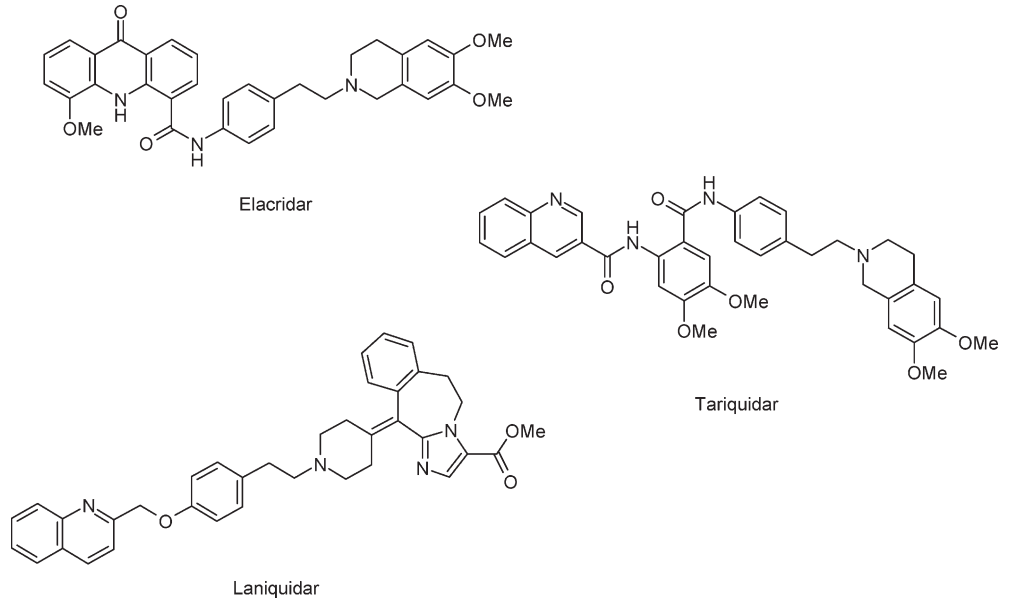
**Chart 1.** P-gp Inhibitors



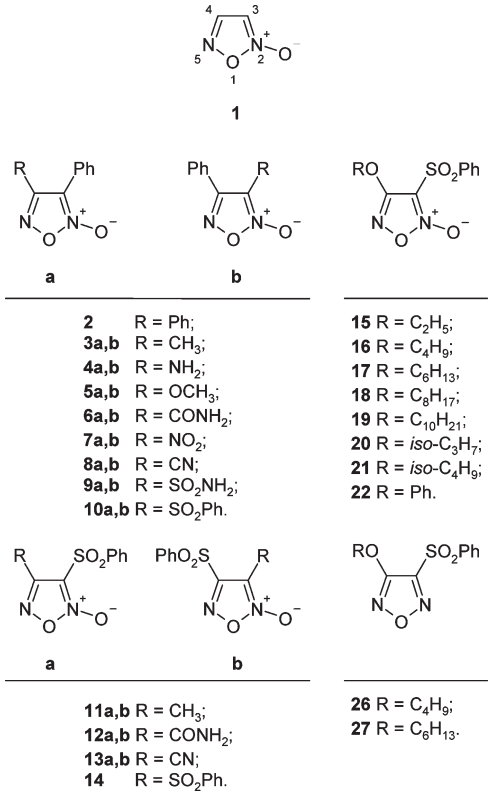




**Figure 1.** Phenylfuroxan, phenylsulfonylfuroxan, and phenylsulfonylfurazan derivatives.

intriguing chemistry and a dispute regarding its structure.[22] In the recent past, there has been renewed interest in furoxan derivatives because it was found that they can release nitric oxide under the action of thiols.[23−25] The mechanism of this release is complex and not yet fully understood. The first step most likely involves interaction of the electrophilic 3-position of furoxan ring with the nucleophilic −SH group, followed by ring opening and then by NO release. A number of biological actions of furoxans are associated, or likely associated, with NO release, following the interaction of these products with free intracellular thiols or thiol groups of proteins.[26−29]

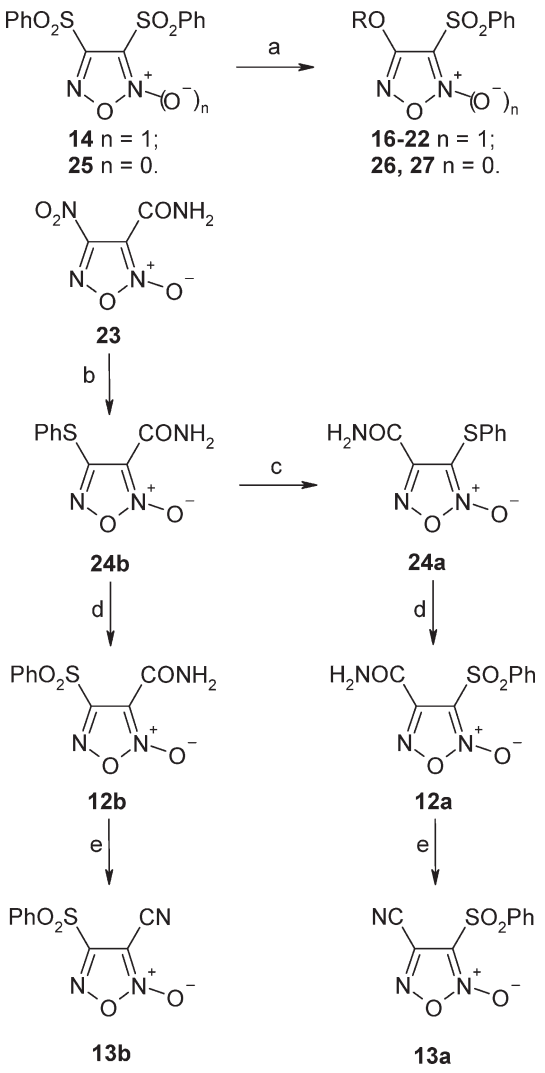
The present work represents a first attempt to develop MDR modulating agents bearing a furoxan ring as the structural determinant. For this purpose, an extended series of furoxan derivatives have been tested for their ability to interact with P-gp and MRP1 transporters in MDCK cells overexpressing these proteins. The structures of the compounds studied are collected in Figure 1.

First, we examined diphenylfuroxan derivative (**2**) and a series of 3-/4-phenylfuroxan isomer pairs (**a**,**b**) bearing at 4-/3-positions R groups with different stereoelectronic and lipophilic properties (**3a**,**b**−**10a**,**b**). From the screening of these products, the isomer pair **10a** and **10b**, bearing as an R substituent the electron-withdrawing and highly lipophilic phenylsulfonyl group, emerged as the most interesting. Consequently, differently substituted phenylsulfonylfuroxan isomer pairs (**11a**,**b**−**13a**,**b**) and bis(phenylsulfonyl)furoxan derivative **14** were considered. In addition, a number of 3-phenylsulfonyl substituted furoxans bearing alkoxy groups at the 4-position (**15**−**21**), characterized by having different sizes, shapes, and lipophilicity, or a phenoxy moiety (**22**) as well as the two furazans (1,2,5-oxadiazoles) **26** and **27** were taken into account.

## Results

**Chemistry.** Most of compounds listed in Figure 1 (**2**, **3a**, **b**−**11a**,**b**, **14**, and **15**) were prepared according to published methods (see Experimental Section). The synthetic pathways used to prepare the remaining products are reported in Scheme 1. The action of thiophenol on the already described 4-nitrofuroxan-3-carboxamide (**23**) in acetonitrile solution afforded the expected phenylthiofuroxan derivative **24b**. This product was partly transformed into the isomer **24a** by irradiation with the full mercury arc of an immersion medium pressure lamp. The isomer mixture was enriched in **24a** by grinding in cold methanol and then resolved by flash chromatography. The two isomers treated with pertrifluoroacetic acid afforded the expected phenylsulfonylfuroxancarboxamides **12a** and **12b**, respectively. The two furoxancarboxamide isomers, dissolved in THF, were treated under nitrogen with pyridine and then with trifluoroacetic

**Scheme 1.** Synthesis of Phenylsulfonylfuroxans **12a**,**b**−**13a**,**b**, **15**−**22**, and Phenylsulfonylfurazans **26** and **27**[a]




[a] Reaction conditions: (a) ROH, DBU, $CH_2Cl_2$, room temp; (b) PhSH, $Et_3N$, MeCN, −10 °C; (c) *hν*, $CH_2Cl_2$, room temp; (d) $H_2O_2$, $CF_3COOH$; (e) $(CF_3CO)_2O$, Py, THF, 0 °C.

anhydride to yield the corresponding furoxancarbonitriles **13a** and **13b**. The structures assigned to these compounds were confirmed by $^{13}C$ NMR spectroscopy, on the basis of the knowledge that in a furoxan isomer pair the $N^+-O^-$ moiety exerts a shielding influence on the resonance of the $^{13}C$ linked to the 3-position of the ring with respect to the corresponding $^{13}C$-linked to the 4-position.[30,31] Consequently, the structure of 4-CN ($-^{13}CN$, 105.9) and of 3-CN ($-^{13}CN$, 103.4 ppm) was assigned to **13a**, and **13b**, respectively.

The preparation of 4-alkoxy-3-phenylsulfonylfuroxans **16**−**21**, phenoxy substituted furoxan **22**, and 3-alkoxy-4-phenylsulfonylfurazans **26** and **27** was carried out in $CH_2Cl_2$ solution by treating the appropriate alcohols with **14** or with **25** in the case of furazans in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The 3-phenylsulfonyl structure was assigned to furoxan products on the basis of the knowledge that **14** undergoes selective nucleophilic displacement of the 4-phenylsulfonyl group by alcohols in THF under basic conditions.[32]

**Biochemical Studies.** P-gp and MRP1 inhibiting activities of tested compounds were performed by fluorescence measurement using calcein-AM fluorescent probes in MDCK-MDR1 and MDCK-MRP1 cell lines.[33] These cells overexpressed only P-gp or MRP1 transporters, respectively, so that the observed biological effects can be ascribed to the inhibition of these pumps.

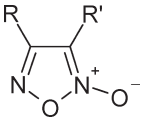
Calcein-AM is a lipophilic P-gp and MRP1 substrate able to cross the cell membrane. Inside the cell compartment it is hydrolyzed by endogenous cytoplasmic esterases, yielding highly fluorescent calcein. This hydrolyzed compound is not a P-gp or MRP1 substrate, and it cannot cross the cell membrane via passive diffusion because it is hydrophilic. Thus, a rapid increase in the fluorescence of cytoplasmic calcein can be monitored. P-gp and MRP1 transporters, present in the cell membrane, rapidly efflux the calcein-AM before its entrance into the cytosol, resulting in a reduction in the fluorescent signal due to a decrease in the accumulation of calcein. Evaluation of P-gp or MRP1 activity in the presence of pumps inhibitors can be performed in a competitive manner. Compounds that block P-gp and MRP1 pumps inhibit calcein-AM efflux, increasing fluorescent calcein accumulation. Calcein measurement into the cells was plotted versus log [drug], and for each compound an $EC_{50}$ value was obtained from nonlinear iterative curve fitting by Prism, version 3.0, GraphPad software.[34]

All compounds were tested for their inhibiting activity against P-gp and MRP1, and the results are reported in Table 1. All 3-phenylfuroxans derivatives **2** and **3a**−**10a** were found to be inactive ($EC_{50} > 100\ \mu M$) toward P-gp and MRP1, with the sole exception of compound **10a** bearing a 4-phenylsulfonyl substituent. This product displayed a moderate effect toward each transporter ($EC_{50} = 46$ and 26.5 $\mu$M, respectively). In the corresponding 4-phenylfuroxan series, compound **10b** displayed the best P-gp inhibiting activity ($EC_{50} = 10\ \mu M$) and a weak MRP1 blocking effect ($EC_{50} = 64\ \mu M$). Also in this series, 3-nitrofuroxan derivative **7b** displayed moderate inhibition toward P-gp ($EC_{50} = 53\ \mu M$), while it was a moderate inducer of the MRP1 pump ($EC_{50} = 45\ \mu M$). Moreover, the 3-CN substituted compound **8b** moderately inhibited the MRP1 pump ($EC_{50} = 42\ \mu M$), while it was inactive toward P-gp ($EC_{50} > 100\ \mu M$). All the other isomers belonging to 4-phenylfuroxan series were found to be inactive vs both P-gp and MRP1 ($EC_{50} > 100\ \mu M$).

Among 3-phenylsulfonyl and 4-phenylsulfonyl derivatives **11a**,**b**−**13a**,**b** and **14**, compounds **11a**−**13a**, belonging to the 3-phenylsulfonyl series, and 3,4-diphenylsulphonyl derivative **14** ($EC_{50}$ from 3.0 to 50 $\mu$M) were found to be more potent with respect to compound **10a** in inhibiting P-gp. In particular, the best results were obtained for compound **14** ($EC_{50} = 3.0\ \mu M$), while 4-carboxamide derivative **12a** displayed comparable activity with respect to **10a** ($EC_{50} = 50\ \mu M$ vs 46 $\mu$M). Moreover, compounds **12a**, **13a**, and **14** displayed unexpected MRP1 inducing activity ($EC_{50}$ from 15.1 to 85.8 $\mu$M), whereas 4-methylsubstituted furoxan **11a** was the best MRP1 inhibiting agent ($EC_{50} = 5.1\ \mu M$). In the corresponding 4-phenylsulfonyl series compounds **11b**−**13b** were found to be less potent than the corresponding isomers ($EC_{50} > 52.6\ \mu M$) in inhibiting the P-gp pump, while only compound **12b** inhibited MRP1 ($EC_{50} = 13\ \mu M$).

In Table 1 the results obtained working with phenoxy or alkoxy substituted furoxans **15**−**22** are reported. With the exception of 4-ethoxy (**15**) and 4-phenoxy (**22**) derivatives, which displayed moderate P-gp inhibitory activity ($EC_{50} = 12$ and 20.5 $\mu$M, respectively), all the other alkoxy derivatives **16**−**21** showed high P-gp inhibitory activity ($EC_{50}$ from 2.15 to 4.60 $\mu$M). This effect is not linearly dependent on their

**Table 1.** P-gp and MRP1 Modulating Activities of Phenylfuroxans (**2**−**10**) and Phenylsulfonylfuroxans (**11**−**22**)



| | | | $EC_{50}$ ($\mu$M) ± SEM[a] | | |
|---|---|---|---|---|---|
| | | | P-gp | MRP1 | |
| compd | R | R′ | | inhibiting activity | inducing activity |
| **2** | Ph | Ph | > 100 | > 100 | |
| **3a** | $CH_3$ | Ph | > 100 | > 100 | |
| **3b** | Ph | $CH_3$ | > 100 | > 100 | |
| **4a** | $NH_2$ | Ph | > 100 | > 100 | |
| **4b** | Ph | $NH_2$ | > 100 | > 100 | |
| **5a** | $OCH_3$ | Ph | > 100 | > 100 | |
| **5b** | Ph | $OCH_3$ | > 100 | > 100 | |
| **6a** | $CONH_2$ | Ph | > 100 | > 100 | |
| **6b** | Ph | $CONH_2$ | > 100 | > 100 | |
| **7a** | $NO_2$ | Ph | > 100 | 98 ± 5.0 | |
| **7b** | Ph | $NO_2$ | 53 ± 2.5 | | 45 ± 9.0 |
| **8a** | CN | Ph | > 100 | 85 ± 4.0 | |
| **8b** | Ph | CN | > 100 | 42 ± 1.5 | |
| **9a** | $SO_2NH_2$ | Ph | > 100 | > 100 | |
| **9b** | Ph | $SO_2NH_2$ | > 100 | > 100 | |
| **10a** | $SO_2Ph$ | Ph | 46 ± 2.5 | 26.5 ± 1.2 | |
| **10b** | Ph | $SO_2Ph$ | 10 ± 0.4 | 64 ± 2.5 | |
| **11a** | $CH_3$ | $SO_2Ph$ | 28.3 ± 5.7 | 5.1 ± 0.8 | |
| **11b** | $SO_2Ph$ | $CH_3$ | > 100 | > 100 | |
| **12a** | $CONH_2$ | $SO_2Ph$ | 50 ± 3.0 | | 85.8 ± 9.0 |
| **12b** | $SO_2Ph$ | $CONH_2$ | 52.6 ± 2.0 | 13 ± 1.2 | |
| **13a** | CN | $SO_2Ph$ | 7.4 ± 0.5 | | 29.5 ± 3.0 |
| **13b** | $SO_2Ph$ | CN | 55.4 ± 2.0 | | 72 ± 8.0 |
| **14** | $SO_2Ph$ | $SO_2Ph$ | 3.0 ± 0.2 | | 15.1 ± 2.0 |
| **15** | $OC_2H_5$ | $SO_2Ph$ | 12 ± 0.4 | | 49.1 ± 5.0 |
| **16** | $OC_4H_9$ | $SO_2Ph$ | 2.26 ± 0.2 | | 7.61 ± 0.8 |
| **17** | $OC_6H_{13}$ | $SO_2Ph$ | 3.35 ± 0.2 | | 12 ± 1.5 |
| **18** | $OC_8H_{17}$ | $SO_2Ph$ | 4.60 ± 0.1 | 8.7 ± 1.0 | |
| **19** | $OC_{10}H_{21}$ | $SO_2Ph$ | 3.31 ± 0.2 | 8.0 ± 0.9 | |
| **20** | O-iso-$C_3H_7$ | $SO_2Ph$ | 2.15 ± 0.5 | | 63 ± 8.0 |
| **21** | O-iso-$C_4H_9$ | $SO_2Ph$ | 2.23 ± 0.2 | | 46 ± 5.0 |
| **22** | OPh | $SO_2Ph$ | 20.5 ± 3.0 | | 13.6 ± 2.0 |
| MC18 | | | 1.50[b] | 2.80[b] | |
| MK571 | | | | 2.85[b] | |

[a] The values are the mean ± SEM from two independent experiments with samples in duplicate. [b] See ref 35.

lipophilicity. The most active products were *n*-butoxy **16** and the two branched alkoxy derivatives **20** and **21**. The behavior of this class of products against MRP1 protein is surprising. Indeed, the highly lipophilic substances **18** and **19**, bearing long aliphatic chains, display high inhibitor activity ($EC_{50}$ = 8.7 and 8.0 $\mu$M, respectively), while the remaining products **15**−**17** and **20**−**22** induced MRP1 activation, similar to compounds **12a**, **13a**, and **14**. This effect, shown in Figure 2 for compounds **14**, **16**, **17**, was seen as lower intracellular calcein accumulation in treated cells than basal levels in untreated cells. The best activation was observed with compounds **16** and **17** ($EC_{50}$ = 7.61 and 12 $\mu$M, respectively).

Compounds **14**, **16**, **17** and **19**−**21**, which displayed the best P-gp inhibitory activity ($EC_{50}$ from 2.15 to 3.35 $\mu$M), were tested at 10 $\mu$M in antiproliferative assay (MTT) employing MDCK-MDR1 cells, which are insensitive to doxorubicin antineoplastic treatment. Products **14**, **16**, **17**, and **19** showed very low antiproliferative action, while **20** and **21** decreased cell viability of 30% and 45%, respectively. For this reason, only **14**, **16**, **17**, and **19** were tested in MTT coadministrated with doxorubicin to check their ability to restore the toxicity of the antineoplastic agent. The results are reported in Figure 3

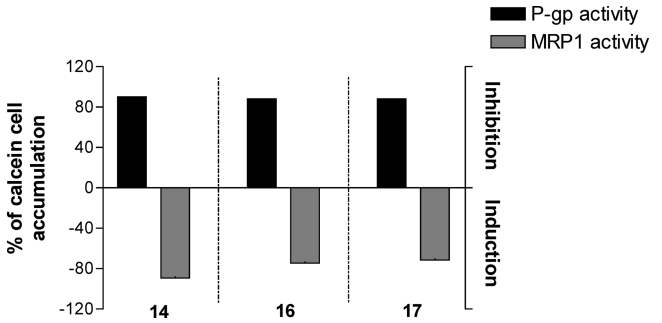



**Figure 2.** Calcein efflux P-gp- and MRP1-mediated. Gray bars indicate the inducer activity of compounds at 50 $\mu$M in MDCK-MRP1 cells, whereas black bars indicate inhibition activity at 50 $\mu$M tested compounds in MCDK-MDR1 cells.

To have a preliminary indication of whether NO could be involved in the mechanism of doxorubicin accumulation induced by 3-phenylsulfonylfuroxan derivatives, MDCK-MDR1

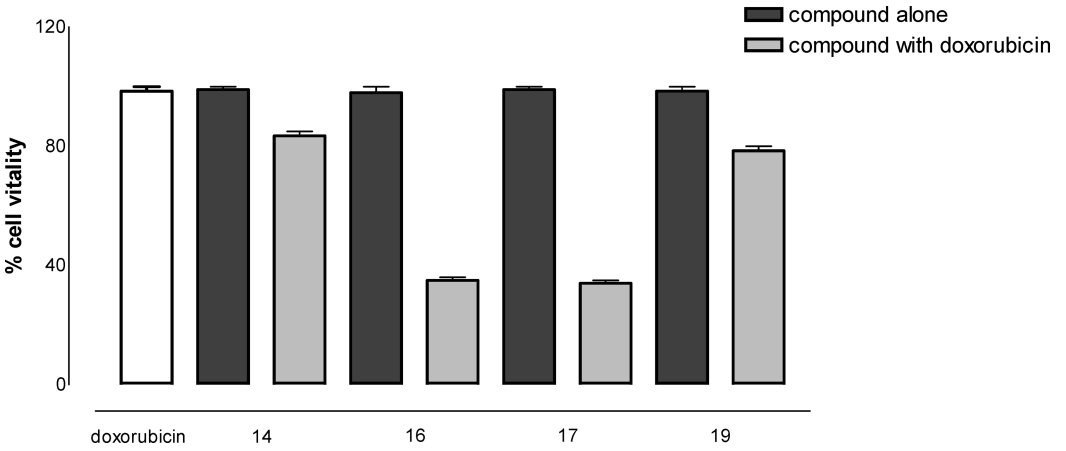



**Figure 3.** Antiproliferative effect of 0.1 $\mu$M doxorubicin (white bar) and of 10 $\mu$M compounds **14**, **16**, **17**, **19** (black bars) at 48 h in MDCK-MDR1 cells. In comparison, each compound (10 $\mu$M) was administered for 24 h. After washout, each compound was coadministrated with doxorubicin (0.1 $\mu$M) at the same concentration for 24 h (gray bars).

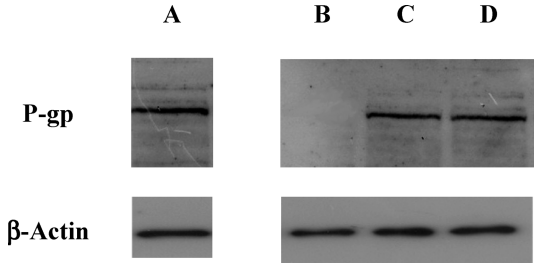


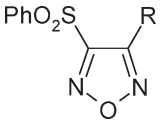

**Figure 4.** Immunoprecipitation and immunoblotting experiments in MCDK-MDR1 cells: detection of P-gp (A) and nitro-Pgp bands in no treated cells (B) and in cells treated with 10 $\mu$M compounds **16** (C) and **17** (D).

cells were incubated for 2 days with either **16** or **17**. The whole cellular lysate was then immunoprecipitated with a specific anti-nitrotyrosine antibody, and immunoprecipitated proteins were subjected to Western blotting, using anti P-gp antibody (Figure 4). In these experiments $\beta$-actin levels were used as protein loading control. Figure 4 indicates a high level of nitrotyrosine residues in the cells treated with the two furoxans.

## Discussion

The activity profile of furoxans as modulators of MRP1 and P-gp (Table 1) is not simple, and its interpretation requires attentive study. The preliminary results obtained in the immunoblotting experiments working with **16** and **17** support the possibility that NO is involved in P-gp inhibitory activity of 3-phenylsulfonylfuroxan derivatives. The NO responsible for nitration could be derived from their interaction with free cellular thiols. Another interesting possibility is that NO might be produced from a nucleophilic attack at the 3-position of the furoxan system by a cysteine residue of the protein transporter. This attack should induce the formation of a tetrahedral intermediate, followed by sequential ring opening to quaternary nitroso derivative, NO production, and the probable release of heterocycle scaffold from protein. Then NO so formed could nitrate the transporter, inducing modulation of its activity. In this case, the whole process should depend not only upon the electrophilic reactivity of the 3-furoxan position but also upon both the docking of the product to the protein and its appropriate alignment with the

**Table 2.** P-gp and MRP1 Inhibiting Activities of Phenylsulfonylfurazans (**26**, **27**)




| | | $EC_{50} \pm SEM^a$ ($\mu$M) | |
|---|---|---|---|
| compd | R | P-gp | MRP1 |
| **26** | $OC_4H_9$ | $49.7 \pm 2.5$ | $>100$ |
| **27** | $OC_6H_{13}$ | $32.2 \pm 1.6$ | $>100$ |
| MC18 | | 1.50[b] | 2.80[b] |
| MK571 | | | 2.85[b] |

[a] The values are the mean $\pm$ SEM from two independent experiments with samples in duplicate. [b] See ref 35.

nucleophilic $-$SH group. A similar mechanism of NO release was recently proposed to explain the capacity of furoxan derivatives to inhibit thioredoxin glutathione reductase (TGR), a selenocysteine-containing flavoenzyme required by trematode flatwarms of the genus *Schistosoma* to maintain proper cellular redox balance.[29] Analogously, the moderate activity of the two furazans **26** and **27** listed in Table 2 ($EC_{50}$ = 49.7 and 32.2 $\mu$M, respectively), which were synthesized for comparison since they were deprived of the capacity to release NO, could arise from the interaction of the $-$SH group of transporter with the carbon of the heteroring bearing the phenylsulfonyl substituent. This interaction should lead to the formation of tetrahedral intermediate followed by the simple displacement of phenylsulfonyl, a good leaving group under the action of thiols in furazan system, without any ring opening and NO release.

The results reported in Figure 3 show that combination treatment of 0.1 $\mu$M doxorubicin with either 10 $\mu$M **14** or **19** partly decreased cell viability (15% and 20%, respectively), while compounds **16** and **17** at the same concentration induce a comparable high viability decrease (65%). These results, combined with those of Western blotting, suggest that NO inhibits the efflux of doxorubicin from MDCK-MDR1 cells and provide proof that furoxan derivatives can reverse drug tolerance through NO-dependent pump inactivation. Likely, NO reduces the number of functionally active transporters modifying the conformation of a site crucial for the drug transport.

Finally, the finding that some furoxans, in particular compounds **16** and **17** (Figure 2), are able to potentiate MRP1 dependent efflux is interesting. Indeed, while P-gp inducers have been already reported and studied,[36] MRPs inducing agents were not as yet described in literature. These products are potential tools to investigate MRP1 induction mechanism and to characterize specific binding sites for substrates, inhibitors, and inducers of this transporter.[37]

## Conclusions

The results obtained in the present work show that the furoxan system can be used to design both P-gp and MRP1 ligands. In particular, 3-phenylsulfonyl substituted furoxan appears to be the most interesting and flexible scaffold for designing inhibitors of both transporters. Substituents at the 4-position of this substructure can modulate the selectivity and the intensity of inhibition. In some cases, they can display interesting MRP1 inducing activity; namely, they can potentiate MRP1 dependent efflux. Results obtained working with selected members of the 3-phenylsulfonylfuroxan series show that the compounds **16** and **17** are able to restore in high degree the antiproliferative activity of doxorubicin when coadministered with this antineoplastic agent. Preliminary immunoblotting studies carried out on these two same compounds indicate that they are capable of nitrating P-gp, which in this form is likely unable to efflux the chemotherapeutic agent.

## Experimental Section

**Chemistry.** $^1$H and $^{13}$C NMR spectra were recorded on a Bruker Avance 300 at 300 and 75 MHz, respectively, using $SiMe_4$ as the internal standard. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM). PE stands for 40−60 petroleum ether. The progress of the reactions was followed by thin layer chromatography (TLC) on 5 cm × 20 cm plates with a layer thickness of 0.2 mm. Anhydrous magnesium sulfate was used as the drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Purities of all new compounds were ≥95% and were determined by elemental analysis and HPLC. Elemental analyses (C, H, N) of the new compounds dried at 20 °C at a pressure of < 10 mmHg for 24 h were performed at the University of Geneva, Switzerland, and the results are within ±0.4% of the theoretical values. HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode array detector (DAD) (model G1315B) integrated in the HP1100 system, and a Zorbax Extend $C_{18}$ column (150 mm × 4.6 mm, 5 $\mu$m particle size). Data analysis was done using a HP ChemStation system (Agilent Technologies). Compounds **9a**,**b**[38] and **23**[39] were synthesized according to the literature. The preparation of products **2**, **3a**,**b**−**11a**,**b**, **14**, **25**, and **15** is reported in a number of references that are collected in the bibliography of reviews 22 and 40.

**4-Phenylthiofuroxan-3-carboxamide (24b).** To a solution of **23** (2.1 g, 12.0 mmol) in $CH_3CN$ (25 mL) a mixture of $Et_3N$ (1.7 mL, 12.0 mmol) and thiophenol (1.3 mL, 13 mmol) in $CH_3CN$ (15 mL) was added dropwise at −10 °C. The reaction mixture was stirred at −10 °C for 1 h, then poured into $H_2O$ (75 mL) and extracted with $CH_2Cl_2$ (2 × 50 mL). The organic solvent was washed with $H_2O$ (30 mL), brine, dried, and evaporated. The obtained solid was washed with cold MeOH, filtered, and used without further purification. Yield 79%. An analytically pure sample was obtained by crystallization, mp = 146−149 °C (dec MeOH). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 7.25−7.56 (m, 3H), 7.67−7.74 (m, 2H) ($C_6H_5$), 7.78 (s, 1H), 8.56 (s, 1H) ($CONH_2$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 111.0, 125.6, 129.8, 130.3, 135.0, 155.7, 157.0. MS (EI) $m/z$ 237 $[M]^+$. Anal. ($C_9H_7N_3O_3S$) C, H, N.

**3-Phenylthiofuroxan-4-carboxamide (24a).** A solution of **24b** (5.5 g, 23.0 mmol) in $CH_2Cl_2$ (100 mL) in a quartz reactor was irradiated with the full mercury arc of an immersion medium pressure lamp (125W, Photochemical Reactors Ltd., Buckinghamshire, HP16 ODR, U.K.) for 1 h at room temperature. The organic solvent was removed under reduced pressure, and the obtained solid was treated with cold MeOH (50 mL). The resulting mixture was filtered and the filtrate was evaporated to give a mixture of two isomers in ∼1:1 molar ratio. The title product was partially purified by flash chromatography (eluent 7/3 PE/AcOEt) and finally crystallized from $CCl_4$ to give pale-yellow crystals. Yield 10%, mp = 115.5−116.5 °C ($CCl_4$). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 7.37−7.45 (m, 5H, $C_6H_5$), 8.28 (s, 1H), 8.55 (s, 1H) ($CONH_2$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 109.9, 127.8, 128.5, 129.5, 130.7, 153.3, 157.6. MS (EI) $m/z$ 237 $[M]^+$. Anal. ($C_9H_7N_3O_3S$) C, H, N.

**4-Phenylsulfonylfuroxan-3-carboxamide (12b).** To a solution of **24b** (0.50 g, 2.1 mmol) in a $CH_2Cl_2/CF_3COOH$ mixture (5 mL/5 mL) a solution of 88% $H_2O_2$ (0.5 mL, 18 mmol) in $CF_3COOH$ (5 mL) was added dropwise at 0 °C. The cooling bath was removed, and the mixture was stirred at room temperature for 2 h. Then it was poured into $H_2O$ (50 mL) and extracted with EtOAc (2 × 30 mL). The organic extract was washed with $H_2O$, brine, dried, and evaporated. The obtained solid was crystallized from MeOH to give the title compound as a white crystalline solid. Yield 68%, mp = 188−188.5 °C (MeOH). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 7.74−7.88 (m, 2H), 7.88−7.93 (m, 1H), 8.10−8.13 (m, 3H), 8.57 ppm (s, 1H) ($C_6H_5 + CONH_2$). $^{13}$C NMR (75 MHz, DMSO- $d_6$) $\delta$ ppm 109.1, 129.3, 129.8, 135.9, 136.6, 153.7, 157.4. MS (CI) $m/z$ 270 $[M + H]^+$. Anal. ($C_9H_7N_3O_5S$) C, H, N.

**3-Phenylsulfonylfuroxan-4-carboxamide (12a).** The product was obtained by the same procedure used to synthesized **12b**, starting from **24a**. Yield 24%, mp = 177.5−178 °C (*i*-PrOH/$H_2O$). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 7.75−7.81 (m, 2H), 7.89−7.94 (m, 1H), 8.08−8.11 (m, 2H) ($C_6H_5$), 8.49 (s, 1H), 8.68 (s, 1H) ($CONH_2$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 115.6, 128.6, 129.6, 136.2, 136.6, 150.5, 156.6. MS (CI) $m/z$ 270 $[M + H]^+$. Anal. ($C_9H_7N_3O_5S$) C, H, N.

**4-Phenylsulfonylfuroxan-3-carbonitrile (13b).** To a solution of **12b** (0.30 g, 1.1 mmol) in dry THF (15 mL) kept under positive $N_2$ pressure, pyridine (0.18 mL, 2.2 mmol) was added at 0 °C followed by $(CF_3CO)_2O$ (0.30 mL, 2.2 mmol). The cooling bath was removed, and the mixture was stirred at room temperature for 1 h. Then it was poured into $H_2O$ (50 mL) and extracted with EtOAc (2 × 25 mL). The organic extract was washed with $H_2O$, brine, dried, and evaporated. The obtained oil was solidified by treating with cold PE and crystallized from hexane to give the title compound as a white crystalline solid. Yield 81%, mp = 89.5−90 °C ($C_6H_{14}$). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 7.70−7.75 (m, 2H), 7.83−7.90 (m, 1H), 8.13−8.16 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$): $\delta$ ppm 93.8, 103.4, 129.4, 130.4, 136.0, 136.6, 157.1. MS (EI) $m/z$ 251 $[M]^+$. Anal. ($C_9H_5N_3O_4S$) C, H, N.

**3-Phenylsulfonylfuroxan-4-carbonitrile (13a).** The product was obtained by the same procedure used to synthesize **13b**, starting from **12a**. Yield 64%, mp = 103−103.5 °C ($C_6H_{14}$). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 7.68−7.73 (m, 2H), 7.83−7.88 (m, 1H), 8.09−8.16 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 105.9, 115.0, 129.0, 129.9, 130.3, 136.4, 136.8. MS (EI) $m/z$ 251 $[M]^+$. Anal. ($C_9H_5N_3O_4S$) C, H, N.

**General Procedure for the Synthesis of 4-Alkyl(aryl)oxy-3-phenylsulfonylfuroxans (16−22).** To a mixture of the corresponding alcohol or phenol (1.5 mmol) and DBU (3.0 mmol) in $CH_2Cl_2$ (15 mL), 3,4-bisphenylsulfonylfuroxan (1.0 mmol) was added in one portion. The reaction mixture was stirred at

room temperature for 2 h. Then the organic solvent was washed with $H_2O$ (20 mL), 1 M HCl (2 × 10 mL), brine, dried, and evaporated. The obtained solid was purified by crystallization.

**4-Butoxy-3-phenylsulfonylfuroxan (16).** Yield 75%, mp = 96−97 °C (*i*-PrOH). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 0.93 (t, 3H, C$H_3$), 1.33−1.45 (m, 2H, $CH_3CH_2$), 1.69−1.79 (m, 2H, $OCH_2CH_2$), 4.40 (t, 2H, $OCH_2$), 7.74−7.79 (m, 2H), 7.89−7.94 (m, 1H), 8.01−8.04 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 13.4, 18.3, 29.9, 71.2, 110.4, 128.3, 130.0, 136.1, 137.2, 158.9. MS (CI) $m/z$ 299 $[M + H]^+$. Anal. ($C_{12}H_{14}N_2O_5S$) C, H, N.

**4-Hexyloxy-3-phenylsulfonylfuroxan (17).** Yield 55%, mp = 72−73 °C (*i*-PrOH/$H_2O$). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 0.87 (t, 3H, C$H_3$), 1.25−1.43 (m, 6H, 3C$H_2$), 1.75−1.84 (m, 2H, $OCH_2CH_2$), 4.34 (t, 2H, $OCH_2$), 7.52−7.57 (m, 2H), 7.66−7.71 (m, 1H), 7.86−8.00 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 14.1, 22.6, 25.4, 28.5, 31.4, 71.8, 110.6, 128.7, 129.8, 135.7, 138.3, 159.2. MS (CI) $m/z$ 327 $[M + H]^+$. Anal. ($C_{14}H_{18}N_2O_5S \cdot 0.25H_2O$) C, H, N.

**4-Octyloxy-3-phenylsulfonylfuroxan (18).** Yield 90%, mp = 78−80 °C (MeOH/$H_2O$). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 0.87 (t, 3H, C$H_3$), 1.24−1.38 (m, 10H, 5C$H_2$), 1.70−1.77 (m, 2H, C$H_2$), 4.39 (t, 2H, $OCH_2$), 7.73−7.80 (m, 2H), 7.88−7.93 (m, 1H), 8.00−8.03 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 13.7, 21.8, 24.8, 27.6, 28.2, 28.3, 30.9, 71.2, 110.1, 128.0, 129.7, 135.9, 137.0, 158.6. MS (CI) $m/z$ 355 $[M + H]^+$. Anal. ($C_{16}H_{22}N_2O_5S$) C, H, N.

**4-Decyloxy-3-phenylsulfonylfuroxan (19).** Yield 88%, mp = 65−67 °C (MeOH/$H_2O$). $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 0.84 (t, 3H, C$H_3$), 1.12−1.35 (m, 14H, 7C$H_2$), 1.72−1.74 (m, 2H, C$H_2$), 4.38 (t, 2H, $OCH_2$), 7.70−7.75 (m, 2H), 7.88−7.93 (m, 1H), 8.00−8.03 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 14.2, 22.4, 25.3, 28.1, 28.8, 29.0, 29.2, 31.6, 71.7, 110.7, 128.6, 130.3, 136.4, 137.6, 159.2. MS (CI) $m/z$ 383 $[M + H]^+$. Anal. ($C_{18}H_{26}N_2O_5S$) C, H, N.

**4-Isopropoxy-3-phenylsulfonylfuroxan (20).** Yield 37%, mp = 101−103 °C (*i*-PrOH). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 1.47 (d, 6H, 2C$H_3$), 5.10 (qi, 1H, OC$H$), 7.60−7.64 (m, 2H), 7.73−7.78 (m, 1H), 8.04−8.07 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 21.1, 76.4, 110.4, 128.3, 129.9, 136.0, 137.2, 158.0. MS (CI) $m/z$ 285 $[M + H]^+$. Anal. ($C_{11}H_{12}N_2O_5S$) C, H, N.

**4-Isobutoxy-3-phenylsulfonylfuroxan (21).** Yield 94%, mp 100−102 °C (*i*-PrOH). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 1.05 (d, 6H, 2C$H_3$), 2.21 (qi, 1H, $CH_2CH$), 4.18 (d, 2H, $OCH_2$), 7.60−7.65 (m, 2H), 7.73−7.79 (m, 1H), 8.05−8.08 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 18.8, 27.8, 77.2, 110.4, 128.5, 129.7, 135.6, 138.3, 159.2. MS (CI) $m/z$ 299 $[M + H]^+$. Anal. ($C_{12}H_{14}N_2O_5S$) C, H, N.

**4-Phenyloxy-3-phenylsulfonylfuroxan (22).** Yield 60%, mp 102−104 °C (*i*-PrOH). $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 7.29−7.34 (m, 3H), 7.42−7.47 (m, 2H), 7.61−7.71 (m, 2H), 7.76−7.81 (m, 1H), 8.08−8.11 (m, 2H) (2$C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 110.8, 119.8, 126.8, 128.6, 129.8, 130.0, 135.8, 138.0, 152.6, 158.5. MS (CI) $m/z$ 319 $[M + H]^+$. Anal. ($C_{14}H_{10}N_2O_5S$) C, H, N.

**General Procedure for the Synthesis of 3-Alkyloxy-4-phenylsulfonylfurazans (26, 27).** To a mixture of the appropriate alcohol (1.5 mmol) and DBU (3.0 mmol) in $CH_2Cl_2$ (15 mL), 3,4-bisphenylsulfonylfurazan (1.0 mmol) was added in one portion, and the reaction mixture was stirred at room temperature for 24 h. Then the organic solvent was washed with $H_2O$ (20 mL), 1 M HCl (2 × 10 mL), brine, dried, and evaporated. The obtained oil was purified by flash chromatography.

**3-Butoxy-4-phenylsulfonylfurazan (26).** Eluent 1/1 PE/$CH_2Cl_2$ v/v, colorless oil. Yield 81%. $^1$H NMR (300 MHz, DMSO-$d_6$) $\delta$ ppm 0.97 (t, 3H, C$H_3$), 1.37−1.50 (m, 2H, $CH_3CH_2$), 1.77−1.86 (m, 2H, $OCH_2CH_2$), 4.37 (t, 2H, $OCH_2$), 7.60−7.65 (m, 2H), 7.73−7.79 (m, 1H), 8.08−8.12 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, DMSO-$d_6$) $\delta$ ppm 13.6, 18.8, 30.5, 73.7, 129.0, 129.6, 135.4, 137.9, 148.8, 161.4. MS (CI) $m/z$ 283 $[M + H]^+$. Anal. ($C_{12}H_{14}N_2O_4S$) C, H, N.

**3-Hexyloxy-4-phenylsulfonylfurazan (27).** Eluent 6/4 PE/$CH_2Cl_2$ v/v, colorless oil. Yield 80%. $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ ppm 0.91 (t, 3H, C$H_3$), 1.30−1.42 (m, 6H, 3C$H_2$), 1.80−1.87 (m, 2H, $OCH_2CH_2$), 4.36 (t, 2H, $OCH_2$), 7.60−7.65 (m, 2H), 7.73−7.78 (m, 1H), 8.08−8.11 (m, 2H) ($C_6H_5$). $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ ppm 14.0, 22.5, 25.2, 28.5, 31.3, 74.0, 129.0, 129.6, 135.4, 137.9, 148.8, 161.4. MS (CI) $m/z$ 311 $[M + H]^+$. Anal. ($C_{14}H_{18}N_2O_4S$) C, H, N.

**Biology. Materials.** Cell culture reagents were purchased from Celbio s.r.l. (Milan, Italy). CulturePlate 96-well plates were purchased from PerkinElmer Life Science; calcein-AM, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), doxorubicin, and all reagents for immunoprecipitation and immunoblotting assays were obtained from Sigma-Aldrich (Milan, Italy). MK571 was purchased from Calbiochem (San Diego, CA).

**Cell Cultures.** MDCK-MDR1 and MDCK-MRP1 are a gift of Prof. P. Borst, NKI-AVL Institute, Amsterdam, The Netherlands. MDCK-MDR1 and MDCK-MRP1 were grown in DMEM high glucose supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin in a humidified incubator at 37 °C with a 5% $CO_2$ atmosphere.

**Calcein-AM Experiment.** These experiments were carried out as described by Feng et al. with minor modifications.[33] Each cell line (50 000 cells per well) was seeded into a black CulturePlate 96-well plate with 100 $\mu$L of medium and allowed to become confluent overnight. An amount of 100 $\mu$L of test compounds was solubilized in culture medium and added to monolayers followed by incubation at 37 °C for 30 min. Calcein-AM was added in 100 $\mu$L of phosphate buffered saline (PBS) to yield a final concentration of 2.5 $\mu$M, and the plate was incubated for 30 min. Each well was washed three times with ice cold PBS. Saline buffer was added to each well, and the plate was read in a Victor3 microplate reader (PerkinElmer) at excitation and emission wavelengths of 485 and 535 nm, respectively. In these experimental conditions calcein cell accumulation in the absence and in the presence of tested compounds was evaluated and fluorescence basal level was estimated in untreated cells. In treated wells the increase of fluorescence with respect to basal level was measured. $EC_{50}$ values were determined by fitting the fluorescence increase percentage versus log [dose].[34]

**Antiproliferative Assay.** Determination of cell growth was performed using the MTT assay at 24 and 48 h.[41,42] On day 1, 20 000 cells/well were seeded into 96-well plates in a volume of 100 $\mu$L. On day 2, the various drugs alone or in combination with doxorubicin were added. In all the experiments, the various drug solvents (ethanol, DMSO) were added to each control to evaluate possible solvent cytotoxicity. After the established incubation time with drugs, 0.5 mg/mL MTT was added to each well, and after 3 h of incubation at 37 °C the supernatant was removed. The formazan crystals were solubilized using 100 $\mu$L of DMSO, and the absorbance values at 570 and 630 nm were determined on a microplate reader Victor 3.

**Immunoprecipitation and Immunoblotting Analysis.** In an immunoprecipitation assay, treated and untreated cells (2 days) were harvested and lysed in ice-cold lysis buffer (0.5 M Tris-HCl, 1.86 g/mL EDTA, 1 M NaCl, 0.001 g/mL digitonin, 4 U/mL aprotinin, 2 $\mu$M leupeptin, and 100 $\mu$M PMSF). Lysates were centrifuged at 13800*g* for 20 min at 4 °C. The protein concentration was determined by the Bradford method. Then 1 mL of clear lysates was incubated with 5 $\mu$L of mouse monoclonal anti-human P-glycoprotein (MDR) antibody overnight with continuous rotation at 4 °C. Protein A-sepharose beads (30 $\mu$L) were then added, and the samples were gently rocked at 4 °C for 3 h. After five washes with lysis buffer, the beads were recovered and resuspended in 40 $\mu$L of 2× SDS sample buffer (4% SDS, 0.125 mol/L Tris-HCl, 20% glycerol, and 0.04% bromophenol blue, pH 6.8) and then boiled for 5 min. The coimmunoprecipitation

(IP) proteins dissociated from the beads were used for immunoblotting analysis.

Immunoblotting was done as described below. Protein samples (25 $\mu$g/lane) and prestained standards were loaded onto 10% sodium dodecyl sulfate (SDS) precast polyacrylamide gels. After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blotting buffer (20 mM Tris/150 mM glycine, pH 8, 20% (v/v) methanol) was used for membrane saturation and blotting. Primary antibodies (mouse monoclonal anti-human P-glycoprotein (MDR), rabbit anti-nitrotyrosine, and $\beta$-actin monoclonal antibody, 1:2000) were used, followed by horseradish peroxidase-linked secondary antibody (goat anti-mouse IgG, goat anti-rabbit, 1:1000) and visualized by chemiluminescence detection.

**Supporting Information Available:** Elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

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