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

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Phenylsulfonyl furoxans as modulators of multidrug resistance-associated protein-1 (MRP1) and P-glycoprotein

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[a] Abbreviations: P-gp, P-glycoprotein; MDCK cells: Madin-Darby canine kidney cells; 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.

Abstract. A series of furoxan derivatives were studied for their ability of interacting with P-gp and MRP1 transporters in MDCK cell overexpressing these proteins. 3-Phenylsulfonyl substituted furoxans emerged as the most interesting compounds. Most of them behaved as inhibitors of both the transporters. Substituents at 4-position of 3-phenylfuroxan scaffold were able to modulate the selectivity and the intensity of inhibition. In some cases, they reverted MRP1 inhibition activity, namely they were capable of potentiating MRP1 dependent efflux. When compounds **16** and **17** were co-administered to doxorubicin they restored in high degree the activity of the antibiotic.

KEYWORDS: furoxans, nitric oxide, multidrug resistance, P-gp, MRP-1, doxorubicin.

Introduction

Multidrug resistance (MDR) determines unresponsive cancer cells 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 some 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 function in several barriers and are involved also 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 the barriers such as the Blood Brain Barrier, Blood-Cerebro Spinal Fluid Barrier, and Blood Testis Barrier.[7] This strategic localization permits to modulate the absorption and excretion of xenobiotics across these barriers.[8]

Moreover, some transporters are overexpressed in several tumor cell lines and tumor tissues causing MDR because they efflux drugs[9,10] reducing the concentration into tumor cells. Among these transporters, ABC-**B1**, better known as P-glycoprotein (P-gp) and ABC-**C1-6**, Multidrug Resistance associated Proteins (MRP_{1-6}) are the most representative pumps involved in MDR. P-gp contains twelve transmembrane helices organized in two Membrane Spanning Domains (MSDs), each containing six

transmembrane helices, and two Nucleotide Binding Domains (NBDs) responsible for ATP binding. MRP_{1-6} transporters differ from P-gp because they display three MSDs and the additional domain contains five transmembrane domains.[11]

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

Recently, it has been reported that a potential mechanism responsible for MDR onset could be mediated by nitric oxide (NO) concentration into the tumor cells. It has been demonstrated that in doxorubicin-sensitive and doxorubicin-resistant cells, displaying a different capacity to produce NO, a reduced synthesis of NO caused the onset of MDR and consequently the restoration of NO production reversed it.[18]

Also 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 μM concentration, markedly reduced the efflux of doxorubicin in doxorubicin-resistent cells (HT29-dx), and induced tyrosine nitration in MRPs transporters.[18] In addition, some studies reported that inadequate supply of oxygen, hypoxia, could induce MDR in solid cancers because several conventional anticancer drugs require oxygen for their maximal activity.[19] It has been demonstrated that MDR by hypoxia could be reverted by low concentrations (from 0.1 nM to 1 μM) of nitric oxide mimetics.[20,21]

Furoxan (**1**) (Figure 1) is an old heterocyclic system well known to chemists for an argument on its structure and for its intriguing chemistry.[22] In the recent past, there was a renewed interest in furoxan derivatives, since 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 should imply

interaction of the electrophilic furoxan ring with the nucleophilic SH group. A number of biological actions of furoxans are associated, or probably 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 furoxan ring as structural determinant. For this purpose, an extended series of furoxan derivatives have been tested to investigate their P-gp and MRP1 inhibitory activity. The structures of the studied compounds are collected in Figure 1.

At first, we examined diphenylfuroxan derivative (**2**) and a series of 3-/4-phenylfuroxan isomer pairs (**a**,**b**), bearing at 4-/3-positions R groups having different stereo-electronic and lipophilic properties (**3a**,**b-10a**,**b**). From the screening of these products, the isomer pair **10a** and **10b**, bearing as R substituent the electron-withdrawing and highly lipohilic 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, also a number of 3-phenylsulfonyl substituted furoxans, bearing at 4-position alkoxy groups (**15-21**), characterised by having different sizes, shapes and lipophilicity, or phenoxy moiety (**22**) were taken into account.

All compounds, were studied for their ability to interact with P-gp and MRP1 transporters in MDCK cell overexpressing these proteins.

Results and Discussion

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. 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 fully 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 anhydride, to yield the corresponding furoxancarbonitriles **13a** and **13b**. The structures assigned to these latter target 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 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 **15-21**, and of the analogue phenoxy substituted furoxan **22**, was carried out in CH_2Cl_2 solution, by treating the appropriate alcohol with **14**, in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU). The 3-phenylsulfonyl structure was assigned to these 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 have been 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 measured biological effects are ascribed to the inhibition towards these pumps.

Calcein-AM is a lipophilic MDR1 and MRP1 substrate able to cross the cell membrane. Into the cell compartment it is hydrolyzed by endogenous cytoplasmic esterases yielding highly fluorescent Calcein. This hydrolyzed compound is not a MDR1 and MRP1 substrate, and since it is hydrophilic, it cannot cross the cell membrane via passive diffusion. Thus, a rapid increase in the fluorescence of cytoplasmic Calcein can be monitored. MDR1 and MRP1 transporters, present in the cell membrane, rapidly effluxes the Calcein-AM before its entrance into the cytosol determining a reduction in the fluorescent signal due to the accumulation of Calcein. Evaluation of MDR1 or MRP1 activity in the presence of pumps inhibitors can be performed in a competitive manner. Compounds that block MDR1 and MRP1 pumps inhibit Calcein-AM efflux increasing fluorescent Calcein accumulation. Calcein measurement into the

cells has been plotted versus log[drug] and for each compound EC_{50} value has been obtained. The EC_{50} values were obtained from non-linear iterative curve fitting by Prism v.3.0, GraphPad software.[34]

Compounds **14**, **16**, **17** that displayed the best P-gp inhibitory activity (EC_{50} from 2.26 μM to 3.35 μM) have been tested in antiproliferative assay (MTT) employing MDCK-MDR1 cells insensitive to antineoplastic treatment, in doxorubicin co-administration to verify the ability of each compound to restore doxorubicin activity. Preliminary, each ligand has been tested alone to subtract its antiproliferative effect measured at 24 h due to intrinsic activity of ligand.

All compounds were tested for their inhibiting activity towards P-gp and MRP1 and the results are reported in Table 1. All 3-phenylfuroxans derivatives **2**, **3a-10a** were found inactive (EC_{50} > 100 μM) towards P-gp and MRP1 excepting for compound **10a** bearing a phenylsulfonyl substituent that displayed moderate effect towards each transporter (EC_{50} = 46 μM and 26.5 μM, respectively). In the corresponding 4-phenylfuroxan series, compound **10b** displayed the best P-gp inhibiting activity (EC_{50} = 10 μM) and moderate MRP1 blocking effect (EC_{50} = 64 μM). In this series compound 4-nitrofuroxan derivative **7b** displayed moderate inhibition towards P-gp (EC_{50} = 53 μM) while it was a feeble inducer towards MRP1 pump (EC_{50} = 45 μM). Moreover, the 3-CN substituted compound **8b** weakly inhibited MRP1 pump (EC_{50} = 42 μM) while it was inactive towards P-gp (EC_{50} > 100 μM). All the other isomers belonging to 4-phenylfuroxan series were found inactive both *vs* P-gp and MRP1 (EC_{50} > 100 μM).

Starting from this finding, 3-phenylsulfonyl and 4-phenylsulfonyl derivatives **11a**,**b-13a**,**b** and **14** have been tested. In 3-phenylsulfonyl series, compounds **11a-13a** and **14** (EC_{50} from 3.0 μM to 50 μM) were found more potent with respect to compound **10a** in inhibiting P-gp. In particular the best results have been obtained for 3,4-diphenylsulfonyl derivative **14** (EC_{50} = 3.0 μM), while 4-carboxamide derivative **12a** displayed comparable activity with respect to **10a** (EC_{50} = 50 μM *vs* 46 μM). Moreover, compounds **12a**, **13a** and **14** displayed an interesting MRP1 inducing activity (EC_{50} from 15.1 μM to 85.8 μM), whereas 4-methylsubstituted furoxan **11a** was the best MRP1 inhibiting agent (EC_{50} = 5.1 μM). In the corresponding 4-phenylsulfonyl series compound **11b-13b** were found lower potent than the

corresponding isomers (EC_{50} > 52.6 μM) in inhibiting P-gp pump, while only compound **12b** moderately inhibited MRP1 (EC_{50} = 13 μM).

In Table 1 the results obtained working with phenoxy or alkoxy substituted furoxans **22**, **15-21** are reported. With exception of 4-ethoxy (**15**) and 4-phenoxy (**22**) derivatives, which displayed moderate P-gp inhibitory activity (EC_{50} = 12 μM and 20.5 μM, respectively), all the other alkoxy derivatives **16-21** triggered high P-gp inhibition activity (EC_{50} from 2.15 μM to 4.60 μM). This effect appear to be not linearly dependent on their lipophilicity. The most active products were *n*-butoxy **16**, and the two branched alkoxy derivatives **18**, **19**. The behaviour of this class of products against MRP1 protein is surprising. Indeed, the highly lipophilic products **18** and **19**, bearing long aliphatic chains, display high inhibitor activity(EC_{50} = 8.7 μM and 8.0 μM, respectively), while the remaining products **15-17**, **20-22** induced MRP1 activation. The best results were found for compounds **16** and **17** (EC_{50} = 7.61 μM and 12 μM, respectively).

Finally, compounds **14**, **16** and **17** were co-administered to 0.1 μM doxorubicin obtaining interesting results. Analysis of the data reported in Figure 2 shows that compound **14** at 10 μM, decreased (15%) cell viability partially restoring the activity of doxorubicin. At the same concentration compounds **16** and **17** displayed high and superimposed decrease of cell viability (65%).

Conclusions

The results obtained in the present work show that furoxan system can be used to plan 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 4-position of this substructure can modulate the selectivity and the intensity of inhibition. In some cases, they can revert MRP1 inhibition activity, namely they can potentiate MRP1 dependent efflux. The results obtained with selected members of the series (**14**, **16**, **17**) in co-administration with doxorubicin, in doxorubicin-resistant cells, indicates that furoxan is an interesting and promising scaffold in developing new MDR inhibitors. In order to rationalize the complex pattern of SAR which arises from this study, including the

role exerted by NO, it is necessary to shed light on the action mechanism(s) of these products. Work is in progress to address this aspect.

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 5×20 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 ≥ 95% were determined by elemental analysis and HPLC. Elemental analyses (C, H, N) of the new compounds dried at 20°C, pressure < 10 mmHg for 24 h were performed at the University of Geneva 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, USA) 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 × 4.6 mm, 5 μm particle size). Data analysis was done using a HP ChemStation system (Agilent Technologies). Compounds **9a,b**[35] and **23**[36] were synthesized according to literature. The preparation of products **2**, **3a,b**-**11a,b**, **14** and **15** is reported in a number of references that are collected in the bibliography of reviews[22,37].

4-Phenylthiofuroxan-3-carboxamide (24b). To a solution of **23** (2.1 g, 12.0 mmol) in CH_3CN (25 mL) 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). Organic solvent was washed with H_2O (30 mL), brine, dried and evaporated. Obtained solid was washed with cold MeOH, filtered and used without further purification. Yield 79%. Analytically pure sample was obtained by crystallization; mp = 146-149°C

(dec. MeOH). ^{1}H NMR (300 MHz, DMSO-d_6) δ 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) δ ppm 111.0, 125.6, 129.8, 130.3, 135.0, 155.7, 157.0. MS (EI) *m/z* 237 $[M]^+$. Anal. $C_9H_7N_3O_2S$ (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 quartz reactor was irradiated with the fully mercury arc of an immersion medium pressure lamp (125W, Photochemical reactors Ltd., Buckinghamshire, HP16 ODR, United Kingdom) for 1 h at rt. Organic solvent was removed under reduced pressure and obtained solid was treated with cold MeOH (50 mL). Resulting mixture was filtered and 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) δ 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) δ ppm 109.9, 127.8, 128.5, 129.5, 130.7, 153.3, 157.6. MS (EI) *m/z* 237 $[M]^+$. Anal. $C_9H_7N_3O_2S$ (C,H,N).

4-Phenylsulfonylfuroxan-3-carboxamide (12b). To a solution of **24b** (0.50 g, 2.1 mmol) in 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. Cooling bath was removed and reaction was stirred at r.t. for 2 h. Then it was poured into H_2O (50 mL) and extracted with EtOAc (2×30 mL). Organic extract was washed with H_2O, brine, dried and evaporated. Obtained solid was crystallized from MeOH to give the title compound as white crystalline solid. Yield 68%, mp = 188-188.5°C (MeOH). ^{1}H NMR (300 MHz, DMSO-d_6) δ 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) δ 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). 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) δ 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) (CON*H*$_2$). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 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). Cooling bath was removed and reaction was stirred at r.t. for 1 h. Then it was poured into H_2O (50 mL) and extracted with EtOAc (2×25 mL). Organic extract was washed with H_2O, brine, dried and evaporated. Obtained oil was solidified by treating with cold PE and crystallized from hexane to give the title compound as white crystalline solid. Yield 81%, mp = 89.5-90°C (C_6H_{14}). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 7.70-7.75 (m, 2H), 7.83-7.90 (m, 1H), 8.13-8.16 (m, 2H) (C$_6$*H*$_5$). ^{13}C NMR (75 MHz, $CDCl_3$): δ ppm 93.8, 103.4, 129.4, 130.4, 136.0, 136.6, 157.1. MS (EI) *m/z* 251 $[M]^+$. Anal. $C_9H_3N_3O_4S$ (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, $CDCl_3$) δ ppm 7.68-7.73 (m, 2H), 7.83-7.88 (m, 1H), 8.09-8.16 (m, 2H) (C$_6$*H*$_5$). ^{13}C NMR (75 MHz, $CDCl_3$) δ ppm 105.9, 115.0, 129.0, 129.9, 130.3, 136.4, 136.8. MS (EI) *m/z* 251 $[M]^+$. Anal. $C_9H_3N_3O_4S$ (C,H,N).

General procedure for the synthesis of 4-alkyl(aryl)oxy-3-phenylsulfonylfuroxans (16-22). To a mixture of 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 and the reaction mixture was stirred at r.t. for 2 h. Then organic solvent was washed with H_2O (20 mL), HCl 1M (2×10 mL), brine, dried and evaporated. 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$_6$) δ 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, OC*H*$_2$), 7.74-7.79 (m, 2H), 7.89-7.94 (m, 1H), 8.01-8.04 (m, 2H) (C$_6$*H*$_5$). ^{13}C NMR (75 MHz, DMSO-d$_6$) δ 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, $CDCl_3$) δ ppm 0.87 (t, 3H, C*H*$_3$), 1.25-1.43 (m, 6H, 3C*H*$_2$), 1.75-1.84 (m, 2H, OCH$_2$C*H*$_2$), 4.34 (t, 2H, OC*H*$_2$), 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, $CDCl_3$) δ 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) δ 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, OC*H*$_2$), 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) δ 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) δ 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, OC*H*$_2$), 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) δ 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-*iso*Propoxy-3-phenylsulfonylfuroxan (20). Yield 37%, mp = 101-103°C (*i*-PrOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.87 (t, 3H, C*H*$_3$), 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, $CDCl_3$) δ 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-*iso*Butoxy-3-phenylsulfonylfuroxan (21). Yield 94%, mp 100-102°C (*i*-PrOH). ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 0.87 (t, 3H, C*H*$_3$), 1.05 (d, 6H, 2C*H*$_3$), 2.21 (qi, 1H, CH$_2$C*H*), 4.18 (d, 2H, OC*H*$_2$), 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, $CDCl_3$) δ 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, $CDCl_3$) δ 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) ($2C_6H_5$). ^{13}C NMR (75 MHz, $CDCl_3$) δ 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).

Biology

Materials. Cell culture reagents were purchased from Celbio s.r.l. (Milano, Italy). CulturePlate 96/wells plates were purchased from PerkinElmer Life Science; Calcein-AM, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide) and Doxorubicin were obtained from Sigma-Aldrich (Milan, Italy). MK571 was purchased from Calbiochem (San Diego, USA).

Cell cultures. MDCK-MDR1 and MDCK-MRP1 are a gift of Prof. P. Borst, NKI-AVL Institute, Amsterdam, Nederland. 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 μg/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 black CulturePlate 96/wells plate with 100 μl medium and allowed to become confluent overnight. 100 μl of test compounds were solubilized in culture medium and added to monolayers. 96/Wells plate was incubated at 37°C for 30 min. Calcein-AM was added in 100 μl of Phosphate Buffered Saline (PBS) to yield a final concentration of 2.5 μM and plate was incubated for 30 min. Each well was washed 3 times with ice cold PBS. Saline buffer was added to each well and the plate was read to Victor3 (PerkinElmer) at excitation and emission wavelengths of 485 nm 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 by 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.[38,39] On day 1, 20000 cells/well were seeded into 96-well plates in a volume of 100 μL. On day 2, the various drugs concentration alone or in combination with doxorubicin were added. In all the experiments, the various drug-solvents (ethanol, DMSO) were added in each control to evaluate a possible solvent cytotoxicity. After the established incubation time with drugs, 0.5 mg/mL MTT was added to each well, and after 3 h incubation at 37 °C, the supernatant was removed. The formazan crystals were solubilized using 100 μL of DMSO and the absorbance values at 570 and 630 nm were determined on the microplate reader Victor 3 from PerkinElmer Life Sciences.

Supporting information available. Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

Chart 1. Examples of P-gp inhibitors.

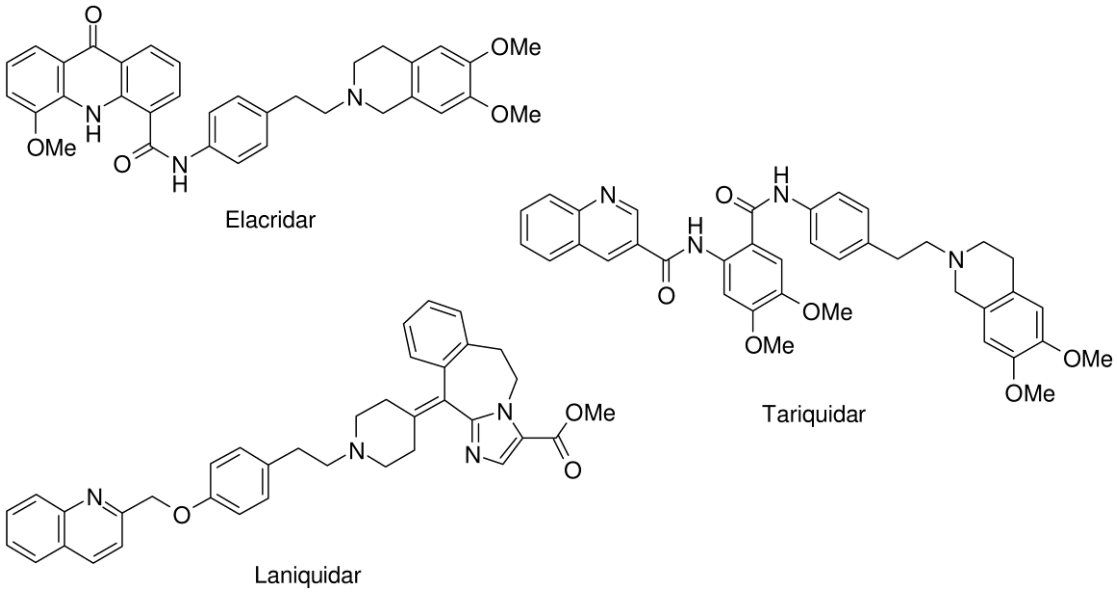
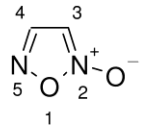


Figure 1. Phenylfuroxan and phenylsulfonylfuroxan derivatives.



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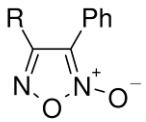
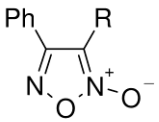
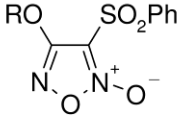


a

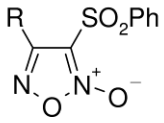


b

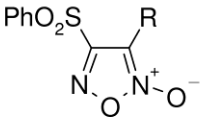


2 R = Ph;
3a,b R = CH_3;
4a,b R = NH_2;
5a,b R = OCH_3;
6a,b R = $CONH_2$;
7a,b R = NO_2;
8a,b R = CN;
9a,b R = $SONH_2$;
10a,b R = SO_2Ph.

15 R = C_2H_5;
16 R = C_4H_9;
17 R = C_6H_{13};
18 R = C_8H_{17};
19 R = $C_{10}H_{21}$;
20 R = iso-C_3H_7;
21 R = iso-C_4H_9;
22 R = Ph.



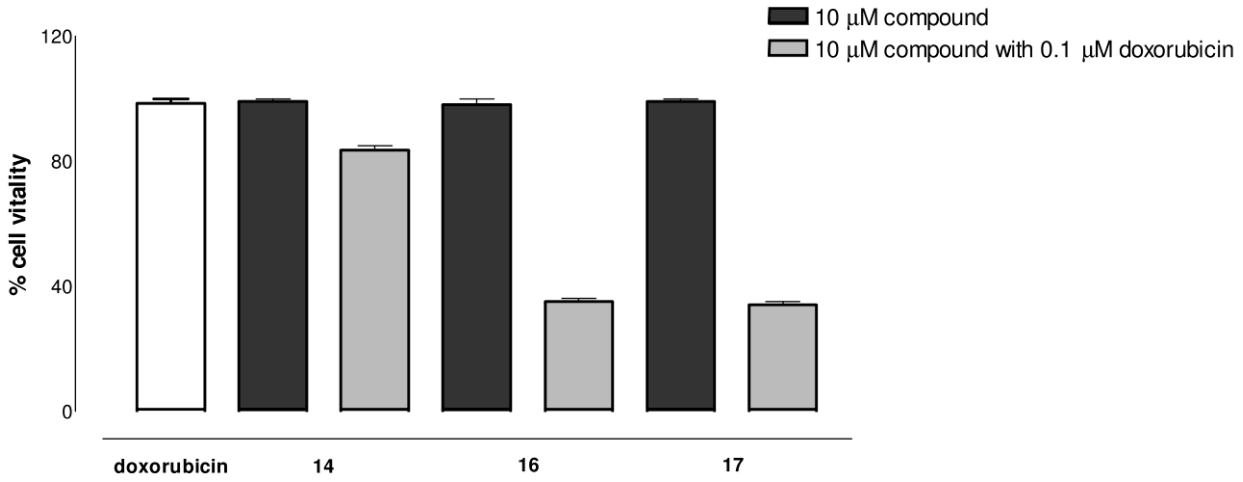
a



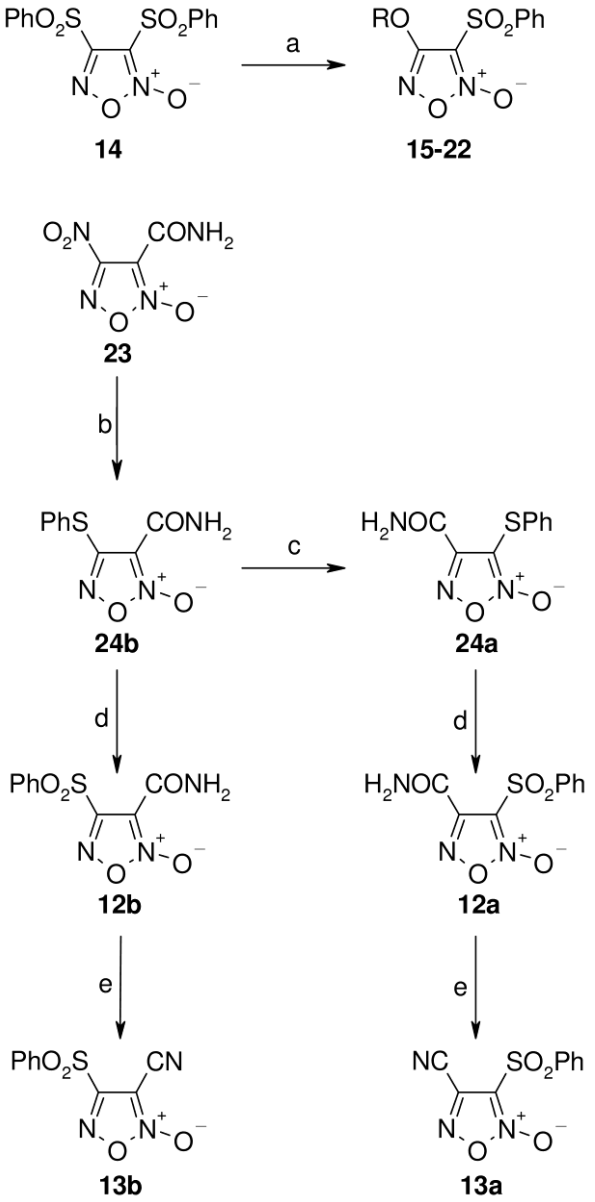
b

11a,b R = CH_3;
12a,b R = $CONH_2$;
13a,b R = CN;
14 R = SO_2Ph.

Figure 2. Antiproliferative effect of 0.1 μM doxorubicin (white bar) and of 10 μM of compounds **14**, **16**, **17** (black bars) at 48 h in MDCK-MDR1 cells. In comparison, each compound (10 μM) was administered for 24 h. After washout, each compound was co-administrated with doxorubicin (0.1 μM) at the same concentration for 24 h (grey bars).

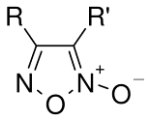


Scheme 1.[a] Synthesis of phenylsulfonylfuroxans **12a**,**b**-**13a**,**b** and **15-22**.



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

Table 1. P-gp and MRP1 inhibiting activities of phenylfuroxans (**2**-**10**) and phenylsulfonylfuroxans (**11**-**22**).



Compound	R	R'	P-gp	MRP1
			EC_{50} (μM) ± SEM[a]	
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[b]
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[b]
12b	SO_2Ph	$CONH_2$	52.6 ± 2.0	13 ± 1.2
13a	CN	SO_2Ph	7.4 ± 0.5	29.5 ± 3.0[b]
13b	SO_2Ph	CN	55.4 ± 2.0	72 ± 8.0[b]
14	SO_2Ph	SO_2Ph	3.0 ± 0.2	15.1 ± 2.0[b]
15	OC_2H_5	SO_2Ph	12 ± 0.4	49.1 ± 5.0[b]
16	OC_4H_9	SO_2Ph	2.26 ± 0.2	7.61 ± 0.8[b]
17	OC_6H_{13}	SO_2Ph	3.35 ± 0.2	12 ± 1.5[b]
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[b]
21	O*iso*-C_4H_9	SO_2Ph	2.23 ± 0.2	46 ± 5.0[b]
22	OPh	SO_2Ph	20.5 ± 3.0	13.6 ± 2.0[b]

a) The values are the mean ± SEM, from two independent experiments with samples in duplicate. b) The value represents the inducing activity of compound.

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SYNOPSIS TOC

PHENYLSULFONYL FUROXANS AS MODULATORS OF MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN-1 (MRP-1) AND P-GLYCOPROTEIN

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