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Oxazolo[3,2-*a*]pyridine. A new structural scaffold for the reversal of multi-drug resistance in *Leishmania*

Esther Caballero [a,*], José Ignacio Manzano [b], Pilar Puebla [a], Santiago Castanys [b], Francisco Gamarro [b,*], Arturo San Feliciano [a]

[a] Departamento de Química Farmacéutica, Facultad de Farmacia, CIETUS, IBSAL, Universidad de Salamanca, 37007-Salamanca, Spain
[b] Instituto de Parasitología y Biomedicina 'López Neyra', IPBLN-CSIC, Granada, Spain

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ABSTRACT

Compounds belonging to three different classes of fused heterocyclic systems, structurally related to Calcium-channel blockers of the 1,4-dihydropyridine family, were evaluated in their ability to overcome leishmanial resistance to common drugs in a MDR *Leishmania tropica* strain. Compounds with the skeletal basis of oxazolo[3,2-*a*]pyridine displayed significant reversion of resistance to daunomycin and miltefosine, with reversion indexes up to 6.7-fold and 8.7-fold, respectively. Most interestingly, the enantiopure compound **20*S*** attained to revert the resistance to both drugs and fairly more significantly than its enantiomer **20*R***.

Leishmaniasis is a poverty-related neglected disease characterized by its high morbidity, being the most important emerging and uncontrolled infectious disease and the second cause of death among the protozoan parasitic diseases, after Malaria. Globally there are an estimated 70,000 deaths each year and 350 million people are at risk of infection. To date there are no protective vaccines, and chemotherapy is the only effective weapon, limited to a few first-line drugs, including miltefosine, paromomycin, amphotericin B and its derivatives, along with pentavalent antimonials, although the latter are no longer effective in endemic areas as in Bihar (India) due to drug resistance. One of the major problems of leishmaniasis chemotherapy is the intrinsic or acquired resistance of parasites to the available drugs. ATP-binding cassette (ABC) transporters are commonly involved in the resistance to different antiparasitic drugs such as antimonials,[1] pentamidine,[2] miltefosine,[3] and sitamaquine.[4] *Leishmania* P-glycoprotein-like transporter (Pgp-like) is included in the ABC subfamily B and its overexpression confers a multi-drug resistance (MDR) phenotype involved in a significantly reduction in intracellular accumulation of drugs, similar to that characterised in mammalian cells overexpressing Pgp.[5,6] Inhibitors of Pgp have thus been advocated as promising candidates for overcoming MDR.

Parasite resistance to drugs has emerged as a major problem in current medicine, and therefore, there is great clinical interest in developing compounds that overcome resistances with lower host toxicity.[7] Several types of compounds with diverse levels of MDR reversal properties have been described.[8] One of the physio-pharmacological categories of drugs displaying such reversion ability is that of Calcium channel blockers represented by verapamil and nifedipine related drugs.[9] Here we report on the evaluation of the MDR reversal abilities of representative members of three types of fused heterocyclic compounds structurally related to nifedipine and 1,4-dihydropyridines, that had previously displayed long-acting antihypertensive effects, presumably due to its probable Ca^{2+}-channel blocker pro-drug character.[10]

The syntheses of the heterocyclic compounds shown in Table 1 were achieved following the known Hantzsch procedure as previously reported (Scheme 1).[11] This methodology afforded compounds **1**–**7** in good yields, starting with enamines of acetoacetate esters obtained from 2-aminoethanol (compounds **1**–**4**, **6** and **7**, Table 1) and 1-amino-2-propanol (**5**). The use of thioethanolamine enamine led to the thiazolopyridine **8** and that of 3-aminopropanol enamine rendered the pyrido-oxazine **9.**

Compounds **1**–**9** include three types of fused heterocyclic systems, with seven diversely substituted oxazolo[3,2-*a*]pyridines, one thiazolo[3,2-*a*]pyridine and one pyrido[2,1-*b*]oxazine, which were initially evaluated in their MDR reversal potentiality on promastigotes of a highly daunomycin-resistant strain of *Leishmania tropica*.[12] Daunomycin (DNM) resistance in this line is related to a decreased intracellular drug accumulation mainly due to the Pgp-like transporter overexpression.[13] This resistant line

* Corresponding authors. Tel.: +34 923294528 (E.C.), +34 958181667 (F.G.).
E-mail addresses: escab@usal.es (E. Caballero), gamarro@ipb.csic.es (F. Gamarro).

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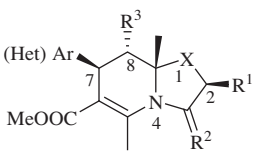
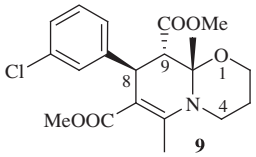
Table 1
Structures and growth inhibition results (%) of wild-type (WT) and MDR *L. tropica* lines by daunomycin (DNM, 150 μM) in the presence of selected heterocyclic compounds



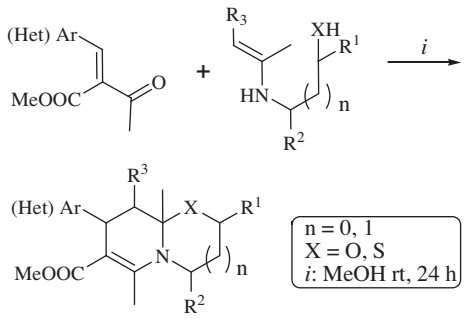
(±) oxa(thia)zolo[3,2-*a*]pyridines



(±) pyrido[2,1-*b*]oxazine

Compound	X	R^1	R^2	R^3	Ar (Het)	[Comp.] = 3 μM		[Comp.] = 6 μM		[Comp.] = 8 μM	
						WT	MDR	WT	MDR	WT	MDR
1	O	H	H,H	CH_2OH	3-NO_2Ph	<10	<10	<10	<10	<10	26.3 ± 0.2
2	O	H	H,H	COOMe	3-NO_2Ph	<10	**75.3 ± 2.3**	<10	**92.3 ± 0.9**	21.8 ± 1.0	**97.6 ± 0.5**
3	O	H	H,H	COOBn	3-NO_2Ph	<10	23.9 ± 1.0	16.4 ± 0.2	71.6 ± 3.7	65.1 ± 3.3	**93.3 ± 0.4**
4	O	H	H,H	COOBn	3-ClPh	<10	<10	<10	<10	<10	<10
5	O	Me	O	COOMe	3-ClPh	<10	<10	<10	<10	<10	<10
6	O	H	H,H	COOMe	2-furyl	<10	<10	<10	<10	<10	<10
7	O	H	H,H	COOMe	2-pyrrolyl	<10	57.3 ± 3.3	<10	**77.8 ± 1.5**	<10	**80.3 ± 4.2**
8	S	H	H,H	COOMe	3-ClPh	<10	43.2 ± 2.5	<10	**78.9 ± 2.0**	<10	**83.8 ± 5.8**
9						<10	18.1 ± 0.2	<10	36.8 ± 1.1	<10	74.8 ± 3.6

Data shown are the average of three independent experiments ± SD, relative to the control, grown in the absence of DNM and compounds. Values above 75% inhibition are boldfaced to facilitate comparisons.



Scheme 1. The synthesis of oxa(thia)zolo[3,2-*a*]pyridines ($n = 0$) and pyrido[2,1-*b*]oxazines ($n = 1$).

has an MDR phenotype similar to tumour cells, with a cross-resistance profile to several unrelated drugs and an overexpressed drug-efflux Pgp-like transporter (LMDR1).[14] The wild-type (WT) *L. tropica* LCR-strain was also included in the assays. To determine the intrinsic toxicity of compounds, the WT and MDR *Leishmania* lines were exposed to compounds in the absence of DNM.[15] The cytotoxicity of compounds for human myelomonocytic THP-1 cells was also determined,[16] and the results (data not shown) showed that these compounds present a significantly low toxicity with IC_{50} values higher than 400 μM.

In order to assess the chemosensitising activity of the compounds, promastigotes of the *L. tropica* MDR line were exposed to both 150 μM DNM and three different concentrations (3, 6 and 8 μM) of the compounds in racemic form. The preliminary evaluation results included in Table 1 showed that several oxazolo[3,2-*a*]pyridines were able to revert the resistance to DNM and that compound **2** achieved a 75% parasite inhibition after 72 h at a low 3 μM concentration. Additionally, compounds **7**, **8** and **9** displayed a moderate MDR reversal activity with lower toxicity.

These encouraging results moved us to design and perform new evaluation experiments focused on establishing IC_{50} values of resistance reversion for the most active compounds. Considering that the MDR *Leishmania* line showed a significant cross-resistance to miltefosine (MLF),[16] the IC_{50} and the reversal index (RI) values to DNM and MLF were determined for compounds **2**, **7**, **8** and **9**. Parasites were exposed to increasing concentrations of drugs in the absence or presence of a 6 μM concentration of the compounds. The RI is defined as the ratio between the IC_{50} values found for a certain drug in absence or in presence of each compound. In other words, a RI value of 3 indicates a 3-fold enhancement of drug effectiveness against the multi-resistant parasite. The calculated maximum RI value with DNM (ratio between IC_{50} values for MDR and parental drug-sensitive parasites) is 64. The maximum RI value with MLF is 22.

As shown in Table 2, the selected compounds showed RI values ranging from 3.9 to 1.3 for DNM, and from 3.5 to 1.5 for MLF. As can be observed, among the oxazolo[3,2-*a*]pyridines, compound **2** displayed the strongest MDR reversal potential with RI values of 3.9 and 3.4 for both drugs DNM and MLF, respectively. These values are of the same order and comparable to those obtained with **Cuzco 5** (1α,2α-diacetoxy-6β,9β-difuroyloxy-4β-hydroxydihydro-β-agarofuran), a known and rather complex natural sesquiterpenoid isolated from *Maytenus canariensis*, being considered up to date as the most promising reversal compound of MDR phenotype in *Leishmania*.[18] Consequently, **Cuzco 5** was used in this research as the positive control of MDR reversion.

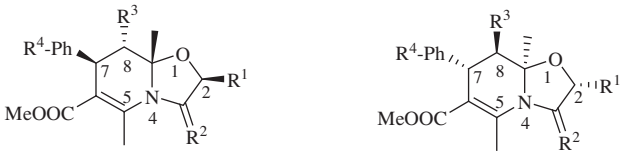
Aiming at discovering better MDR reversal agents of this type, we evaluated a larger number of oxazolo[3,2-*a*]pyridines, including several enantiomerically pure compounds. The structures of the evaluated compounds **10–25** are shown in Table 3. The enantioselective synthesis of 2,3,8,8a-tetrahydro-7*H*-oxazolo[3,2-*a*]pyridines

Table 2
Quantitative effects of compounds **2**, **7**, **8,** and **9** on the IC_{50} values for daunomycin (DNM) and miltefosine (MLF) in the MDR *L. tropica* line

Compound (6 μM)	IC_{50}(μM) for DNM	RI_{DNM}	IC_{50}(μM) for MLF	RI_{MLF}
—	179.8 ± 1.1	—	239.5 ± 2.1	—
2	47.0 ± 6.4	**3.9**	70.8 ± 3.9	**3.4**
7	138.6 ± 4.4	1.3	123.2 ± 2.7	1.9
8	83.0 ± 4.0	2.2	69.2 ± 6.3	**3.5**
9	55.7 ± 0.4	**3.2**	161.2 ± 2.8	1.5
Cuzco5	38.3 ± 0.6	**4.7**	39.7 ± 2.1	**6.0**

IC_{50} values for the WT line in absence of compounds were: 2.8 ± 0.1 (DNM), and 10.9 ± 2.1 (MLF). RI values above 3.0 are boldfaced for comparison purposes. Data are expressed as average ± SD ($n = 3$) of concentrations of DNM and MLF necessary to inhibit parasite growth by 50%.

Table 3
Resistance reversal effects for further oxazolo[3,2-*a*]pyridine derivatives on the MDR line of *L. tropica*



Compound (6 μM)	R^1	R^2	R^3	R^4	IC_{50} (μM) for DNM	IC_{50} (μM) for MLF
–					179.8 ± 1.1	239.5 ± 2.1
10(*r*)	H	H,H	CH_2OAc	3-NO_2	179.7 ± 4.2	131.7 ± 0.0
11(*r*)	H	H,H	COOMe	3-Cl	189.0 ± 2.0	154.2 ± 4.2
12(*r*)	H	H,H	COOEt	3-NO_2	187.6 ± 2.1	185.1 ± 5.4
13(*r*)	H	H,H	COOEt	3-Cl	206.7 ± 11	124.5 ± 1.4
14(*r*)	H	H,H	COOAllyl	3-Cl	**34.2 ± 0.4**	71.1 ± 2.4
15(*r*)	H	H,H	COO*t*Bu	3-NO_2	187.9 ± 2.2	238.3 ± 8.4
16(*r*)	H	H,Et	COOMe	3-NO_2	54.2 ± 0.8	58.1 ± 0.6
17(*r*)	H	H,Et	COOMe	3-Cl	56.9 ± 2.1	**38.7 ± 0.3**
18*S*	Me	H,H	CH_2OAc	3-Cl	50.7 ± 0.3	55.1 ± 2.3
19(*r*)	Me	H,H	COOMe	3-NO_2	173.5 ± 3.5	133.7 ± 7.1
19*R*	Me	H,H	COOMe	3-NO_2	172.6 ± 5.1	171.3 ± 2.5
20*R*	Me	H,H	COOMe	3-Cl	79.6 ± 4.6	84.3 ± 2.0
20*S*	Me	H,H	COOMe	3-Cl	**26.7 ± 4.2**	**45.6 ± 3.6**
21(*r*)	Me	H,H	COOEt	3-NO_2	172.1 ± 8.4	151.6 ± 0.4
22*R*	Me	H,H	COOEt	3-Cl	**42.1 ± 2.0**	55.5 ± 2.9
22*S*	Me	H,H	COOEt	3-Cl	57.9 ± 3.2	**45.8 ± 2.8**
23*R*	Me	H,H	COO*t*Bu	3-Cl	51.4 ± 2.3	**27.7 ± 2.2**
24*R*	Me	H,H	COOBn	3-Cl	**45.7 ± 1.4**	50.9 ± 3.6
24*S*	Me	H,H	COOBn	3-Cl	61.2 ± 0.2	**46.0 ± 3.3**
25*R*	Me	O	COOEt	3-Cl	88.8 ± 0.7	62.1 ± 4.3
Cuzco5					**38.3 ± 0.6**	**39.7 ± 2.1**

IC_{50} values for the WT line in absence of compounds were: 2.8 ± 0.1 μM (DNM) and 10.9 ± 2.1 μM (MLF). Data are expressed as average ± SD (n = 3) of concentrations of DNM and MLF necessary to inhibit parasite growth by 50%. IC_{50} values similar as or better than those found for **Cuzco 5** are boldfaced for comparison purposes.

was carried out with enamines prepared from homochiral 1-amino-2-propanols as previously reported by some of us.[19] It is noticeable that the formation of three new stereocenters in the bicyclic system is highly controlled by the presence of a single chiral carbon atom in the starting enamine reagent, and placed in the end product three, four and five bonds away from the newly created stereocenters. When racemic 1-amino-2-propanol and 2-aminobutanol were used, racemic **16**, **17**, **19** or **21** were obviously produced.

The results of the MDR reversal evaluation are shown in Table 3. As it can be seen, the tested compounds behave differently depending on their respective structures and on the antileishmanial drug being considered. Thus, while most compounds reverted *Leishmania* resistance to one or both drugs in a similar manner as the mentioned above for compounds **2** and **8**, several of them resulted practically inactive and even one compound (**13**) increased the resistance to DNM. These experimental facts support the existence of fair structure/resistance–reversal relationships that can also be extended to the stereochemical aspects, as in the case of the enantiomers **20*R*** and **20*S***.

A first observation related to the influence of the *m*-substituted phenyl group attached to position C-7 reveals that the 3-chlorophenyl group is preferred to the nitro group at the same position.

It can also be observed that compounds **14** and **20*S*** significantly revert the IC_{50} values for DNM, by more than 5- and 6-fold, respectively, with higher RI values than the reference **Cuzco5** (Table 3, Fig. 1). Indeed, among the tested compounds, **20*S*** showed the highest potency for DNM-resistance reversion. Additionally, compound **23*R*** reverts around 8.7-folds the IC_{50} value for MLF, with higher RI than **Cuzco 5** for this drug (Fig. 1). Interestingly, both compounds **20*S*** and **23*R*** present a low toxicity for human THP-1 cells, with IC_{50} values higher than 400 μM (data not shown). Therefore, within the series of oxazolo[3,2-*a*]pyridine derivatives tested, compound **20*S*** which produces a significant reversion of resistance to both drugs DNM and MLF, is considered as a good candidate for further studies as reversal agent of *Leishmania* MDR. Similarly, compound **23*R***, with the highest RI against the modern antileishmanial agent MLF would play a similar role. Complementarily, we attempted to determine the MDR reversal activity of these compounds on intracellular amastigotes, however due to the high intrinsic cytotoxicity of MLF and DNM for the human THP-1 cells, with IC_{50} values: 26.9 ± 3.1 μM (MLF) and 7.0 ± 0.04 μM (DNM), it was difficult to quantify the resistance reversion.

In order to assess the expectancy of these oxazolo-pyridines as potential lead compounds is important to note that several representative compounds of this series were tested already in vivo, having demonstrated their antihypertensive efficacy with low toxicity.[10] However, such assays were performed under intraperitoneal administration only, and some doubts emerged about their bioavailability under oral administration, the ordinary route of administering MLF and DNM. At this respect, the expectancy for the potential clinical use of compounds **17**, **20**, **22** and **23** results reinforced by fulfilling of the Lipinski rules, and complemented with favourable theoretical physico-chemical, ADME and toxicological calculations and predictions (data not shown) obtained for those compounds. Consequently, compounds **20*S*** and **23*R*** could be considered as drug leads for MDR reversal in the treatment of resistant leishmaniasis. Furthermore, the good results found for **20*S***, **14** and other compounds of the series in the reversal of resistance to DNM, a well established and clinically used anticancer drug, suggest the potential interest of these family of compounds for the treatment of anthracycline-resistant neoplastic diseases. In this sense, in vitro MDR reversal studies on human neoplastic cells have been initiated.

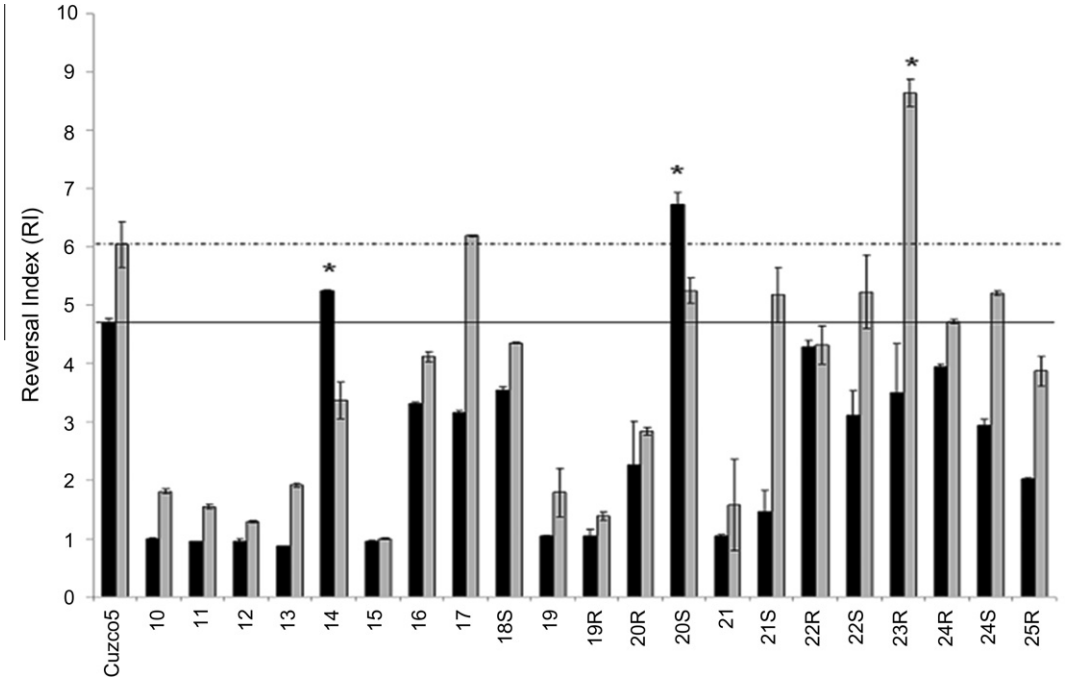


Figure 1. Reversal indexes of oxazolo[3,2-*a*]pyridine derivatives, showing their comparative effects on the resistance to DNM and MLF by the MDR line of *L. tropica*. Reversal index (RI) values for a compound and drug (DNM: black; MLF: grey) correspond to the ratio IC_{50} (without)/IC_{50} (with compound). Continuous (DNM) and dotted (MLF) lines define the RI values for **Cuzco 5** to allow an easy comparison of compounds with the positive reference. Bars represent the average of three independent experiments ± SD (*p <0.05).

As a conclusion, the results here shown constitute a first report which justifies the interest of the oxazolo[3,2-*a*]pyridine moiety as a good structural base for MDR reversal. Accordingly, it seems recommendable to continue the research with evaluation of a wider family of compounds, including further variants in substituents and arrangements all around the fused heterocyclic system, and to extend the research to other therapeutic areas. In order to bring about this task, experimental studies of in vivo efficacy and acute toxicity, and the establishment of the mechanism of action with identification of the target molecule(s) or the biochemical pathway(s) of MDR reversion used by this type of compounds will be necessary.

Acknowledgments

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15. *Parasite growth control:* After 72 h of incubation at 28 °C, the viability of promastigotes was determined by the colorimetric MTT assay and absorbance was read at 540 nm using a microplate reader (Beckman Biomek 2000). Growth inhibition respect to non-treated parasites was quantified by the ratio of absorbance at a given compound concentration with respect to control.
16. *Human cells toxicity:* THP-1 cells were grown at 37 °C and 5% CO_2 in RPMI-1640 supplemented with 10% iFBS, 2 mM glutamate, 100 U/mL penicillin and 100 μg/mL streptomycin. 3×10^4 cells /well, in 96 wells-plate, were differenced to macrophages with 20 ng/mL of PMA treatment for 48 h followed to 24 h of culture in fresh medium. The MTT-based assay procedures were the same as for *Leishmania* parasites with the exception of incubation temperature, 37 °C in this case.
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