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Tricyclic Isoxazoles are Novel Inhibitors of the Multidrug Resistance Protein (MRP1)

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Abstract—Tricyclic isoxazoles were identified from a screen as a novel class of selective multidrug resistance protein (MRP1) inhibitors. From a screen lead, SAR efforts resulted in the preparation of LY 402913 (**9h**), which inhibits MRP1 and reverses drug resistance to MRP1 substrates, such as doxorubicin, in HeLa-T5 cells ($EC_{50} = 0.90$ μM), while showing no inherent cytotoxicity. Additionally, LY 402913 inhibits ATP-dependent, MRP1-mediated LTC_4 uptake into membrane vesicles prepared from the MRP1-overexpressing HeLa-T5 cells ($EC_{50} = 1.8$ μM). LY 402913 also shows selectivity (~22-fold) against the related transporter, P-glycoprotein, in HL60/Adr and HL60/Vinc cells. Finally, when dosed in combination with the oncolytic MRP1 substrate vincristine, LY 402913 delays the growth of MRP1-overexpressing tumors in vivo. © 2002 Published by Elsevier Science Ltd.

The multidrug resistance protein (MRP1) is a member of the family of ATP-binding cassette (ABC) transporters and is structurally and functionally related to the multidrug resistance (MDR) protein, P-glycoprotein (P-gp).[1] ABC transporters are known to have a number of functions in normal human physiology. While the P-gp pump has been linked to the removal of toxic xenobiotics,[2] MRP1 seems to play an important role in the transport of leukotrienes.[3–7] The most thoroughly studied MRP1 substrate is leukotriene C_4 (LTC_4), which is transported by MRP1 in an ATP-dependent fashion with high efficiency (K_m approx. 100 nM).[4,5] Like P-gp, MRP1 has been shown to confer cellular resistance to various natural product oncolytics by transporting the agents out of the cells. The resulting lower intracellular concentration of the oncolytic produces a drug-resistant phenotype.[8] These drug-resistant cells require higher concentrations of the oncolytic to achieve cytotoxicity. MRP1-associated oncolytics include the anthracyclines (doxorubicin and daunarubicin), epipodophylotoxins (etoposide), and vinca alkoloids (vincristine). While P-gp confers resistance to taxanes, MRP1 is apparently not involved in taxane resistance.[8]

Drug resistance is a tremendous problem in the treatment of many types of cancer. In the clinical setting, patients who overexpress multidrug resistance proteins such as P-gp and MRP1 in their tumors are usually not responsive to associated anticancer agents.[9] Thus, many of these patients progress to advanced disease and have poorer prognoses. It has been hypothesized that inhibition of MDR transporters can restore sensitivity to some oncolytics, allowing patients with drug-resistant tumors to become responsive to their drug therapy. This hypothesis is presently being tested in the clinic for P-gp.[10] However, the search for MRP1 modulators just began a few years ago and few potent and selective modulators are known.[11,12] We would like to report here on the discovery of tricyclic isoxazoles, a new class of selective MRP1 modulators.

A screen of potential MRP1 modulators uncovered the tricyclic isoxazole indane **1**, which had an EC_{50} of 10 μM in the MRP1-transfected cell line HeLa-T5 (when

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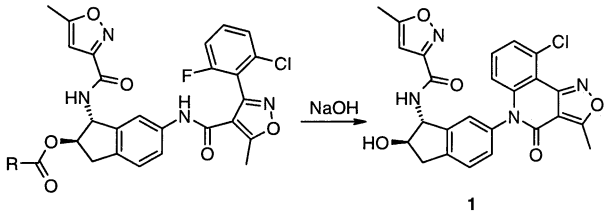
dosed in the presence of a sublethal dose of doxorubicin).[13] The tricyclic moiety was formed, somewhat serendipitously, during the base-promoted deprotection of the indane alcohol functionality (Scheme 1). Under the saponification conditions, the 2-chloro-6-fluorophenyl-isoxazole functionality reacted via an intramolecular nucleophilic aromatic substitution reaction to give the tricyclic compound **1**. It was later determined that this tricyclic functionality was absolutely required, as all non-cyclized materials were inactive in our assays. From this lead compound, we began to explore the SAR of tricyclic isoxazoles and related compounds.

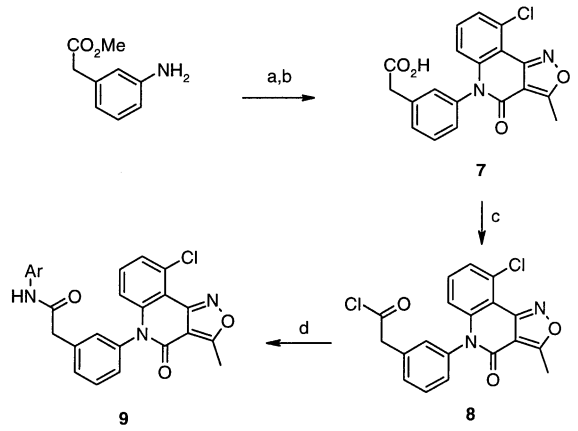


Scheme 1.

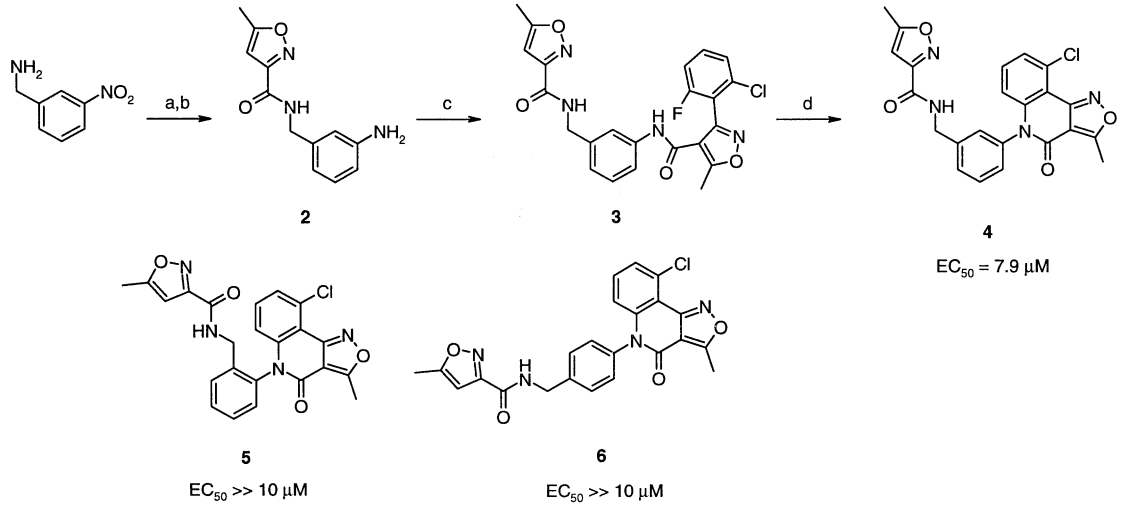
At first, we investigated the importance of the indane functionality by preparing the simplified compound **4** (Scheme 2). The synthesis began with 3-nitrobenzylamine. Acylation with the isoxazole acid chloride, followed by reduction with tin (II) chloride, resulted in the aniline **2**. Treatment of this material with the commercially available 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazoyl chloride resulted in the acyclic compound **3**. When this material was treated with sodium hydroxide in DMF at room temperature, the intramolecular nucleophilic aromatic substitution reaction occurred to give the tricyclic product **4**. The related *ortho* and *para* substituted analogues **5** and **6** were prepared in similar fashion. While the *meta* material **4** had an EC_{50} value (7.9 μM) similar to the indane, both *ortho* and *para* compounds were inactive (EC_{50} >> 10 μM).

At the same time, we explored a route towards the isomeric amide series, where anilines could be reacted with the corresponding acid chloride (Scheme 3). While similar to the earlier route, we were able to employ efficient parallel synthesis techniques in the final step of this sequence. Specifically, by treating the acid chloride **8** with excess aniline and triethylamine, all amine-containing impurities could be easily removed by passing the crude mixture down a strong cation exchange (SCX) column. The resulting solutions could be concentrated to give extremely pure products in >80% yield. Early results on the aniline series were promising, both from a chemistry and biological activity perspective. Thus, it seemed logical to pursue an initial SAR around the more synthetically friendly phenyl linker region with aniline substitution.

Using the method outlined in Scheme 3, an SAR was conducted and the highlights are summarized in Table 1. While the activities of most compounds ranged from about 1 to >10 μM, one compound, **9h** (LY 402913), demonstrated activity below 1 μM (EC_{50} = 0.90 μM). Thus, the 3,4,5-trimethoxyphenyl substitution was

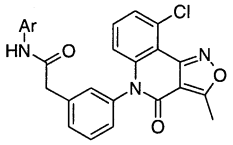


Scheme 3. (a) 3-(2-Chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl chloride, pyr, DMAP (cat.), CH_2Cl_2, 25 °C, 3 h (94%); (b) 2 N NaOH/methanol, DMF, 25 °C, 2 h (53%); (c) $(COCl)_2$, CH_2Cl_2, 1 h (100%); (d) selected aniline, Et_3N, CH_2Cl_2, 15 h, purified on SCX column (80–95%).



Scheme 2. (a) 5-Methyl-3-isoxazolyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (79%); (b) $SnCl_2 \cdot 2H_2O$, THF, 25 °C, 18 h (86%); (c) 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl chloride, pyr, DMAP (cat.), CH_2Cl_2, 25 °C, 3 h (90%); (d) 2 N NaOH/methanol, DMF, 25 °C, 2 h (97%).

Table 1. Structures and in vitro activities of tricyclic isoxazoles in the MRP1-transfected HeLa-T5 cell line in the presence of doxorubicin



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Compd	Ar	EC_{50} (μM)
9a	Ph	2.3
9b	4-Me-Ph	3.7
9c	3-OMe-Ph	1.8
9d	4-OMe-Ph	>10
9e	3,5-$(OMe)_2$-Ph	2.1
9f	3,4-$(OMe)_2$-Ph	4.1
9g	3,4-(-OCH_2O-)-Ph	4.0
9h	3,4,5-$(OMe)_3$-Ph	0.90 (±0.03)[a]
9i	3,5-$(OMe)_2$-4-OH-Ph	1.3
9j	4-F	3.2
9k	4-*t*-Bu	>10
9l	4-CN	4.9
9m	4-CF_3	>10
9n	4-CO_2Me	>10
9o	4-SO_2NH_2	>10

[a]Standard error for **9h** ($n=81$), which was the control compound for all experiments. Other compounds were tested in duplicate.

pursued in subsequent SAR studies. It should be pointed out that compounds were tested for cytotoxicity in this cell line and all were found to have no inherent cytotoxicity. This is important to note, as additive cytotoxic effects would confound the interpretation of these data.

Several amide replacements were pursued. Initially, we prepared **10**, which contained our original amide orientation, using the procedure shown in Scheme 2. We also prepared ether isostere **11**[14] and the reduced amide isostere **12**.[15] All three of these compounds showed good activity (EC_{50} in the low μM range in the HeLa-T5 assay), comparable to LY 402913. One change in this region that did result in a slight increase in potency was the *N*-methyl analogue **13** ($EC_{50}=0.64$ μM) (Fig. 1).[16]

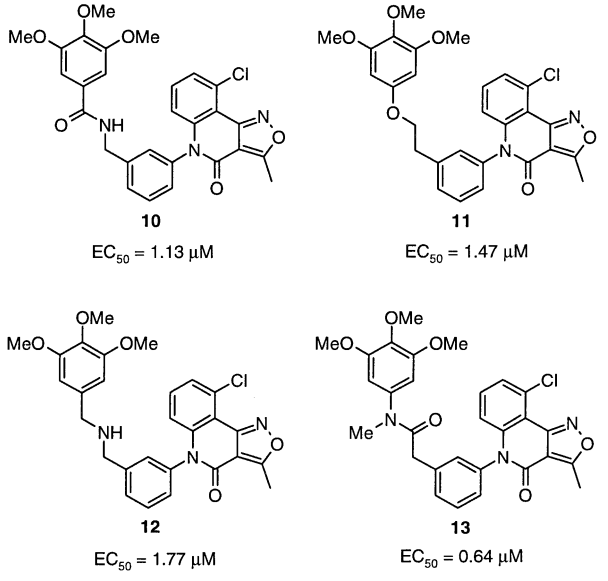

Figure 1. Amide isosteres of LY 402913.

Based on the in vitro data in the HeLa-T5 cellular assay, several compounds were evaluated for their ability to (a) inhibit the target directly in an LTC_4 transport assay and (b) selectively inhibit the desired target (MRP1) in preference to the related transporter (P-gp). These data are summarized in Table 2. The LTC_4 transport assay measures the ability to specifically inhibit the transport of the known substrate (LTC_4) by MRP1 in an ATP-dependent fashion.[17] Compounds that can reverse the drug-resistant phenotype in a cellular assay (such as the HeLa-T5 assay) by some other mechanism will not show activity in this assay. Thus, for our program, it was critical that compounds display activity here. Selectivity was determined in the HL60 panel of drug selected cells, similar to the HeLa-T5 experiments. The HL60/Adr cells overexpress MRP1, while the HL60/Vinc cells overexpress P-gp.[18,19] Therefore, the ability for a compound to reverse drug resistance in the HL60/Adr cell line, but not in the HL60/Vinc cell line, would be indicative of selective MRP1 inhibition. A selectivity ratio was calculated using the EC_{50} values in these two assays. While all of the compounds showed good activity and selectivity in these assays, LY 402913 demonstrated the best profile of potency in the cell lines, specificity at the target and selectivity for MRP1 versus P-gp.

We developed an MRP1-dependent in vivo model by implanting the drug-resistant HeLa-T5 cell line into nude mice.[20] This produced tumors that were resistant to the MRP1-associated oncolytic vincristine. It should be pointed out that the vector control cell line HeLa-C1, which does not overexpress MRP1, was found to be responsive to vincristine treatment. Thus, a successful MRP1 inhibitor should have a synergistic effect on reducing tumor growth when dosed in combination with vincristine in the HeLa-T5 in vivo model. Figure 2 shows the results of an in vivo study using LY 402913 and vincristine. Animals were dosed orally with 10 mg/kg LY 402913, 30 min before and after a bolus iv infusion (0.5 mg/kg) of vincristine, for 5 days. Additionally, several controls were included in this study. First, the vehicle control shows the tumor growth with no therapy and, second, the vincristine-treated animals (0.5 mg/kg,

Table 2. Specificity and selectivity of MRP1 inhibitors

Compd	LTC_4 transport	EC_{50} (μM)[a]		Selectivity ratio[b]
		HL60/Adr	HL60/Vinc	
9h (LY 402913)	1.8	0.07 (±0.01)	1.54 (±0.08)	22
9i	1.7	0.19 (±0.08)	2.58 (±0.42)	14
11	4.4	0.16 (±0.02)	2.34 (±0.63)	14
13	4.2	0.32 (±0.11)	1.33 (±0.15)	4

[a]Standard errors are shown in parentheses.
[b]Selectivity ratios were determined by calculating the ratio of average EC_{50} values in the HL60/ADR and HL60/Vinc cell lines.

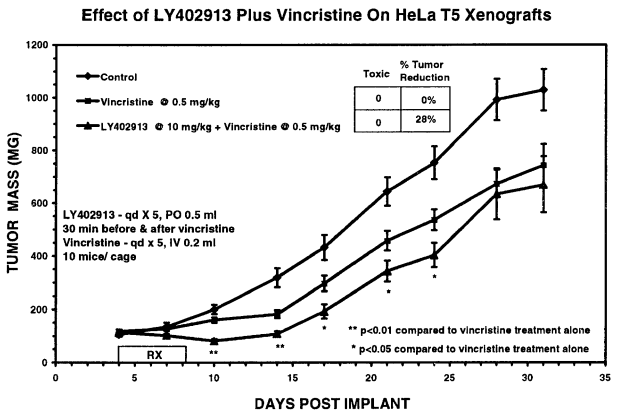

Figure 2. In vivo activity of LY 402913.

iv) showed the effect of drug without inhibitor. A final control was performed where the animals were dosed with inhibitor alone (same regimen). This curve, which overlaps the vehicle control curve (data not shown), indicates that the inhibitor has no antitumor activity of its own. As shown in Figure 2, the combination treatment of vincristine and LY 402913 gave a statistically significant improvement in efficacy, relative to the controls. We believe that this is the first report of a selective MRP1 modulator with in vivo efficacy in an MRP1-dependent animal model.

In summary, these studies show that the tricyclic isoxazoles are a promising series of selective MRP1 inhibitors. As more is learned on the tissue specificity of ABC transporters, and their relationship to clinical drug resistance, we feel that it is essential that selective drug resistance modulators be developed. Due to the fact that most of the known MRP1 modulators also inhibit the related transporter, P-gp, this series is particularly exciting.

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13. Compounds were tested against the HeLa-T5 cell line in vitro using an MTT-like assay. A fixed concentration of doxorubicin (0.5 μg/mL) and varying concentrations of test compounds, starting at 10 μM, were added to triplicate wells of a 96-well plate and incubated for 72 h at 37 °C. EC_{50} values were calculated from level of inhibition of cell growth using curve fitting software. Each compound was also tested for inherent toxicity in the same assay in the absence of doxorubicin. Test compounds showed <20% growth inhibition at 10 μM.
14. Ether isostere **11** was prepared using the following conditions: (a) 3,4,5-trimethoxyphenol, 3-nitrophenethyl alcohol, DEAD, PPh_3, toluene, 1 h (75%); (b) H_2/Pd on C, methanol, 30 psi, 2 h (98%); see Scheme 2 (c) and (d).
15. Reduced amide **12** was prepared according to Scheme 2, beginning with BOC-protected 3-nitrobenzylamine. Upon formation of the tricyclic moiety, the BOC group was removed (TFA, 100%) and reductive amination with 3,4,5-trimethoxybenzaldehyde ($NaCNBH_3$, methanol, 87%) gave the product.
16. The *N*-methyl analogue **13** was prepared according to Scheme 2 and was methylated after the first acylation (NaH, MeI, DMF, 1 h, 90%).
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19. Modulator EC_{50} in HL60/Adr and HL60/Vinc cells were determined by assessing the concentration-dependent ability of the modulator to enhance the antiproliferative response of the cells to an IC_{20} concentration of doxorubicin using alamarBlue® reduction as a surrogate measure for cell number. The selectivity ratio was calculated by dividing the average EC_{50} for HL60/Vinc cells by the average EC_{50} for HL60/Adr cells.
20. The ability of the compounds to increase the antitumor activity of co-administered vincristine in the HeLa-T5 xenograft model was tested. Test compounds were orally administered for 5 consecutive days to female nude mice with and without intravenous administration of vincristine sulfate. Caliper measurements of tumor growth were taken at various time points and plotted to indicate antitumor response.