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Cyclohexyl-linked tricyclic isoxazoles are potent and selective modulators of the multidrug resistance protein (MRP1)

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Abstract—Structure–activity relationship (SAR) studies on the tricyclic isoxazole series of MRP1 modulators have resulted in the identification of potent and selective inhibitors containing cyclohexyl-based linkers. These studies ultimately identified compound **21b**, which reverses drug resistance to MRP1 substrates, such as doxorubicin, in HeLa-T5 cells (EC_{50} = 0.093 μM), while showing no inherent cytotoxicity. Additionally, **21b** inhibits ATP-dependent, MRP1-mediated LTC_4 uptake into membrane vesicles prepared from the MRP1-overexpressing HeLa-T5 cells (EC_{50} = 0.064 μM) and shows selectivity (1115-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, **21b** showed tumor regression and growth delay in MRP1-overexpressing tumors in vivo.

The effective treatment of many types of cancer continues to be a significantly unmet medical need. Oftentimes, the failure of a specific course of therapy is a result of the tumor cells developing resistance to the agent(s) used in the therapy.[1] In the clinic, it is quite common for cancer patients to initially respond to chemotherapy, but ultimately experience relapse, due to the fact that their tumors have developed resistance. In some cases, patients develop multidrug resistance (MDR). These patients are resistant to several classes of oncolytics in addition to the treatment drug.[1] We have been interested in understanding the molecular mechanisms by which tumor cells become resistant to chemotherapeutic agents.[2,3] One mechanism involves the efflux of the oncolytic (or a metabolite) out of the tumor cell, resulting in a lower intracellular concentration of the agent. It has been shown that P-glycoprotein (Pgp, ABCB1)[4] and the multidrug resistance protein (MRP1, ABCC1)[5,6] can transport a variety of chemotherapeutics out of cancer cells and these transport proteins are often overexpressed in MDR tumors. Thus, one approach to improving treatment would be to inhibit the action of these transporters, thus restoring sensitivity to the tumor cells. This should result in an improved response to therapy.

The Pgp transporter has been studied over the past two decades and numerous modulators of Pgp have been described.[7] Many of these agents are presently being tested in the clinic to determine the role of Pgp in clinical resistance. The related transporter, MRP1, was discovered much later.[8] Thus, the number of selective modulators of MRP1 is far less than for Pgp.[9] We recently reported on a novel class of selective MRP1 modulators called tricyclic isoxazoles, characterized by **1** and **2**.[3] These compounds demonstrated reversal of MRP1-mediated drug resistance (in the presence of a sublethal dose of doxorubicin) in the MRP1-transfected cell line, HeLa-T5 (EC_{50} = 0.90 and 1.13 μM, respectively). This is a direct measure of a compound's ability to reverse the MRP1-mediated resistance in a relevant cell line which overexpresses MRP1. Additionally, **1** demonstrated selectivity for MRP1 versus Pgp in cells and inhibited ATP-dependent, MRP1-mediated uptake of LTC_4 into membrane vesicles (Fig. 1). Finally, when dosed in combination with the MRP1 substrate vincristine, **1** delayed the growth of MRP1-overexpressing tumors in vivo. This was the first description of an enhanced antitumor effect

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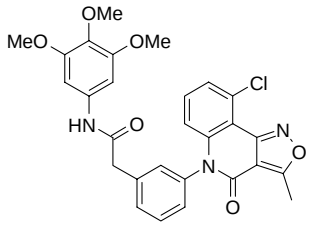
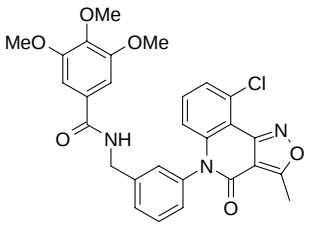


1
HeLa-T5 EC_{50} = 0.90 μM
HL60/ADR EC_{50} = 0.07 μM
HL60/Vinc EC_{50} = 1.54 μM
MRP1/P-gp Selectivity Ratio = 22-fold
LTC_4 Uptake inhibition IC_{50} = 1.8 μM



2
HeLa-T5 EC50 = 1.13 μM

Figure 1. Aryl-linked tricyclic isoxazole inhibitors of MRP1.

with a selective MRP1 modulator and represented the beginning of an SAR effort to find molecules which demonstrated greater potency and selectivity at the target.

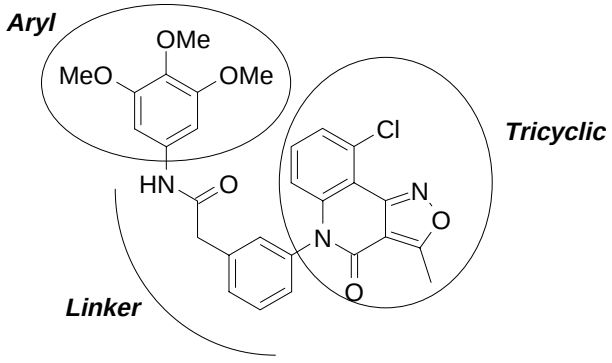
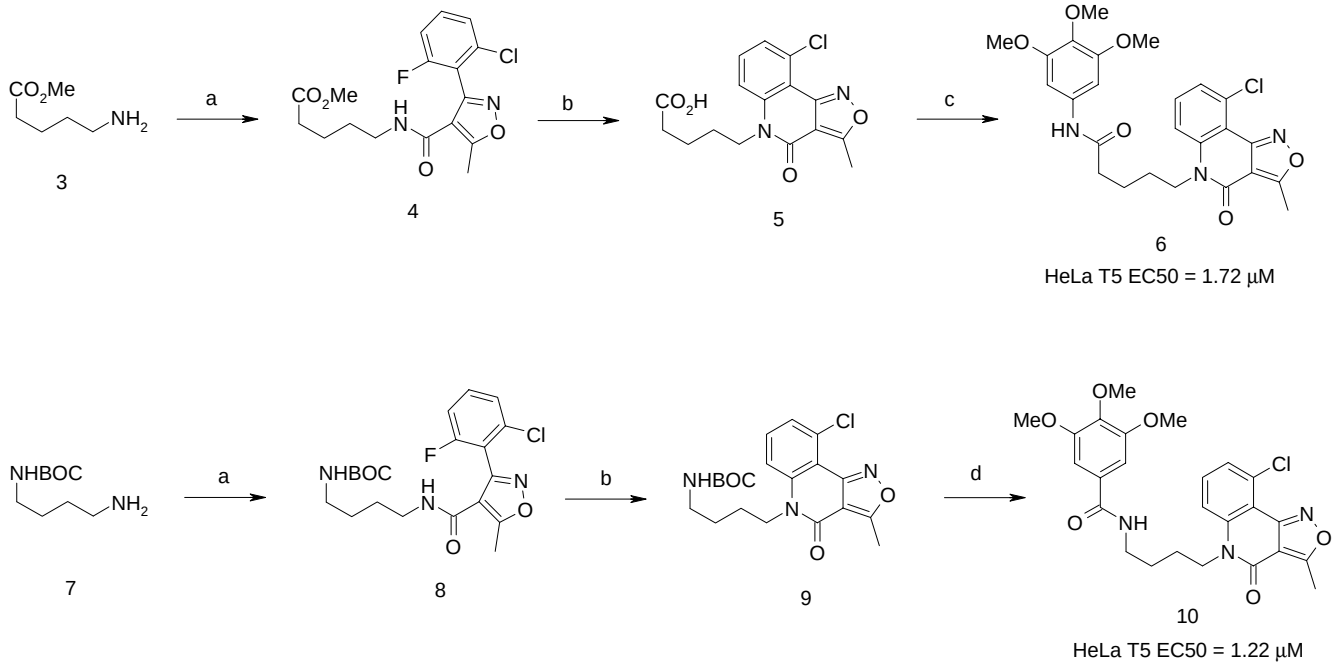


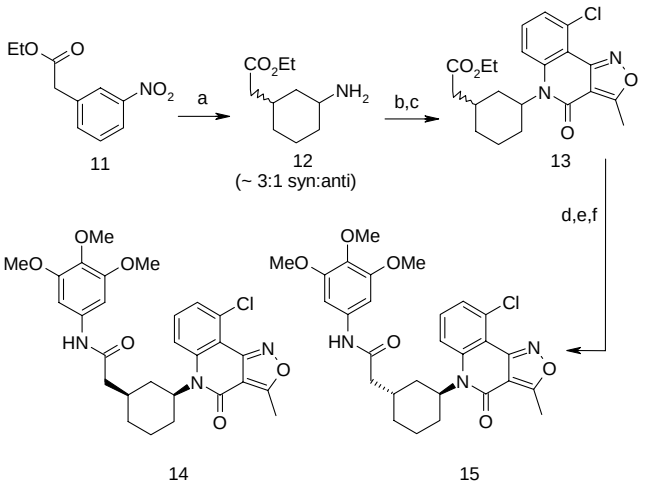
Figure 2. Tricyclic isozazoles. SAR regions.

One can imagine this class of molecules as having three specific regions: tricyclic, linker, and aryl regions (Fig. 2). Our early SAR efforts indicated that the aryl substitution had very significant effects on the activity of these compounds. Additionally, we have shown that the tricyclic functionality is required for activity. Thus, we have hypothesized that the primary purpose of the linker region is to present the aryl and tricyclic moieties in the proper three-dimensional space for optimal binding to the MRP1 protein. At first, we tried the simplest linker replacements—straight carbon chains. The chemistry to prepare these compounds is shown in Scheme 1.[10] Beginning with either 5-aminovaleric acid–HCl (**3**) or BOC-1,4-diaminobutane (**7**), the targets (**6** and **10**) were prepared as previously described,[3] with the key synthetic step being an intramolecular nucleophilic aromatic substitution reaction to form the tricyclic isoxazole. Interestingly, these flexible linker analogs **6** and **10** had HeLa-T5 EC_{50} values similar to those of the phenyl linker compounds **1** and **2**.

In an effort to continue to explore alternative linker functionalities, we considered saturated, 1,3-disubstituted carbocyclic linkers. We envisioned that these molecules would be significantly less flexible than the straight chain linkers **6** and **10**, but with a slightly different presentation of the aryl and tricyclic moieties, relative to the aryl linkers, such as **1** and **2**. Additionally, due to the sp^3 nature of the saturated carbocyclic linkers, we sought to explore both cis and trans analogs of the cyclohexyl linkers. Due to the expected 1,3-diequatorial orientation of the cis and 1,3-axial/equatorial orientation of the trans isomers, we felt that the cyclohexyl



Scheme 1. Reagents and conditions: (a) 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (85%); (b) 2 N NaOH/MeOH, DMF, 25 °C, 2 h (94%); (c) i—$(COCl)_2$, CH_2Cl_2, 1 h (100%); ii—3,4,5-trimethoxyaniline, Et_3N, CH_2Cl_2, 15 h (SCX column) (89%); (d) i—TFA, 1 h; ii—3,4,5-trimethoxybenzoyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (81%).



Scheme 2. Reagents and conditions: (a) H_2, Rh on C, 60 psi, EtOH, 60 °C, 18 h (72%); (b) 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (85%); (c) KO-*t*-Bu, DMF, 25 °C, 18 h (90%); (d) 1 N NaOH, MeOH (98%); (e) $(COCl)_2$, CH_2Cl_2, 1 h (100%); (f) 3,4,5-trimethoxybenzoyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (86%).

linkers would provide a significant harvest of ligand orientation information.

We prepared the cyclohexyl analogs **14** and **15**, starting with ethyl 3-nitrophenylacetate (**11**, Scheme 2).[11] Saturating hydrogenation conditions provided the cyclohexyl amino ester **12** as a 3:1 mixture (*syn*:*anti*) of inseparable isomers.[12] Acylation of the amine mixture, followed by intramolecular nucleophilic aromatic substitution, produced the tricyclic ester **13**. Standard saponification conditions, followed by acid chloride formation and amide preparation, provided the targets **14** and **15** as a 3:1 mixture of diastereomers. These materials were easily separated by flash chromatography. Additionally, each diastereomer was separated into its pure enantiomers (**14a** and **b**; **15a** and **b**) via chiral HPLC (Chiralpak AD).

The in vitro data on cyclohexyl-linked compounds **14** and **15** are shown in Table 1.[13] While all four compounds inhibited MRP1, **14b** showed greater potency in the HeLa-T5 assay and significantly greater activity in the transport assay, which is a direct measure of the compound's ability to inhibit the ATP-dependent transport of an MRP1 substrate into membrane vesicles. Additionally, we noted a clear enantiospecificity in the in vitro results. Specifically, **14b** was ~4× more potent in cells and up to 23× more potent in the LTC_4 transport assay, relative to **14a**.

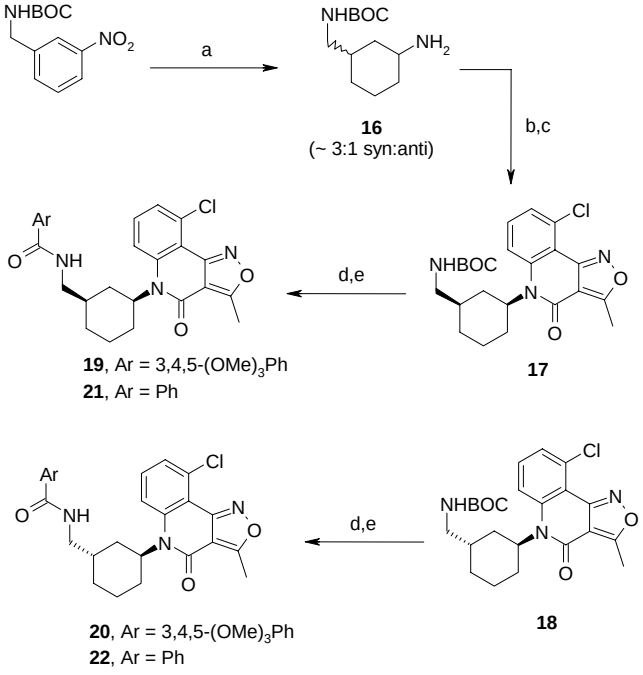
Table 1. In vitro properties of **14** and **15**[a]

Compound	EC_{50} (μM) HeLa-T5	IC_{50} (μM) LTC_4 transport
14a	1.10 (±0.13)	1.86 (±0.545)
14b	0.256 (±0.021)	0.079 (±0.038)
15a	1.63	0.849
15b	0.344	0.637

[a] Standard errors are shown in parentheses. Others tested in duplicate.

We became interested in pursuing a different amide orientation in the linker region. Thus, we prepared targets **19**–**22** (Scheme 3) in a similar fashion and separated the enantiomers of the more active cis analogs.[11,12] The in vitro data are shown in Table 2.[13]

Once again, cis diastereomers **19** and **21** proved more potent than the trans isomers **20** and **22** in both HeLa-T5 and LTC_4 transport assays. Additionally, we found that in this amide series, simple phenyl was more potent than the trimethoxyphenyl substitution. For both series, the cis diastereomers were separated into their enantiomers **19a/b** and **21a/b**.[14] In addition to its exquisite activity in these assays, the more active phenyl substituted enantiomer **21b** showed excellent selectivity (>1000-fold) in the HL60 panel of MRP1- overexpressing (HL60/Adr) and Pgp-overexpressing (HL60/Vinc) cells.[15]



Scheme 3. Reagents and conditions: (a) H_2, Rh on C, 60 psi, EtOH, 60 °C, 18 h (81%); (b) 3-(2-chloro-6-fluorophenyl)-5-methyl-4-isoxazolyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (82%); (c) KO-*t*-Bu, DMF, 25 °C, 18 h (86%); (d) TFA, 1 h (100%); (e) aroyl chloride, Et_3N, CH_2Cl_2, 25 °C, 1 h (71–86%).

Table 2. In vitro properties of **19**–**22**[a]

Compound	EC_{50} (μM) HeLa-T5	IC_{50} (μM) LTC_4 transport
19 (racemic)	0.25	0.27
20 (racemic)	0.42	NT
21 (racemic)	0.18 (±0.016)	0.038 (±0.004)
22 (racemic)	0.64	1.014
19a	0.56	0.78
19b	0.16	0.17 (±0.07)
21a	0.43 (±0.015)	0.545 (±0.159)
21b	0.093 (±0.004)	0.064 (±0.022)

[a] Standard errors are shown in parentheses. Others tested in duplicate.

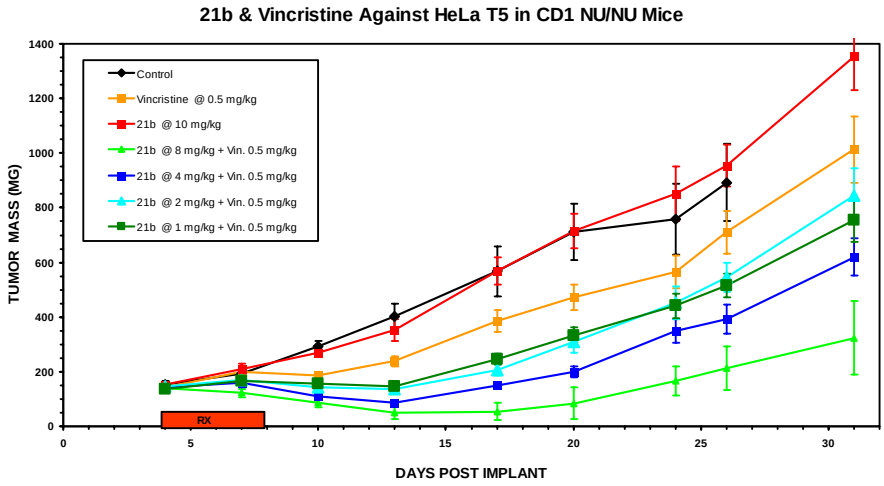


Figure 3. Efficacy of **21b** and vincristine in HeLa-T5 antitumor model. Compound **21b**: BID × 5, PO, 30 min before and after vincristine dosing. Vincristine: qd × 5, iv.

21b

HL60/Adr	EC_{50} = 0.002 μM
HL60/Vinc	EC_{50} = 2.23 μM
Selectivity = 1115×	

Due to its interesting in vitro properties, we decided to explore **21b** in our in vivo model for MRP1-mediated drug resistance. As previously described,[3] we have developed an MRP1-dependent in vivo model by implanting the drug resistant HeLa-T5 cell line into nude mice, producing tumors which are resistant to the MRP1-associated oncolytic, vincristine.[13] The vector control cell line HeLa-C1, which does not overexpress MRP1, was similarly implanted into nude mice and 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 3 shows the results of an in vivo study using **21b** and vincristine. Animals were dosed orally with 8, 4, 2, and 1 mg/kg **21b**, 30 min before and after a bolus iv infusion (0.5 mg/kg) of vincristine, for five days. Additionally, several controls were included in this study. The vehicle control shows the tumor growth with no therapy, while the vincristine-treated animals (0.5 mg/kg, iv) showed the effect of drug without inhibitor. A final control was performed where the animals were dosed with inhibitor alone. This curve indicates that the inhibitor has no antitumor activity of its own. As shown in Figure 3, the combination treatment of vincristine and **21b** gave a statistically significant improvement in efficacy, relative to the controls, at all doses. The effect was dose responsive. Even more noteworthy is the demonstration of tumor regression at the higher doses of **21b**: 63% regression at the 8 mg/kg dose (green line) and 40% regression at the 6 mg/kg dose (blue line).

In conclusion, we have shown that tricyclic isoxazoles continue to be a promising scaffold for selective MRP1 modulation. Specifically, compounds, such as **21b**, are useful tools, which may hasten the understanding of MRP1-mediated drug resistance.

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12. The relative stereochemistry of the *syn* and *anti bis*-substituted cyclohexyl derivatives (**14**, **15**, and **19**–**21**) was determined using spectroscopic methods and will be described in a forthcoming publication.
13. Assay conditions have been previously described. See Ref. 3 (and references cited therein).
14. An enantiospecific synthesis has been developed and the absolute stereochemistries have been determined for compounds in the cyclohexyl-linked series. This work will be described in a forthcoming publication.

15. 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 IC_{20} concentrations for doxorubicin used are 1.0 μg/ml for HL60/Adr line and 0.3 μg/ml for the HL60/Vinc line. The dox IC_{50} values for these cell lines are 4.0 μg/ml for HL60/Adr and 1.0 μg/ml for HL60/Vinc. The selectivity ratio was calculated by dividing the average EC_{50} for HL60/Vinc cells by the average EC_{50} for HL60/Adr cells.