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Discovery of potent LPA_2 (EDG4) antagonists as potential anticancer agents

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Abstract—The LPA_2 protein is overexpressed in many tumor cells. We report the optimization of a series of LPA_2 antagonists using calcium mobilization assay (aequorin assay) that led to the discovery of the first reported inhibitors selective for LPA_2. Key compounds were evaluated in vitro for inhibition of LPA_2 mediated Erk activation and proliferation of HCT-116 cells. These compounds could be used to evaluate the benefits of LPA_2 inhibition both in vitro and in vivo.

The lysophosphatidic acid receptor 2 (LPA_2, also known as endothelial differentiation gene 4, EDG4) is a G-protein coupled receptor (GPCR) activated by lysophosphatidic acid (LPA, 1- or 2-acyl-*sn*-glycerol-3-phosphate, general structure represented by **1** in Fig. 1).[1,2] LPA is known to mediate cell adhesion and migration,[3] promote cancer cell proliferation,[4] and elevated levels of LPA are often found in some cancer patients.[1b,5] LPA_2 has been shown to be overexpressed in cells from many tumor types (breast,[6] colorectal,[7] gastric,[8] ovarian,[1b,9] and kidney).[10] Recently, siRNA knock-down of LPA_2 in HCT-116 colon cancer cells led to growth arrest and apoptosis of cells in vitro and in xenograph models.[11] Computational modeling has been used to develop LPA_2 agonists[12] and several LPA_2 agonists are reported,[13] including some that induce cell migration and proliferation.[13c] In an effort to explore novel cancer treatment pathways, we sought to develop a small molecule LPA_2 antagonist as a potential anticancer therapeutic.

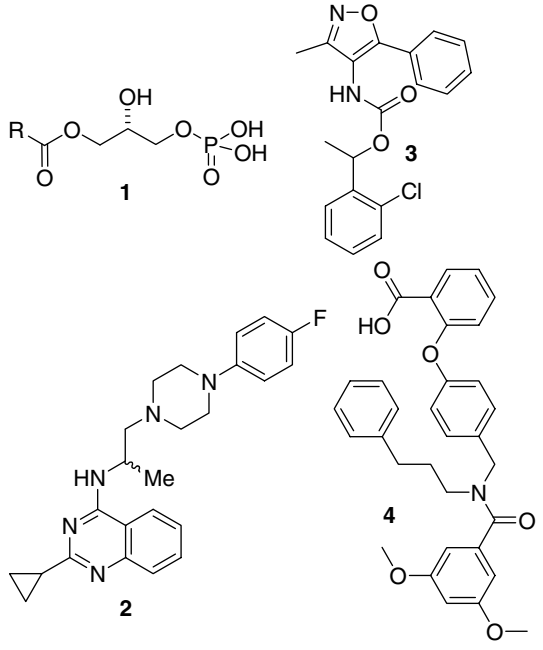

Figure 1. Literature compounds and HTS hit.

Here we report the first potent and selective small molecule antagonists for LPA_2. Using a cell-based Ca^{2+} flux

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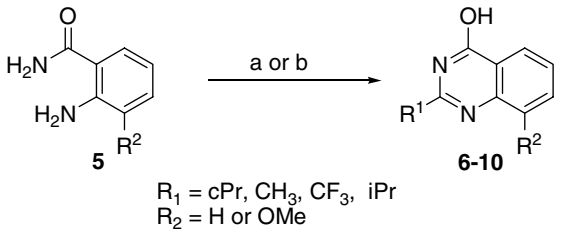
assay in a high throughput screening of our compound library,[14] we identified compound **2** as an inhibitor of LPA-induced Ca^{2+} flux (IC_{50} = 1.7 μM). Some compounds have been reported as antagonists for the LPA receptors LPA_1 (EDG2) and LPA_3 (EDG7) (**3**[17] and **4**,[18] respectively). Due to the sequence similarity among LPA_1, LPA_2, and LPA_3,[1c–e] these compounds were examined for their LPA_2 potency but were found to be inactive.

In order to explore the SAR of this initial hit, we needed a reliable method to prepare 2-alkyl-quinazolines. We performed several attempts to prepare the desired substituted quinazolines (Scheme 1). Our most successful efforts employed 2-amino benzamides **5**. Unfortunately, the amides **5** failed to reproducibly afford the desired quinazolines **6** under basic conditions (NaOEt, EtOH, 90 °C) when reacted with either cyclopropylethyl ester or cyclopropyl acid chloride. However, we discovered that under microwave heating conditions, we could quickly and reliably prepare the desired quinazolines **6**–**10** in moderate yields by using neat alkyl carboxylic acids (Table 1).

With a convenient route to 2-alkyl-quinazolines in place, we examined several linker and substituted phenyl derivatives for antagonist activity. The analogues were prepared by converting the 4-hydroxyquinazolines **6**–**10** to the corresponding chlorides **11** (Scheme 2). S_NAr installation of the amino acid derivatives followed by amide coupling and then reduction of the amide provided analogues **13**–**19**. Pyrimidyl ring reduction was a major side-product of this sequence. All biologically active compounds were characterized by ^1H NMR, LC/MS and their purity was determined to be greater than 95% by reverse phase HPLC.[19]

Compounds **13**–**19** were tested for LPA_2 activity using a calcium mobilization (aequorin) assay and the results

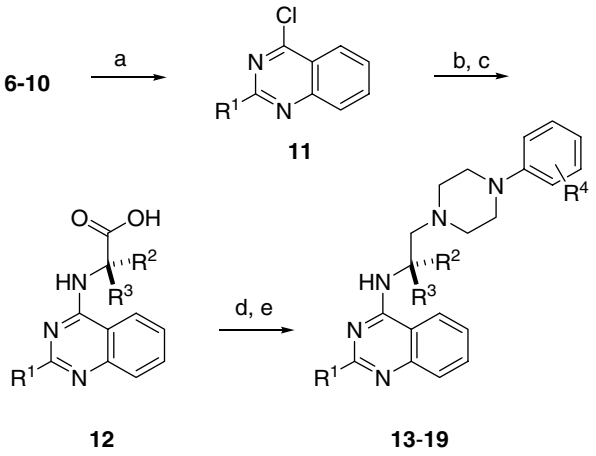


Scheme 1. Preparation of quinazolines. Reagents and conditions: (a) R_1CO_2Et or R_1CO_2Cl, NaOEt, EtOH, 90 °C, 24 h; (b) RCO_2H, μ-wave, 300 °C, 10 min.

Table 1. Preparation of 2-alkyl-quinazolines **6**–**10** using microwave[a]

Compound	R^1	R^2	Yield (%)
6	*c*-Pr	H	52
7	Me	H	69
8	*i*-Pr	H	37
9	CF_3	H	75
10	CF_3	OMe	29

[a] Isolated yields are reported in table.



Scheme 2. Preparation of linker analogues. Reagents and conditions: (a) $POCl_3$, 100 °C, 4 h, 80%; (b) Na_2CO_3, $NH_2C(R_2, R_3)CO_2Me$, MeCN, 12 h; (c) LiOH, 90% over two steps; (d) R_4-aryl piperazine, HOBt, EDC, CH_2Cl_2, 50–100%; (e) BH_3, THF, 20–100%.

are shown in Table 2. 2-Trifluoromethyl-quinazoline **14** was preferred over the 2-cyclopropyl derivative **13**. Analogues **14** and **15** illustrated that (*S*) is the preferred stereochemistry within the linker region. Only the monomethyl linkers **14** and **19** were found to be active. Other groups such as ethyl **17**, dimethyl **18**, and unsubstituted **16** linker regions resulted in greatly reduced potency. Several substituents on the phenyl group were explored with the 2-methyl **19** being the most potent derivative in this series.

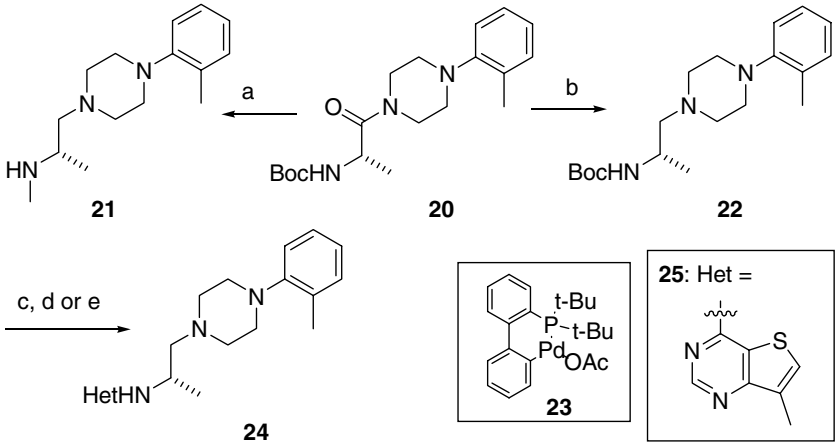
Having established the SAR of the linker region, our effort then shifted to finding the optimal heterocyclic tail functionality for LPA_2 activity. To this end, *N*-Boc protected L-alanine was coupled with 2-tolylpiperazine under standard conditions to afford **20** (Scheme 3). Mild reduction of **20** with borane at room temperature afforded amine **22**. Under reflux heating, **20** was converted to the undesired methyl amine **21**. The amine of carbamate **22** was then unmasked and coupled to various heterocyclic chlorides via S_NAr displacement (Cs_2CO_3) or Pd catalyzed coupling (**23**,[20] NaOtBu). Many heterocycles were well tolerated as tail moieties for LPA_2 antagonists; however, the thienopyrimidine **25** was found to be superior with an IC_{50} of 0.26 μM in the aequorin assay.

Since the thienopyrimidine **25** was found to be the optimal tail functionality for potency, we explored the SAR

Table 2. LPA_2 activity of compounds **13**–**19**[a]

Compound	R^1	R^2	R^3	R^4	IC_{50}
13	*c*-Pr	CH_3	H	2-OCH_3	3.2
14	CF_3	CH_3	H	2-OCH_3	1.3
15	CF_3	H	CH_3	2-OCH_3	>30
16	CF_3	H	H	2-OCH_3	>30
17	CF_3	CH_2CH_3	H	2-OCH_3	>30
18	CF_3	CH_3	CH_3	2-OCH_3	>30
19	CF_3	CH_3	H	2-CH_3	0.73

[a] IC_{50} values in μM (n = 2) have been determined by Ca^{2+} flux in RH7777 cells co-expressing LPA_2, chim, G_{i4}-protein, and aequorin.

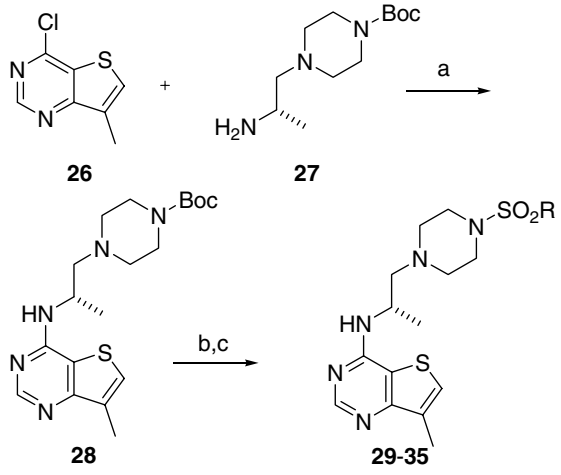


Scheme 3. Preparation of quinazoline replacements. Reagents and conditions: (a) BH_3–THF, THF, reflux, 100%; (b) BH_3–THF, rt; then $NH_2CH_2CH_2NH_2$, MeOH, reflux, 43%; (c) HCl, dioxane, CH_2Cl_2, 72%; (d) HetCl, Cs_2CO_3, CH_3CN, 85 °C; (e) HetCl, **23**, NaOtBu, toluene, 80 °C, 20–89%.

of the head region with this advanced tail in place. The compounds were prepared by coupling chloride **26** with the amine linker **27**, thus affording **28** (Scheme 4). After amine deprotection several functional groups were then examined (amide, urea, carbamate, and alkyl). Aryl sulfonamides were found to be the most active.

Compounds **29**–**35** were examined for LPA_2 activity in the aequorin assay and the results are shown in Table 3. Phenyl analogues **31**–**35** were found to be optimal over alkyl **29** and benzyl **30** substituted derivatives for potency. In addition, 4-methyl **33** was more active than 2-methyl **31** and 3-methyl **32**. The 3-chloro derivative **34** provided a very potent compound with 3,4-dichloro **35** being the most active analogue of this series.

The LPA_2 selectivity of the most potent compounds was tested against LPA_1 and LPA_3 along with known LPA_1 and LPA_3 antagonists (**3** and **4**) and the results are shown in Table 4. Heterocycle **3** was found to be a potent LPA_1 antagonist while exhibiting some LPA_3 activity but no LPA_2 activity. Amide **4** was found to be a select and potent LPA_3 antagonist showing no activity in either LPA_1 or LPA_2 receptor assay. Tolyl analogue **25** and 3,4-dichloro derivative **35** are analogues of different chemotypes and were found to be inactive against the LPA_1 and LPA_3 receptors. Thus, these analogues of different chemotypes are potent and selective LPA_2 antagonists.[21]



Scheme 4. Preparation of sulfamide replacements. Reagents and conditions: (a) Cs_2CO_3, CH_3CN, 93%; (b) HCl, dioxane, 100%; (c) RSO_2Cl, py, CH_2Cl_2, 31–81%.

Table 3. LPA_2 activity of compounds **29**–**35**[a]

Compound	R	IC_{50}
29	Butyl	6.61
30	Benzyl	1.86
31	2-Me-Ph	0.73
32	3-Me-Ph	0.31
33	4-Me-Ph	0.17
34	3-Cl-Ph	0.035
35	3,4-Cl_2-Ph	0.017

[a] IC_{50} values μM ($n = 2$) have been determined by Ca^{2+} flux in RH7777 cells co-expressing LPA_2, chim, G_{i4}-protein, and aequorin.

Table 4. Selectivity for LPA_2, LPA_1, and LPA_3 receptors[a]

Compound	LPA_2	LPA_1	LPA_3
25	0.26	>50	>50
35	0.017	>50	>50
3	>50	0.05–0.30	12.00
4	>50	>50	0.04–0.08

[a] IC_{50} values μM ($n = 2$) have been determined by Ca^{2+} flux in RH7777 cells co-expressing LPA_1, LPA_2, or LPA_3, chim. G_{i4}-protein, and aequorin.

Encouraged by these results we sought to further examine the biological effect of our selective LPA_2 antagonists. Since activation of LPA_2 by LPA may lead to phosphorylation of Erk in LPA responsive cells, we investigated the effect of **35** on phosphorylation of Erk. An in-Cell Western blot analysis of cell lysates from HCT-116 cells treated with **35** was performed. The data shown in Figure 2 demonstrates that **35** inhibited the phosphorylation of Erk induced by LPA in a concentration dependent manner. As a specificity control, **35**

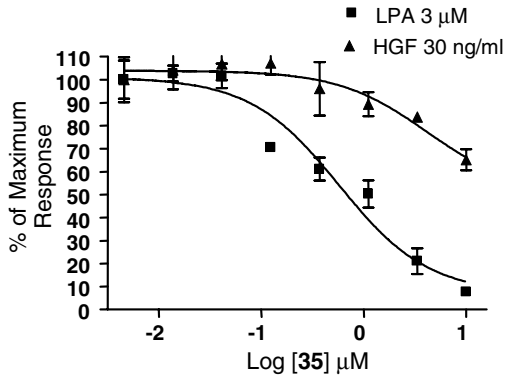


Figure 2. **35** inhibits LPA induced phosphorylation of Erk.[22]

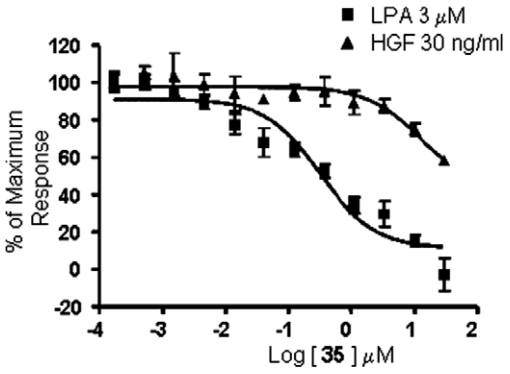


Figure 3. Compound **35** inhibits LPA induced proliferation of HCT-116 colon cancer cells.[23]

showed only a minor effect on ERK phosphorylation stimulated by HGF. Thus, an LPA_2 specific antagonist can inhibit LPA induced phosphorylation of Erk in the HCT-116 colon cancer cells.

Since LPA has been known to promote proliferation of some cancer cells,[6] we examined the effect of **35** on LPA induced proliferation of HCT-116 colon cancer cells. As shown in Figure 3, cell proliferation caused by LPA was inhibited by **35** in a doses dependent manner. This effect appears to be specific since HGF induced proliferation was only slightly inhibited at high concentrations of **35**. These data suggest that LPA_2 antagonism can potentially inhibit proliferation of cancer cells.

In summary, a high-throughput screen and a subsequent medicinal chemistry effort identified the first reported compounds that are selective and highly potent LPA_2 antagonists. Cell based functional assays showed that the potent LPA_2 antagonist **35** inhibited LPA induced Erk activation and proliferation of HCT-116 colon cancer cells. These compounds could be used as tool compounds to evaluate the anticancer effects of blocking LPA_2 mediated signaling.

Acknowledgment

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14. Rat hepatoma cells (RH7777) were transiently transfected with aequorin, chim. G_{i4}-protein, and LPA_2 using Fugene 6 transfection reagent (Roche Biosciences). In this assay, the reduction of LPA-induced receptor activation by library compounds in a 384-well based assay was monitored by flash luminescence detection. For experimental details, see Refs. 15,16.
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21. Compounds **19** and **35** were also inactive against the S1P receptor $S1P_1$ (also known as EDG1).
22. HCT-116 cells at 90% confluence in a 96-well plate were serum-starved for 2 h. Compound **35** was added at various concentrations and incubated with the cells for 1 h at 37 °C. The cells were then treated with LPA at 3 μM or HGF at 30 ng/ml for 12 min at 37 °C followed by fixation and permeabilization. The plate was then blocked for 2 h in Odyssey blocking buffer. After primary and secondary antibodies' incubation, phosphorylated Erk (p-Erk) and total Erk were simultaneously detected by LI-COR system (LI-COR Biosciences). p-Erk signal was normalized using the total ERK signal from each well.
23. HCT-116 cells were plated in 96-well plates (5000 cells/well) in serum free DMEM with 0.11% BSA. Compound **35** was added at various concentrations. LPA or HGF was then added to a final concentration of 3 μM or 30 ng/ml respectively. After 24-h incubation, viable cells were determined by using the CellTiter-Glo Assay kit (Promega).