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Pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amides as novel anti-proliferative agents: parallel synthesis for lead optimization of amide region

Ariamala Gopalsamy,[a,*] Hui Yang,[a] John W. Ellingboe,[a] Hwei-Ru Tsou,[a] Nan Zhang,[a] Erick Honores,[a] Dennis Powell,[a] Miriam Miranda,[b] John P. McGinnis[b] and Sridhar K. Rabindran[b]

[a]*Chemical and Screening Sciences, Wyeth Research, Pearl River, NY 10965, USA*
[b]*Oncology, Wyeth Research, Pearl River, NY 10965, USA*

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Abstract—A novel series of p21 chemoselective agents containing a pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amides were identified by high throughput screening. Optimization of the amide region by parallel synthesis and the iterative design toward understanding structure–activity relationship to improve potency are described. The isopropyl carbamate derivative **34** was identified as a highly chemoselective agent displaying a potency of 51 nM in the p21 deficient cell line.

Deregulation of cell proliferation, or a lack of appropriate cell death, has a wide range of clinical implications, including cancers, restenosis, angiogenesis, hyperplasia, endometriosis, lymphoproliferative disorders, graft rejection and the like. Such cells may lack the normal regulatory control of cell division, therefore fail to undergo appropriate cell death. Progression from one phase of the cell division cycle to the next phase is controlled by a series of sensors and arresting mechanisms called checkpoints.[1] Loss of checkpoint control is a hallmark of tumor cells, as it increases the mutation rate and allows a more rapid progression to the tumorigenic state.[1,2] However, inactivation of these checkpoints results in aberrant responses to cellular damage. This failure of checkpoint responses in malignant cells can be exploited in cancer drug discovery. Identification of compounds that selectively kill checkpoint-deficient cells compared with checkpoint-proficient cells can be expected to preferentially target tumor cells, while sparing normal cells.[3,4]

The p53 tumor suppressor gene is the major regulator of the DNA damage checkpoint and one of the most commonly mutated genes in human cancer (50–70%).[5,6] As a downstream effector of p53, p21 inhibits the cyclin-dependent kinases (CDKs) and arrests cell cycle progression in response to DNA damage.[7,8] Disruption of this checkpoint by deletion of the p21 protein, leads to the failure of the cell to arrest in response to DNA damage, endoreduplication, and ultimately, apoptosis.[9] These p21-deficient cells show increased chemosensitivity[10] compared with the isogenic p21-proficient parental cells to a variety of DNA damaging agents, including clinically used anti-neoplastic drugs, validating the role of checkpoints in determining chemosensitivity. We have used this isogenic pair of cell lines (p21+/+ and p21−/−) to identify novel molecules that preferentially induce apoptosis in the p21-deficient cells.

High throughput screening[11] using the above cell lines lead to the identification of pyrazolo[1,5-*a*]pyrimidin-7-yl phenyl amide **1** (Fig. 1), which had an IC_{50} of 0.4 μM in a p21 deficient cell line (80S14)[12] and an IC_{50} of 12 μM in a p21 proficient cell line (HCT116)[12] with a selectivity index of 30 (ratio of IC_{50}s of p21-proficient and p21-deficient cells). The parallel synthesis effort carried out to understand the structure–activity

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* Corresponding author. Fax: +1 845 602 3045; e-mail: gopalsa@wyeth.com

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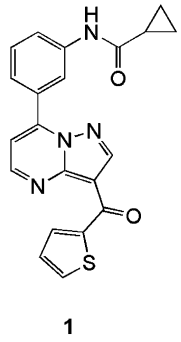

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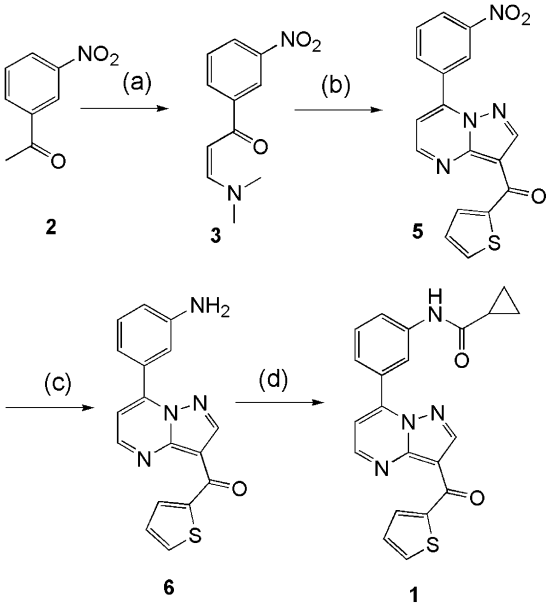
Figure 1. High throughput screen lead for identification of p21 chemoselective agent.

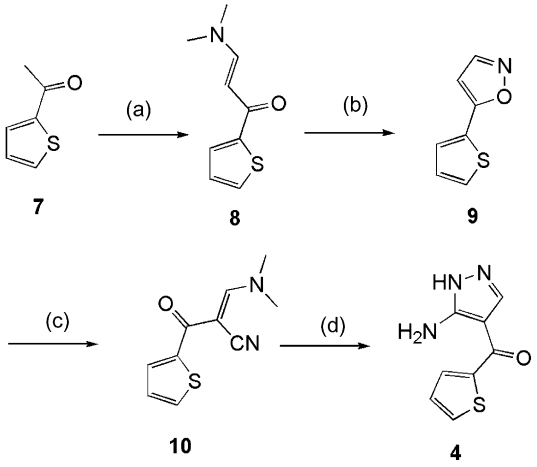
relationship of the amide region of this novel lead is described below.

The compounds for this series were readily synthesized[13] utilizing parallel solution phase synthesis as shown in Schemes 1–3. The common intermediate amine **6** required for varying the amide region of the molecule was synthesized starting from *m*-nitro acetophenone **2**. Conversion of the ketone **2** to enamine **3** followed by condensation with amino pyrazole derivative **4** (synthesis shown in Scheme 2) afforded the nitro compound **5**. Reduction of **5** afforded the common intermediate amine **6**. Reaction of the amine with cyclopropyl carbonyl chloride gave the lead compound **1**.[14]

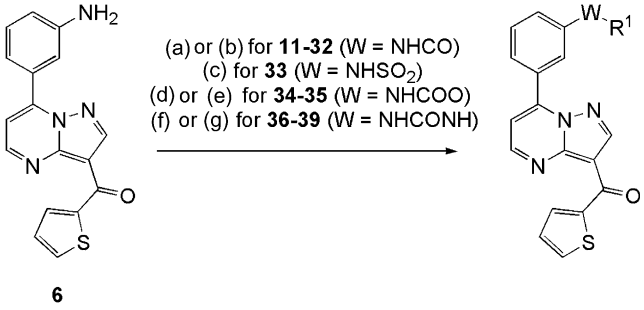
A number of amides **11**–**32** were synthesized using various acid chlorides or acids with the appropriate activation. To vary the amide region of the molecule to sulfonamide, carbamate, or urea, intermediate amine **6** was reacted with the required sulfonyl chlorides, chloroformates, or isocyanates (Scheme 3). Converting the



Scheme 1. Reagents and conditions: (a) $Me_2NCH(OMe)_2$, reflux, 18 h, 89%; (b) **4**, acetic acid, reflux, 82%; (c) Fe, NH_4Cl, MeOH, H_2O, 61%; (d) cyclopropyl carbonyl chloride, pyridine, 0 °C to rt, 6 h, 82%.



Scheme 2. Reagents and conditions: (a) $Me_2NCH(OMe)_2$, reflux, 18 h, 92%; (b) $NH_2OH{\cdot}HCl$, reflux, 3 h, 65%; (c) $Me_2NCH(OMe)_2$, reflux, 18 h, 74%; (d) $NH_2NH_2{\cdot}H_2O$, 60%.

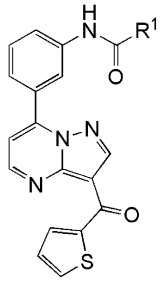


Scheme 3. Parallel synthesis. Reagents and conditions: (a) R^1COCl, pyridine, 0 °C to rt, 6 h; (b) R^1COOH, EDCI, HOBT, Et_3N, DMF, rt, 12 h; (c) R^1SO_2Cl, pyridine, 0 °C to rt, 6 h; (d) R^1OCOCl, pyridine, 0 °C to rt, 6 h; (e) 1: (4-NO_2Ph)OCOCl, pyridine, 0 °C to rt, 3 h, 2: R^1OH, rt, 18 h; (f) R^1NCO, pyridine, 0 °C to rt, 6 h; (g) 1: (4-NO_2Ph)OCOCl, pyridine, 0 °C to rt, 3 h, 2: R^1NH_2, rt, 18 h.

amine **6** into the 4-nitrophenyl carbamate derivative and reacting it with various amines also afforded ureas. Similarly, carbamates were also synthesized by reacting the 4-nitrophenyl carbamate of **6** with various alcohols. *N*-Alkylation of **1** using sodium hydride and ethyl iodide afforded **40**.

Our initial effort toward understanding the SAR of the lead molecule was focused on replacing the cyclopropyl group with other cycloalkyl groups. As seen from the data on Table 1, going to larger rings was very well tolerated and compounds **11**–**13** displayed similar activity and selectivity. At this point we decided to incorporate simple alkyl groups in the place of cyclopropyl to explore the spatial requirements for this region and also to see if flexible alkyl groups can take the place of the rigid cycloalkyl rings. The smaller methyl group **14** abolished the activity and the ethyl derivative **15** showed slightly weaker activity. The activity was regained as we went to the larger *n*-propyl and *n*-butyl groups (**16**–**17**). However, the longer and flexible pentyl and hexyl groups (**18**–**19**) showed a loss of potency, indicating the spatial constraint for this region of the molecule.

Table 1. Activity comparison of amide derivatives in p21 proficient and deficient cell lines

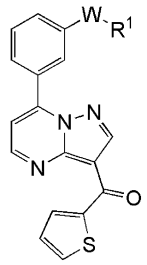


Ex	R^1	HCT116 IC_{50}(μM)	80S14 IC_{50} (μM)	Ratio
1	Cyclopropyl	12	0.40	30
11	Cyclobutyl	11	0.19	58
12	Cyclopentyl	6.9	0.2	35
13	Cyclohexyl	8.4	0.25	34
14	Methyl	>20	12	>2
15	Ethyl	29	1.1	26
16	*n*-Propyl	8.0	0.4	20
17	*n*-Butyl	5.4	0.35	15
18	*n*-Pentyl	9.1	1.0	9
19	*n*-Hexyl	13	4.3	3
20	*i*-Propyl	16	0.6	27
21	*i*-Butyl	6.4	0.14	46
22	*t*-Butyl	4.7	0.33	14
23	Neopentyl	7.7	0.29	27
24	Homoallyl	4.0	0.19	21
25	Buty-3-nyl	7.9	0.25	32
26	Phenyl	8.3	0.21	40
27	Benzyl	6.1	0.27	23
28	2-Furyl	15	0.44	34
29	2-Thiophenyl	15	0.47	32
30	$–CH_2NH_2$	>20	>20	1
31	$–CH_2N(Me)_2$	>20	11.5	>2
32	$–CH_2NHCO–$cyclopropyl	>20	>20	1

Branched alkyls (**20**–**23**) performed on par or slightly better when compared to the corresponding straight chain analogs. In particular, the isobutyl derivative **21** showed improved potency as well as selectivity. Unsaturation (**24**–**25**) was well tolerated in this region of the molecule but did not significantly improve the activity or selectivity. Similarly, replacing the cyclopropyl group with aryl groups (**26**–**27**) or heteroaryl groups like furan **28** or thiophene **29** maintained the biological activity. A dramatic drop in activity was seen as we introduced polar amino groups (**30**–**31**) at this region of the molecule. Also separating the cyclopropyl moiety with a glycine spacer (**32**) decreased the potency significantly (Table 1).

Although we optimized the lipophilic requirements using the amide linker, it was of interest to see if any other linkers would have an impact on improving the biological profile of the lead. The results of this study are summarized in Table 2. Replacing the amide with sulfonamide decreased the potency (**33**). However, a carbamate linker was more favorable, leading to a several fold improvement in potency while retaining the selectivity in the case of isopropyl carbamate **34**. When urea

Table 2. Activity comparison for derivatives with various linkers in p21 proficient and deficient cell lines



Ex	R^1	W	HCT116 IC_{50} (μM)	80S14 IC_{50} μM	Ratio
33	*i*-Propyl	$NHSO_2$	12	2.1	6
34	*i*-Propyl	NHCOO	1.1	0.051	22
35	*n*-Butyl	NHCOO	14	0.15	93
36	*i*-Propyl	NHCONH	2.7	0.089	30
37	*n*-Butyl	NHCONH	4.4	0.36	12
38	Neopentyl	NHCONH	3.9	0.14	28
39	Cyclopropyl	NHCONH	13	1.6	8
6	H	NH	>20	>20	1
40	Cyclopropyl	N(Et)CO	>20	>20	1

was used as the linker, as in the case of amide and carbamate series, the isopropyl analog **36** was found to be the most potent analog. The intermediate amine **6**, which lacks the lipophilic capping group, was completely inactive. Similarly, removing the hydrogen bond donor from the amide by alkylating the amide NH, as in the case of compound **40**, rendered the molecule inactive. This observation lead to the conclusion that a hydrogen bond donor–hydrogen bond acceptor sequence is preferred for the linker region.

The most potent compounds with the three linkers of interest, amide (**21**), carbamate (**34**), and urea (**36**) were selected for further characterization in the colon cell line panel (Table 3). These compounds displayed good potency across the panel and with no significant difference in potency based on the variation in the linker.

In conclusion, we have identified pyrazolo[1,5-*a*]pyrimidine derivatives as a novel class of p21 chemoselective anti-proliferative agents. We have explored the structure–activity requirement for the amide region of the molecule. For the lipophilic region of the molecule a small branched aliphatic group like isopropyl or isobutyryl group was found to be ideal to achieve potency as well as selectivity. Of the linkers explored, carbamate and urea linkers were slightly more potent than an amide linker. This effort has defined a promising strategy to discover new agents with anti-proliferative

Table 3. Activity comparison in the colon cell line panel

Ex	LoVo IC_{50} (μM)	SW620 IC_{50} (μM)	DLD1 IC_{50} (μM)	HT29 IC_{50} (μM)
21	0.021	0.045	0.042	0.036
34	0.017	0.022	0.028	0.023
36	0.024	0.052	0.046	0.052

properties and led to the identification of several analogs with sub-micromolar activity in a panel of colon cell lines.

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11. *Cytotoxicity assays*: p21+/+ and p21−/− cells isogenic cells and other carcinoma cell lines (LoVo, SW620, DLD1, HT29) were plated in 96-well tissue culture plates. The following day, dilutions of compounds were added and cells were cultured for an additional 4 days (colon cell panel) or 5 days (isogenic cell lines). Cell survival was determined using sulforhodamine B, a protein binding dye. The concentration of the compound that inhibits cell proliferation by 50% (IC_{50}) was estimated from inhibition curves. IC_{50}s shown are the mean values compiled from independent experiments (ranging from 2 to 67), except for compounds **27**, **33**, and **40**.
12. HCT116 is a human colon carcinoma cell line containing an intact p21 checkpoint (p21+/+). 80S14 cells are identical to HCT116 cells, except that the p21 checkpoint was disrupted by targeted deletion of the p21 gene (p21−/−) (Ref. 9a).
13. Dusza, J. P.; Tomcufcik, A. S.; Albright, J. D.; Beer, B. U.S. Patent 6,399,621, 2002.
14. Parallel solution phase synthesis was carried out in 8 mL vials in 0.1 mmol scale using an orbital shaker. Reaction completion was monitored by LC/MS and the solvent was removed in Savant evaporator and compounds were purified by HPLC and the purity was >90%. LC Conditions: HP 1100, 23 °C, 10 μL injected; Column: YMC-ODS-A 4.6 × 50 mm, 5 μm; gradient A: 0.05% TFA/water; B: 0.05% TFA/acetonitrile; time 0 and 1 min: 98% A and 2% B; 7 min: 10% A and 90% B; 8 min: 10% A and 90% B; 8.9 min: 98% A and 2% B; post time 1 min; flow rate 2.5 mL/min; detection: 215 and 254 nm, DAD. Semi-Prep HPLC: Gilson with Unipoint software; Column: Phenomenex C18 Luna 21.6 mm × 60 mm, 5 μm; solvent A: water (0.02% TFA buffer); solvent B: acetonitrile (0.02 % TFA buffer); solvent gradient: time 0: 5% B; 2.5 min: 5% B; 12 min: 95% B; hold 95% B 3 min; flow rate: 22.5 mL/min; detection: 215 and 254 nm. Compounds integrity was further determined by HRMS or CHN elemental analysis.