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Tricyclic azepine derivatives: Pyrimido[4,5-*b*]-1,4-benzoxazepines as a novel class of epidermal growth factor receptor kinase inhibitors

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Abstract—A novel class of pyrimido[4,5-*b*]-1,4-benzoxazepines is described as inhibitors of epidermal growth factor receptor (EGFR) tyrosine kinase. Two compounds display potent EGFR inhibitory activity of less than 1 μM in cellular phosphorylation assays (IC_{50} 0.47–0.69 μM) and are highly selective against a small kinase panel. Such compounds demonstrate anti-EGFR activity within a class that is different from any known EGFR inhibitor scaffolds. They also provide a basis for the design of kinase inhibitors with the desired selectivity profile.

Growth factor interactions with cell surface receptors induce the activation of signaling pathways that mediate a wide range of responses such as proliferation, survival, differentiation, and metabolism. The epidermal growth factor receptor (EGFR) has served as a paradigm in the study of how these tyrosine kinase receptors function and the signal transduction pathways involved. EGFR belongs to the ErbB family that includes EGFR (ErbB1), HER2/neu (ErbB2), HER3 (ErbB3), and HER4 (ErbB4).[1] These receptors regulate intracellular signaling pathways mediating cell proliferation, differentiation, migration, survival, and adhesion.[2] In oncogenesis, EGFR overexpression is a common feature in tumors of epithelial origin, including cancers of the lung, breast, head and neck, colon, and bladder. Moreover, patients expressing high levels of EGFR usually have poor prognosis.[3] Finally, studies have implicated EGFR in the development of several human tumors, thus establishing EGFR as a target for cancer therapy.[1] Therefore, a strategy to inhibit and suppress the unregulated activation of the tyrosine kinase domain of EGFR by targeting the ATP-binding pocket of EGFR with a small molecule would be beneficial.

The work presented below addresses the ongoing need to develop kinase inhibitors which have high activity and efficacy while demonstrating improved safety profiles. The majority of EGFR inhibitors reported in the literature belong to the quinazoline class as exemplified by *Iressa* (**1a**),[4] which has recently been approved for the treatment of non-small cell lung cancer, but has been shown to have major toxicity side-effects in a broad patient population.[5] An alternative compound series would potentially have a different pharmacokinetic and toxicity profile as well as kinase selectivity. In this paper, we report our initial progress toward optimizing a novel kinase inhibitor template. Specifically, we describe preliminary structure–activity relationships of pyrimido-1,4-benzoxazepines[4,5] as EGFR tyrosine kinase inhibitors (Fig. 1).

Keywords: Epidermal growth factor receptor; Kinase inhibitor; Benzoxazepine; Kinase selectivity.
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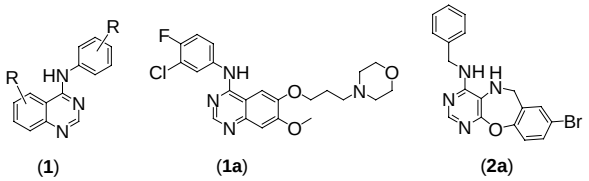
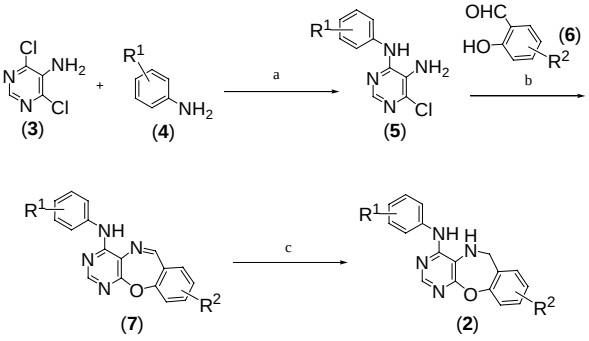


Figure 1. Quinazoline general scaffold (**1**), *Iressa* (**1a**), and benzoxazepine HTS hit (**2a**).

High-throughput screening identified compound (**2a**) as a small molecule inhibitor of EGFR with enzymatic IC_{50} of 6 μM.[6] The activity of (**2a**) in a cellular phosphorylation assay was shown to be weakly inhibitory with an IC_{50} equal to 30 μM.[7] The tricyclic structure (**2a**) provides a novel framework to perform SAR studies. Analysis of the literature suggested the tricyclic core of (**2a**) may play a vital role in positioning substituents in the correct orientation (**1**).[8] Hence, we kept the benzoxazepine core unchanged while varying substituents around it. Efforts therefore focused on substitution of both the amine group on the pyrimidine ring and substituents on the benzoxazepine core benzene ring of (**2a**) while retaining the seven-membered ring structural feature. Access to such compounds was via the general route summarized in Scheme 1, which is a modification of previously reported procedures.[9] 5-Amino-4,6-dichloropyrimidine (**3**) was reacted with an aniline (**4**) resulting in an intermediate (**5**) with moderate to good yields. A variety of hydroxybenzaldehydes (**6**) were reacted with (**5**) to provide benzoxazepine precursors (**7**). Reduction of (**7**) led to the final product (**2**).[10] We have prepared a variety of benzoxazepine analogs (**2**) with some representatives disclosed in Table 1.[11]

To explore how a substitution on the aniline phenyl ring would affect activity, a variety of groups were examined (Table 1). Noteworthy was the original HTS hit (**2a**) and its direct analogs (**2b**, **2c**) that possess a benzylic amine rather than an anilino moiety seen in more active analogs in Table 1. The unsubstituted compounds (**2d**, **2e**, and **2f**) showed little activity, similar to that seen in the original hit (**2a**), while several examples of *para* substitution (**2g**, **2h**) likewise exhibited discouraging results. In contrast, compounds with *meta* substitution in the aniline ring demonstrated a dramatically



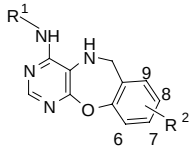
Scheme 1. Reagents and conditions: (a) DMF, 80 °C; (b) NaH, DMF, 80 °C; (c) $LiBH_4$, THF.

improved activity over *para* analogs. *Meta*-bromo (**2i**–**2m**) and *meta*-bromo-*para*-fluoro (**2n**–**2r**) compounds showed enzymatic activity reaching 0.9 μM and 15- to 30-fold increase in cellular potency when compared to the initial HTS hit (**2a**). However, some large groups such as a hydrophobic phenyl group are not well tolerated at the *meta* position on the aniline ring (see examples **2s**, **2t**).

Another region that is amendable to structure–activity relationship (SAR) studies is the benzene ring adjacent to the seven-membered oxazepine ring. The availability of a substituted hydroxyl aldehyde played a key role in our initial SAR effort. We observed that, in general, having a 7-methoxy, 8-methoxy or 8-halogen substituent (**2i**–**2r**) was advantageous over a 9-methoxy substituent (**2u**). Analogs substituted with other functional groups such as carboxylic acid, amine, amide, and sulfonamide groups (**2v**–**2ab**) were, on average, less active or equipotent in enzymatic assay to 7-methoxy, 8-methoxy or 8-halogen-substituted compounds (**2i**–**2r**). The unsubstituted benzoxazepines (**2ae**) and (**2af**) demonstrated potency similar to that of 7- or 8-substituted derivatives (including methoxy analogs), suggesting that these two positions are amenable to further modifications. This observation indicates that both 7-methoxy and 8-methoxy groups are favorable for the inhibition of kinase activity.[12] With the indication that two methoxy groups on the benzene ring could improve activity, several bis-methoxy analogs were prepared (Table 1). This modification proved to be very fruitful, resulting in compounds with 40- to 60-fold improvement in cellular potency (**2ag**, **2ah**). However, the 7,9-dimethoxy analog (**2ai**) did not retain the activity seen with the 7,8-disubstituted compounds (**2ag** and **2ah**). The cellular activity of (**2ag**) proved to be superior to all previously prepared benzoxazepine compounds. With this lead compound in hand, future efforts will be directed toward refining the benzoxazepine template.

The synthetic route presented in Scheme 1 creates an opportunity to examine some structural aspects of the benzoxazepine core in EGFR inhibition. Intermediates (**7**) were also isolated and tested for activity. The results of enzymatic and cellular testing showed that derivatives of the template (**7**) were virtually inactive in our screens.[8] The dramatic difference in activity between dihydrobenzoxazepine analogs (**2**) and their precursors (**7**) can be rationalized by looking at the overall geometry of the molecule. Compounds of general structure (**7**) possess a rigidified tricylic core. Final products (**2**) exhibit a less rigid structure that, apparently, is required for the binding to the EGFR kinase ATP pocket. This conclusion was further confirmed by the synthesis and biological evaluation of compound (**11**) which contained a benzoxazepine core having been rigidified by an amide group (Scheme 2). The amide analog (**11**) showed no activity when tested in both enzymatic and cellular assays compared to the benzoxazepine derivative (**2ae**). The flexible benzoxazepine core structure desirable for activity in our compound class is contrary to the quinazoline template where a flat aromatic bicycle is generally required for activity.[12]

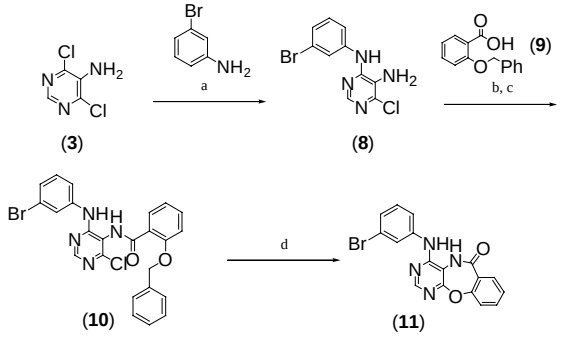
Table 1. Enzymatic and cellular activities of (**2a**–**2ai**) toward EGFR kinase[6,7]



Compound	R^1	R^2	Enzymatic, IC_{50} (μM)[a]	Cellular phosphorylation, IC_{50} (μM)[a]
2a	CH_2 Ph	8-Br	6.0	30
2b	CH_2 Ph	8-F	8.9	>30
2c	CH_2 Ph	8-OCH_3	2.0	8.5
2d	Ph	8-Br	10.0	nt
2e	Ph	8-F	7.0	nt
2f	Ph	8-OCH_3	7.5	nt
2g	*p*-Cl Ph	8-F	>10	nt
2h	*p*-Cl Ph	8-OCH_3	5.8	nt
2i	*m*-Br Ph	8-Br	1.0	7.2
2j	*m*-Br Ph	8-Cl	3.4	>30
2k	*m*-Br Ph	8-F	1.5	6.9
2l	*m*-Br Ph	7-OCH_3	3.0	1.9
2m	*m*-Br Ph	8-OCH_3	3.1	1.0
2n	*m*-Cl, *p*-F Ph	8-Br	2.0	8.4
2o	*m*-Cl, *p*-F Ph	8-Cl	1.0	4.3
2p	*m*-Cl, *p*-F Ph	8-F	1.0	7.2
2q	*m*-Cl, *p*-F Ph	7-OCH_3	1.0	8.9
2r	*m*-Cl, *p*-F Ph	8-OCH_3	0.9	2.4
2s	*m*-C_6H_5 Ph	8-F	2.0	9.9
2t	*m*-C_6H_5 Ph	8-OCH_3	>10	9.8
2u	*m-Br Ph*	9-OCH_3	>10	nt
2v	*m*-Br Ph	8-OCF_3	>10	nt
2w	*m*-Br Ph	8-COOH	1.0	>30
2z	*m*-Cl, *p*-F Ph	8-COOH	4.4	>30
2y	*m*-Br Ph	8-NH_2	1.7	>30
2z	*m*-Cl, *p*-F Ph	8-NH_2	3.7	9.1
2aa	*m*-Br Ph	8-$NHSO_2CH_3$	4.1	7.0
2ab	*m*-Cl, *p*-F Ph	8-$NHSO_2CH_3$	>10	nt
2ac	*m*-Br Ph	8-NHCOCH=CH_2	5.7	5.4
2ad	*m*-Cl, *p*-F Ph	8-NHCOCH=CH_2	4.9	8.9
2ae	*m*-Br Ph	H	3.4	8.1
2af	*m*-Cl, *p*-F Ph	H	1.1	2.7
2ag	*m*-Br Ph	7,8-$(OCH_3)_2$	0.34	0.47
2ah	*m*-Cl, *p*-F Ph	7,8-$(OCH_3)_2$	1.2	0.69
2ai	*m*-Br Ph	7,9-$(OCH_3)_2$	6.3	>30

nt, not tested; enzymatic activity is too low to be tested in cellular assay.

[a] The compounds showing greater than 5 μM enzymatic activity were not generally tested in cellular assay.



Scheme 2. Reagents and conditions: (a) DMF, 80 °C; (b) oxalyl chloride, DMF, THF, 0 °C; (c) Et_3N, THF; (d) HBr, AcOH, 80 °C.

The compounds described in this manuscript were cross-screened against a panel of tyrosine and serine/threonine kinases (HER2, KDR, CDK2, Raf1, Flt1, InR, Akt, IGF1R, c-Met, Flt3, FGFR1, and c-Kit). We were delighted to discover that most of the compounds in Table 1 are highly selective for the EGFR kinase within this cross-screening panel when tested at 10 μM compound concentration. In particular, (**2ag**) selectively inhibited EGFR over HER2, a closely related kinase family member.[12,13]

Some compounds reported in this study display little correlation between enzymatic activity and cellular activity. A possible explanation for this discrepancy could be poor solubility of test compounds at the cellular assay conditions. Thus, attaching solubilizing groups at various positions (compare to quinazoline (**1a**)) could improve both solubility and possibly change the selectivity profile to pick up cross-reactivity with some therapeutically relevant kinases. Our on-going efforts to optimize the compound properties and to demonstrate in vivo potency will be reported in due course. In

summary, the data presented here describe optimization efforts of a novel series of EGFR tyrosine kinase inhibitors with good cellular potency (improved IC50 from double-digit micromolar to nanomolar range) and excellent selectivity against related kinases.[13] As such, benzoxazepine derivatives should serve as a useful chemotype for kinase inhibitors and may provide a basis for the design of novel therapeutic agents.[14]

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6. See Ref. 7 for EGFR enzymatic assay conditions.
7. EGFR cellular phosphorylation assay: DiFi cells were plated in 96-well Costar plates (2.5×10^5 cells per well) and incubated for 4 h. The cells were then starved in serum-free medium overnight. The next morning test compounds were added to individual wells. Following a 2 h incubation, cells were lysed in 100 μL lysis buffer (150 mM NaCl, 50 mM Hepes, 0.5% Triton X-100, 10 mM NaPPi, 50 mM NaF, 1 mM Na_3VO_4, and protease inhibitors, pH 7.5) and rocked for 1 h at 4 °C. The autophosphorylation level of EGFR was then analyzed by ELISA using anti-phosphotyrosine antibodies. ELISA: 96-well ELISA plates were coated with 100 μL/well of 1 μg/mL anti-EGFR antibodies (*Erbitux*) and incubated overnight at 4 °C. The anti-EGFR antibodies were prepared in a buffer made with Na_2CO_3 (0.2 M, 16 mL) and $NaHCO_3$ (0.2 M, 34 mL), and the pH was adjusted to 9.6. Prior to adding cell lysates to the wells, the plates were washed 3 times with PBS + 0.1% Tween 20 and blocked by 3% BSA in PBS (200 μL for 1 h incubation). 80 μL of cell lysates was transferred to the coated wells and incubated for 1 h at 4 °C. After incubation, the plates were washed three times with PBS + 0.1% Tween 20. To detect autophosphorylated EGFR (tyrosine residues), 100 μL of anti-phosphotyrosine antibodies (RC20:HRP, Transduction Laboratories) was added per well (final concentration 0.5 μg/mL in PBS) and incubated for 1 h. The plates were then washed six times with PBS + 0.1% Tween 20. Enzymatic activity of HRP was detected by adding 50 μL/well of equal amounts of the Kirkegaard & Perry Laboratories (KPL) substrate A and substrate B (KPL cat. #54-61-0). The reaction was stopped by adding 0.1 N H_2SO_4 (50 μL/well) and absorbance was detected at 450 nm.
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