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Novel tricyclic azepine derivatives: Biological evaluation of pyrimido[4,5-*b*]-1,4-benzoxazepines, thiazepines, and diazepines as inhibitors of the epidermal growth factor receptor tyrosine kinase

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Abstract—Novel tricyclic derivatives containing an oxazepine, thiazepine, or diazepine ring were studied for their EGFR tyrosine kinase inhibitory activity. While the oxazepines were in general more potent than thiazepines, the diazepines displayed somewhat different structure–activity relationships. Moreover, the diazepines, in contrast to the oxazepines, showed appreciable inhibitory activity against the KDR tyrosine kinase. Furthermore, both oxazepines and diazepines demonstrated significant ability to inhibit autophosphorylation of EGFR in DiFi cells (generally, IC_{50} values in the single-digit micromolar to submicromolar range).

Receptor tyrosine kinases (RTKs) are involved in cellular signal transduction pathways which influence cell growth, differentiation, survival, and proliferation.[1] Many human cancers are characterized by an up-regulation of some of these RTKs.[2] Consequently, they have become targets of intense drug discovery efforts to identify novel anti-cancer agents.[3] In particular, *Erbitux*, an antibody which targets the epidermal growth factor receptor (EGFR), has been approved for the treatment of irinotecan-refractory colorectal cancer and squamous-cell carcinoma of the head and neck.[4] *Iressa*[5] (**1a**) and *Tarceva*[6] (**1b**), both of which belong to the same chemical class of quinazolines, inhibit the EGFR kinase and are being used to treat locally advanced, or metastatic non-small cell lung cancer and certain types of pancreatic cancer. We have reported a new class of non-quinazoline oxazepine derivatives as inhibitors of EGFR kinase, exemplified by structure (**1c**).[7] It is interesting that bromoanilino and dimethoxy moieties of (**1c**) have also been described as desirable functionalities in quinazoline-type EGFR kinase inhibitors,[8] despite a significant difference in the core ring systems between the two structural classes (Fig. 1). In this report, we detail structure–activity relationship results pertaining to the azepine class of compounds, encompassing not only oxazepines but also thiazepine and diazepine scaffolds.

Since the dimethoxy substitution on the tricyclic phenyl ring of (**1c**) appeared to be advantageous for activity, our goal became to modify the aniline moiety, the pyrimidine portion, and the seven-membered ring in an attempt to enhance the kinase inhibitory activity of the compounds. Select oxazepines were synthesized as outlined in Scheme 1.[9]

Thus, 4,6-dichloro-5-aminopyrimidine (**5**) was coupled with a diverse set of anilines (**6**) giving rise to diaminopyrimidine intermediates (**7**), which were reacted with 2,4,5-trihydroxybenzaldehyde to form the requisite tricyclic core structure upon displacement of the chlorine atom and concomitant imine formation.[10] The remain-

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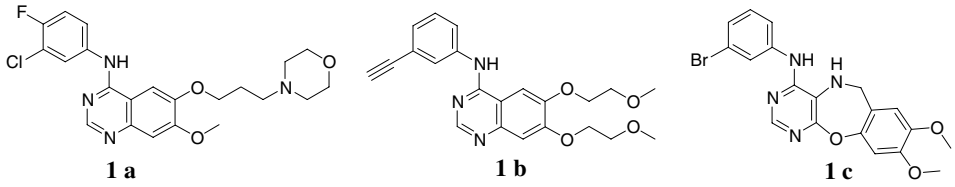
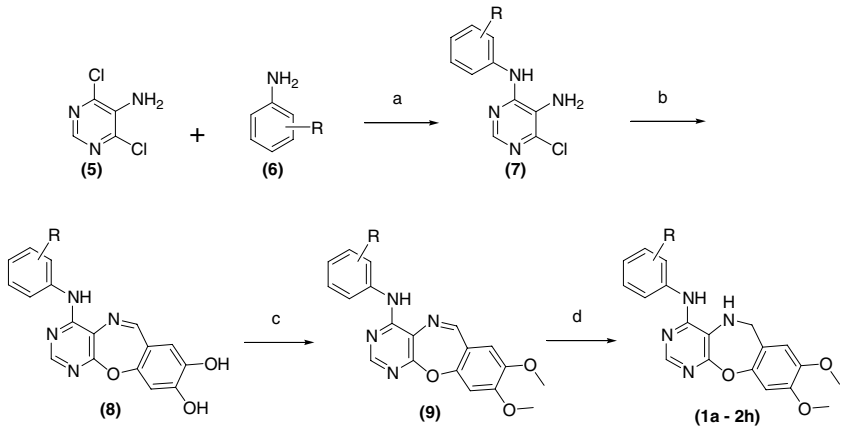


Figure 1. *Iressa*™ (**1a**), *Tarceva*™ (**1 b**), and benzoxazepine EGFR kinase inhibitor (**1c**).



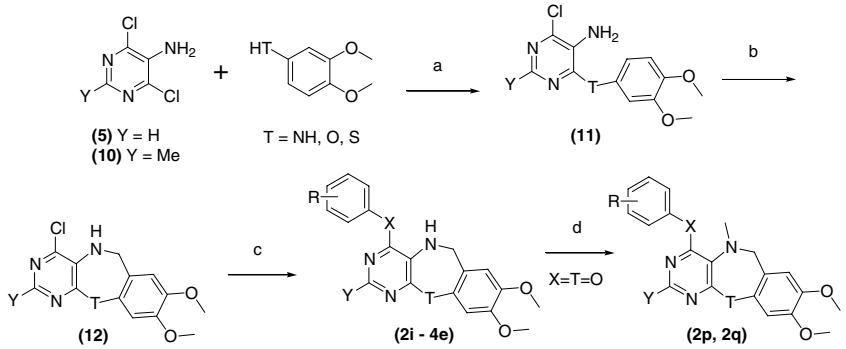
Scheme 1. Reagents and conditions: (a) DMF/120 °C, or EtOH/H_2O/HCl, 100 °C, 59–72%; (b) 2,4,5-trihydroxybenzaldehyde, DMF, 100–120 °C, 21–72%; (c) DMF, NaH or K_2CO_3, MeI or Me_2SO_4, 25–80 °C, 3–93%; (d) $NaBH_4$, THF, EtOH or MeOH, 40 °C-reflux, 2–68%.

ing hydroxy moieties were then methylated and the imine bond reduced to afford the desired target compounds (**1a**–**2k**).

Some additional oxazepines, thiazepines, and diazepines were prepared by a modified procedure described in Scheme 2. In this more convergent approach, a dichloropyrimidine (**5** or **10**) was reacted with 3,4-dimethoxyphenol or thiophenol under basic condition, or with 3,4-dimethoxyaniline under acidic condition, affording an intermediate (**11**) which then underwent a cyclization reaction[11] in the presence of paraformaldehyde to form an oxazepine, thiazepine or diazepine (**12**),[12] respectively. Subsequently, the remaining chloro group was displaced with different anilines, amines, phenols, and thiophenols to yield the target compounds.

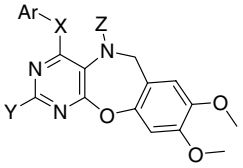
The EGFR tyrosine kinase inhibitory activity of the compounds was determined by measuring the phosphorylation level of poly-Glu-Ala-Tyr-biotin peptide in a homogeneous time-resolved fluorescence (HTRF) at an ATP concentration of 2.0 μM.[13] To ensure quality control, a literature EGFR tyrosine kinase inhibitor (*Iressa*™, **1a**) was included as an internal standard (IC_{50} = 20 ± 10 nM).[14] Most of the compounds were also assayed[13] for their ability to inhibit the autophosphorylation level of EGFR in DiFi cells (**1a**, IC_{50} = 55 ± 15 nM). Results for enzymatic and cellular assays were reported as an IC_{50} expressed in μM.

Table 1 summarizes the biochemical and cell-based inhibitory activity of oxazepines with various modifications. Substitution of the aniline portion of the oxazepine core afforded modest results. For example, replacing the bromine of the parent compound (**1c**) with a methyl (**2a**) led to a dramatic drop in potency. Furthermore, introducing an ethynyl group (**2b**) resulted in a reduction in enzymatic activity while maintaining



Scheme 2. Reagents and conditions: (a) K_2CO_3/DMF/60 °C or EtOH/H_2O/HCl/reflux, 24–84%; (b) CH_2Cl_2, trifluoroacetic acid, $MgSO_4$, $(CHO)_n$, 40 °C, 18–76%; (c) Anilines/amines, HCl, 2-propanol or DMF, 94–150 °C, or neat/120–150 °C, or K_2CO_3/DMF/65–80 °C for phenols and thiophenols, 8–79%; (d) Cs_2CO_3, MeI, DMF, 70 °C, 33–38%.

Table 1. Inhibition results for oxazepines[9]



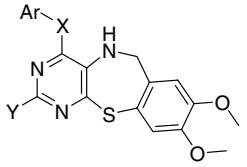
Compound	Ar	X	Y	Z	Enzymatic IC_{50}[a] (μM)	DiFi cell IC_{50}[a] (μM)
1c	3-Br–Ph	NH	H	H	0.3	0.5
2a	3-Me–Ph	NH	H	H	7.4	4.4
2b	3-Ethynyl–Ph	NH	H	H	3.2	0.9
2c	4-Br–Ph	NH	H	H	8.7	>100
2d	4-F–Ph	NH	H	H	7.1	7.6
2e	3-Cl–4-F–Ph	NH	H	H	1.2	0.7
2f	3-Cl–2-F–Ph	NH	H	H	0.3	4.6
2g	5-Cl–2-F–Ph	NH	H	H	1.1	3.9
2h	2-Cl–4-F–Ph	NH	H	H	3.4	>100
2i	6-Indazolyl	NH	H	H	1.2	nt
2j	2-Naphthyl	NH	H	H	0.5	2.3
2k	6-Benzthiazolyl	NH	H	H	5.6	nt
2l	3-Br–Ph	NH	Me	H	>100	nt
2m	3-Cl–2-F–Ph	NH	Me	H	>100	nt
2n	3-Br–Ph	O	H	H	0.7	1.8
2o	3-Cl–4-F–Ph	O	H	H	1.2	6.1
2p	3-Br–Ph	O	H	Me	4.6	nt
2q	3-Cl–4-F–Ph	O	H	Me	10.8	nt
2r	3-Br–Ph	S	H	H	1.3	1.2
2s	3-Cl–Ph	S	H	H	1.0	2.4

nt, not tested; generally, enzymatic activity too low to test in cellular assay.
[a] Data are reported as means of $n \geqslant 2$ determinations.

submicromolar cell-based potency. A substitution at the *para* position (**2c**, **2d**) did not confer any benefit as well. Among the dihalo-substituted analogs (**2e**–**2h**), (**2f**) was the most active analog. Interestingly, compound (**2e**), with the same substitution as *Iressa*, was more potent in the cellular assay than in the enzymatic assay, essentially being equipotent to (**1c**). Of the bicyclic anilines (**2i**–**2k**), only the naphthyl analog (**2j**) was comparable to (**1c**) in activity. Compounds (**2l**) and (**2m**) clearly showed that further substitution on the pyrimidine ring was undesirable. We also evaluated the necessity of the aniline *NH*- in inhibiting EGFR. While the *O*-linked analog (**2n**) was comparable to the corresponding *NH*-linked compound (**1c**), having twofold lower activity, the *S*-linked compound (**2r**) was somewhat less potent. Lastly, we established that the *NH*- of the seven-membered ring was necessary for good activity. When methylated there was a significant loss (>6.5-fold) in inhibitory activity (**2p**, **2q**).

Table 2 lists the thiazepines prepared and tested for enzymatic inhibitory activity. Compound (**3a**), in comparison to (**1c**), was at least 10-fold less active. Similarly, other analogs (**3b**–**3e**) were less potent than their counterparts in the oxazepine series (**2e**, **2f**, **2i**, and **2k**, respectively). Steric hindrance of the sulfur atom over oxygen is a possible explanation for decrease in activity seen in thiazepines. Substituting the bridge nitrogen with a methyl group (**3f**, **3g**) decreased activity. As was the case with the oxazepines, adding a methyl substituent

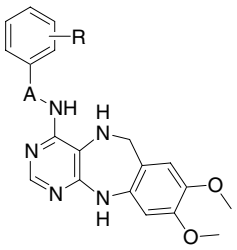
Table 2. Inhibition results for thiazepines[9]



Compound	Ar	X	Y	Enzymatic IC_{50}[a] (μM)
3a	3-Br–Ph	NH	H	5.9
3b	3-Cl–4-F–Ph	NH	H	8.2
3c	3-Cl–2-F–Ph	NH	H	62.9
3d	6-Indazolyl	NH	H	14.1
3e	6-Benzthiazolyl	NH	H	>100
3f	3-Br–Ph	NMe	H	>100
3g	3-Cl–Ph	NMe	H	27.0
3h	3-Br–Ph	NH	Me	30.7
3i	3-Br–4-Me–Ph	NH	Me	>100
3j	3-Cl–4-F–Ph	NH	Me	45.9
3k	3-Cl–2-F–Ph	NH	Me	>100
3l	4-Cl–3-Me–Ph	NH	Me	>100
3m	2-Naphthyl	NH	Me	17.0
3n	5-Benzimidazolyl	NH	Me	14.5
3o	6-Benzthiazolyl	NH	Me	15.2
3p	6-Indazolyl	NH	Me	24.6
3q	5-Indazolyl	NH	Me	16.2
3r	3-Br–Ph	O	H	21.7
3s	3-Cl–2-F–Ph	O	H	19.3
3t	3-Br–Ph	S	H	1.6
3u	3-Cl–Ph	S	H	2.9

[a] Data are reported as means of $n \geqslant 2$ determinations.

Table 3. Inhibition results for diazepines[9]



Compound	R	A	Enzymatic IC_{50}[a] (μM)	DiFi cell IC_{50}a (μM)
4a	3-Br	—	1.5	nt
4b	3-Cl–4-F	CH_2	0.3	1.6
4c	3-MeO	CH_2	0.8	11.7
4d	H	CH_2	0.2	0.9
4e	H	(*R*)-MeCH	0.15	0.5

nt, not tested; enzymatic activity too low to be tested in cellular assay.
[a] Data are reported as means of $n \geqslant 2$ determinations.

to the pyrimidine ring led to poorly active compounds (**3h**–**3q**). Replacing the bridge nitrogen with an oxygen (**3r**, **3s**) also significantly decreased activity. Having a sulfur bridge as in (**3t**) and (**3u**) actually maintained some of the potency relative to (**3a**), (**2r**) and (**2s**). Because of their overall moderate enzymatic potencies, only a handful of thiazepines were assayed for their cellular potency in DiFi cells. Analogs that were tested showed weak activity ($IC_{50} \geqslant 17$ μM).

Table 3 summarizes the activity profiles of the diazepines. Compound (**4a**) showed reduced activity compared to the oxazepine counterpart (**1c**). However, when the nitrogen bridge was extended by one methylene unit (A = CH_2), the activity was less sensitive to the substituent variation on the phenyl ring such that even a methoxy group (**4c**) or lack of a substituent (**4d**) led to enzymatic potencies comparable to that of (**1c**). Indeed, an additional methyl moiety on the bridge (**4e**) did not adversely affect potency at all. When assayed in the DiFi cells, (**4e**) emerged as the most potent compound among the diazepines, and equipotent to (**1c**).

Since KDR (or VEGFR-2), another receptor tyrosine kinase, has long been within our area of interest,[15] some of the test compounds were also assayed for their enzymatic inhibitory activity against the KDR kinase.[16] While the oxazepines in general showed very weak KDR activity ($IC_{50} > 10$ μM), diazepines (**4b**–**4e**) displayed appreciable potency with IC_{50} in the range of 2–5 μM. It is perceivable that the diazepine nitrogen between two aromatic rings, and the adjacent pyrimidine nitrogen together form hydrogen bonding interactions at the active site, analogous to that proposed for AEE788 which is a pyrrolo[2,3-*d*]pyrimidine reported to be an inhibitor of both EGFR and KDR.[17] This is in contrast to the oxazepines and thiazepines that lack a potential hydrogen bond donating group present in the diazepine scaffold.

As seen in our earlier report, some compounds in this study display little correlation between enzymatic activity and cellular activity. Poor solubility of such compounds is a possible explanation for these discrepancies. In summary, we have identified and studied the structure–activity relationships of a novel series of non-quinazoline compounds containing a tricyclic oxazepine, thiazepine or diazepine ring system. While the oxazepines were in general more potent EGFR kinase inhibitors than thiazepines, the diazepines showed somewhat different SAR, and were moderately potent inhibitors of the KDR kinase as well. Furthermore, both oxazepines and diazepines demonstrated significant ability to inhibit cell-based phosphorylation in DiFi cells (generally, IC_{50} values in the single-digit micromolar to submicromolar range). Mono- and multi-kinase targeting and orally efficacious analogs of these compound series have been obtained and will be reported in due course.

Acknowledgments

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13. Enzymatic assay conditions: EGFR tyrosine kinase inhibition was determined by measuring the phosphorylation level of poly-Glu-Ala-Tyr-biotin (pGAT-biotin) peptide in a homogeneous time-resolved fluorescence (HTRF) assay. Into a black 96-well Costar plate was added 2 μl/well of 25× compound in DMSO (final compound concentration in the 50-μl kinase reaction was typically 1 nM to 10 μM). Next, 38 μl of reaction buffer (25 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, and 2 mM DTT) containing 1.5 pmol pGAT-biotin and 1–2 ng EGFR enzyme was added to each well. After 5–10 min preincubation, the kinase reaction was initiated by the addition of 10 μl of 10 μM ATP in reaction buffer, after which the plate was incubated at room temperature for 45 min. The reaction was stopped by the addition of 50 μl KF buffer (50 mM Hepes, pH 7.5, 0.5 M KF) containing 100 mM EDTA and 0.23 μg/ml PY20K (Eu-cryptate labeled anti-phosphotyrosine antibody, CIS Biointernational). After 30 min, 100 μl of 5 nM SV-XL (modified-APC-labeled Streptavidin, CIS Biointernational) in KF buffer was added, and after an additional 2-h incubation at room temperature, the plate was read in a RUBYstar HTRF Reader.
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 of 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 three times with PBS + 0.1% Tween 20 and blocked by 3% BSA in PBS (200 μl for 1 h incubation). Eighty microliters 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 auto-phosphorylated 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.
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