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INHIBITION OF THE HER2 TYROSINE KINASE AND CHARACTERIZATION OF A HYDROPHOBIC SITE NEAR THE NUCLEOTIDE BINDING DOMAIN[1]

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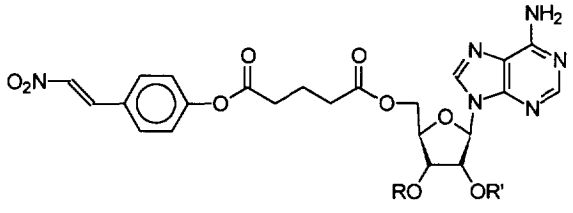
Abstract: A series of compounds was prepared to investigate the hydrophobic character of the HER2 receptor tyrosine kinase active site. These bisubstrate analogs contained hydrophobic moieties in place of the polar triphosphate and nucleoside fragments of the natural ATP ligand. Despite these modifications, good affinity was observed as measured by inhibition of receptor autophosphorylation. © 1997 Elsevier Science Ltd.

Receptor tyrosine kinases (TKs) are one of the chief transducers of external stimuli to intracellular signalling pathways.[2] Of the many TKs that have been identified and characterized, those of the epidermal growth factor receptor (EGFR) family are especially important, having been implicated in a variety of ectopic cell proliferative processes.[3-6] Consequently, there has been enormous interest recently in developing inhibitors of TKs,[7] and of EGFR family TKs in particular,[8-10] because of their prospective utility as anticancer agents. One member of the EGFR family, HER2 (also called *erb*B2 or *neu*), plays a pivotal role in the intrafamily communication network because of its propensity to heterodimerize with other family members.[11] Differences in the recruitment of downstream proteins along the signalling pathway that result from this crosstalk permit a subtle degree of control on cellular growth regulation. Perhaps as a result of this finely balanced equilibrium, HER2 overexpression is directly associated with increased incidence of, and/or poor prognosis for, numerous cancers, including breast, ovarian, gastric, and colon.[3-5,12]

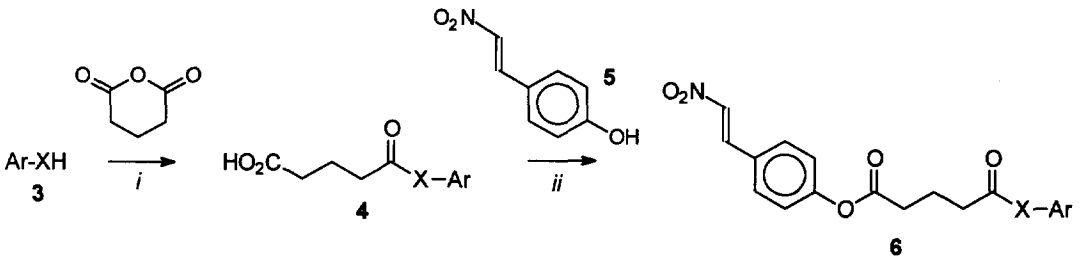
Among the inhibitors of the EGFR family TKs are various nitrostyrene derivatives of adenosine 5′-glutarates **1**.[9] These bisubstrate-type inhibitors are noteworthy in that the highly polar, anionic triphosphate bridge linking the nucleoside and tyrosine binding sites has been replaced by a lipophilic hydrocarbon chain, yet high affinity for the TK active site is retained. Moreover, we noted that the affinity of the EGFR TK for a protected intermediate in the synthesis of **1**, *viz.* 2′,3′-isopropylidene **2**, was greater than for **1** itself, suggesting that the sugar binding site of the TK domain also had hydrophobic character. This hydrophobicity is



1, R = R' = H

2, R-R' = $C(CH_3)_2$

Scheme 1



See Table 1 for definition of **X**, **Ar**. Reagents: (i) diisopropyl ethyl amine/dioxane; (ii) dicyclohexylcarbodiimide/tetrahydrofuran.

Table 1
Inhibition of Autophosphorylation in EGFR/HER2 Chimeric and HER2 Receptors[13]

Compound	X[a]	Ar[a]	IC_{50} (μM) EGF-Stimulated Chimera[b]	IC_{50} (μM) Basal HER2[c]
6a	$NHCH_2$	C_6H_5	4.8	ND[d]
6b	O	4-FC_6H_4	>10	ND
6c	NH	C_6H_5	>10	ND
6d	O	3-$CH_3OC_6H_4$	9.5	ND
6e	OCH_2CH_2	C_6H_5	>10	ND
6f	O	2-$CH_3OC_6H_4$	>10	ND
6g	OCH_2	C_6H_5	>10	ND
6h	OCH_2	2-furyl	0.29	3.8
6i	O	C_6H_5	0.42	4.4
6j	O	4-$CH_3OC_6H_4$	1.0	6.6
2	—	—	2.0	4.5

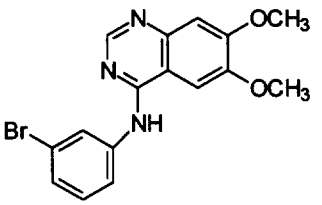
[a]See Scheme 1 for structures; [b]Inhibition of ligand-stimulated autophosphorylation in the EGFR/HER2 chimeric receptor; [c]Inhibition of basal autophosphorylation in the HER2 receptor; [d]ND = not determined

potentially extremely significant, because hydrophobic interactions typically provide the major driving force for ligand binding.[14] Also, by taking advantage of any unique hydrophobic character of the EGFR TK family active site, enhanced selectivity may result. Since in unrelated work we had successfully exploited hydrophobic pockets

contiguous to sugar binding sites for design of high avidity inhibitors of nucleoside binding enzymes,[15] we decided to investigate the nature and extent of the hydrophobicity of this site in the homologous HER2 TK.

Scheme 1 depicts the synthesis of the first iteration of compounds, prepared to test the hypothesis that the entire nucleoside portion of the nitrostyryl glutarates could be replaced with hydrophobic aryl moieties. Initial attempts to apply the methodology developed by Séquin, Traxler, et al.[9] for synthesis of adenosyl derivatives **1** failed. Their procedure involved condensation of the nitrostyrylphenol to glutaric anhydride, followed by conversion of this monoester intermediate to the acyl chloride, which was then treated with a protected adenosine to obtain **2**. In the present case the phenols, more highly nucleophilic than the corresponding nucleosides, coupled to the nitrostyryl glutaryl chlorides as expected, but this was followed by addition of a second equivalent of the phenol in a Michael reaction with the nitroolefin. An inverted sequence worked quite well however: treatment of glutaric anhydride with the phenolic nucleoside surrogates **3** afforded glutarate monoacids **4**, and reaction of these compounds with nitroolefin **5** produced the desired materials **6**.[16] For the final transformation, we found DCC coupling of the acid more convenient than use of the corresponding glutaryl chloride. All target compounds had satisfactory spectroscopic and analytical data.[17]

Compounds were evaluated for inhibition of EGF-stimulated autophosphorylation in membrane extracts of recombinant fibroblasts expressing a chimeric receptor consisting of the extracellular domain of the EGFR, and the TK domain of HER2 (Table 1).[13] The most potent compounds were further evaluated for inhibition of basal TK activity against the HER2 TK purified from HER2-overexpressing lines, using compound **2** as a positive control (Table 1).[13] Thus, both ligand-induced and basal TK activity were evaluated. As can be seen, replacement of the nucleoside in several cases results in compounds having affinity for the kinase comparable to the control. It is remarkable that an active site evolved for recognition of such a polar species as ATP nonetheless binds exceedingly hydrophobic ligands that necessarily lack many of the polar contacts that might be assumed important; indeed, both the triphosphate and adenosine are simultaneously dispensable without adversely affecting binding affinity. In this regard, it is interesting to speculate that the EGFR inhibitor PD 153035,[10] which has similar affinity for the HER2 TK as the present compounds and, at least in the case of the EGFR, is known to bind at the ATP site, may be functioning as an adenosine surrogate one of whose aryl rings, perhaps the bromophenyl moiety, occupies the same hydrophobic site that we are characterizing. It remains to be determined whether this site is an unusually hydrophobic sugar binding motif, or, alternatively, a hydrophobic pocket accessible from the sugar site. We anticipate that enzyme affinity of the current series of compounds could be improved by extension into the purine binding pocket, *e.g.*, by addition of a suitable hydrophobic substituent onto the aromatic ring. Alternatively, if the most crucial polar contacts to the native substrate can be identified, than incorporation of select polar functionality into these inhibitors should also



PD 153035

augment affinity. Synthetic tests of these two conjectures are in progress.

Acknowledgement: We thank Dr. Paolo P. Di Fiore (National Cancer Institute) for providing the NIH3T3-*erb*B2 and NIH3T3-EGFR/*erb*B2 cell lines.

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13. *Assay Description:* Transfected NIH3T3 cell lines expressing the HER2 receptor (NIH3T3-*erb*B2) or a chimeric EGFR/HER2 receptor (NIH3T3-EGFR/*erb*B2) were obtained from the Di Fiore laboratory. Cells were grown in

Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Membrane extracts were prepared from subconfluent NIH3T3 fibroblasts containing either HER2 or chimeric EGFR/HER2 receptors as described in detail by Levitzki et al.[18] The protein concentrations for the stocks were 1.36 mg/mL for the chimeric receptor, and 1.72 mg/ml for the HER2-containing membranes. Monoclonal antibodies to the EGFR (EGFR Ab-5; Oncogene Sciences) and c-*neu* (c-*neu* Ab-5; Oncogene Sciences) reacted with the appropriate membrane stock; no cross-reactivity was noted.

Autophosphorylation of both receptors was measured using methods described by Levitzki et al.[18] and Osherov et al.[19] with slight modifications. The reactions contained either 2.15 ng HER2 or 2.72 ng EGFR/HER2, [γ-^{32}P]ATP (45 μM; 2μCi/assay), divalent cations (90 μM $MnCl_2$, 500 μM $MgCl_2$), and a protein tyrosine phosphatase inhibitor. (To activate the EGFR/HER2 receptors, membrane extracts were preincubated with EGF.) After electrophoresis (SDS-PAGE), the gels were washed and the radioactivity in the appropriate molecular weight band was counted.

Assays were run at 4 °C and were linear for 5 min. To stay within this linear range, both inhibitor screens were run for 4 min. To ensure that the response seen in the p188 band (EGFR/HER2 receptor) was ligand stimulated, a time course was run with and without EGF preincubation. The number of counts in the unstimulated p188 band was less than 10% of that in the EGF-stimulated band.

2′,3′-*O*-Isopropylidene-5′-adenosyl 4-[(*E*)-2-nitroethenyl]phenyl glutarate **2** was used as a positive control during the assays. Measured IC_{50}s for this compound (4.5 μM for HER2; 1.8 μM for the EGFR/HER2 chimera) are comparable to the published values for this agent against EGFR TK[9] (published data for the HER2 TK were unavailable).

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16. Typical reaction procedures were as follows: (a) Formation of the monophenyl glutarates **4**: p-methoxyphenyl glutarate **4j**. Reactions were run at ambient temperature under argon using anhydrous solvents, the reaction vessel shielded from light. To 2.317 g (18.670 mmol) 4-methoxyphenol dissolved in 40 mL dioxane was added with stirring 78 mL diisopropyl ethyl amine. After 1.25 h, 2.130 g (18.670 mmol) glutaric anhydride in 40 mL dioxane was added dropwise over ~25 min. The clear, colorless solution was stirred for 1.25 h, at which time tlc (cyclohexane:ethyl acetate, 3:1) showed no remaining phenol and a single spot corresponding to product. The solution was concentrated in vacuo, taken up in ~40 mL water, the milky suspension acidified with acetic acid, then extracted with ethyl acetate (2 X 100mL). The organic layer was backwashed with water (1 X 50 mL), dried over sodium sulfate, filtered, and concentrated in vacuo over P_2O_5 to yield the desired product (4.16 g, 94%). The material was used without further purification, though an aliquot was recrystallized from boiling water for characterization.

(b) Formation of phenyl nitrovinylphenyl diglutarates **6**: 4-methoxyphenyl 4′-(2-(*E*)-nitroethenyl)phenyl glutarate **6j**. To 18 mL THF under argon at ambient temperature was added with stirring 0.414 g (1.738 mmol) **4j**. The solution was cooled to -20 °C, whereupon 0.273 g (1.706 mmol) *p*-(2-(*E*)-nitrovinyl)phenol[9] was added in one portion, followed by 0.364 g (1.764 mmol) dicyclohexylcarbodiimide. The yellow solution was stirred 15 min at -20 °C, then allowed to warm to room temperature and stirred overnight; with time turbidity developed, and a fine precipitate of dicyclohexylurea appeared. A few drops of acetic acid was added, the mixture concentrated to dryness, the solid triturated with chloroform and the insoluble material filtered and washed with $CHCl_3$. The solution was purified by flash chromatography on ~90 cm^3 silica gel ($CHCl_3$ eluant), yielding 0.377 g analytically pure **6j**.

17. **Benzylamide 6a:** NMR ($CDCl_3$) δ 7.99 (d, 1, *J* = 14 Hz, -**HC**=CHNO$_2$), 7.57 (m, 2, *J* = 9 Hz, styr-phenyl Hs), 7.55 (d, 1, *J* = 14 Hz, -CH=**CH**NO$_2$), 7.32 (m, 5, phenyl Hs), 7.20 (m, 2, *J* = 9 Hz, styr-phenyl Hs), 5.76 (br s, 1, NH), 4.46 (d, 2, *J* = 6 Hz, NH**CH$_2$**), 2.70 (t, 2, *J* = 7 Hz, -CH$_2$C=O), 2.38 (t, 2, *J* = 7 Hz, -CH$_2$C=O), 2.13 (quint, 2, *J* = 7 Hz, -**CH$_2$**CH$_2$C=O). FABMS (M+H)$^+$ 369; Anal.Calcd. for $C_{20}H_{20}N_2O_5$: C, 65.20; H, 5.47; N, 7.60. Found: C, 65.06; H, 5.58; N, 7.52.

***p*-Fluorophenyl ester 6b:** NMR ($CDCl_3$) δ 7.99 (d, 1, *J* = 14 Hz, -**HC**=CHNO$_2$), 7.58 (m, 2, *J* = 9 Hz, styr phenyl Hs), 7.56 (d, 1, *J* = 14 Hz, -CH=**CH**NO$_2$), 7.21 (m, 2, *J* = 9 Hz, styr-phenyl Hs), 7.07 (s, 2, *p*-F-phenyl Hs), 7.05 (s, 2, *p*-F-phenyl Hs), 2.76 (t, 2, *J* = 7 Hz, -CH$_2$C=O), 2.74 (t, 2, *J* = 7 Hz, -CH$_2$C=O), 2.20 (quint, 2, *J* = 7 Hz, -**CH$_2$**CH$_2$C=O). FABMS (M+H)$^+$ 374; Anal.Calcd. for $C_{19}H_{16}FNO_6$: C, 61.11; H, 4.32; N, 3.75. Found: C, 61.13; H, 4.41; N, 3.83.

Anilide 6c: NMR ($CDCl_3$) δ 8.00 (d, 1, *J* = 14 Hz, -**HC**=CHNO$_2$), 7.58 (m, 2, *J* = 9 Hz, styr-phenyl Hs), 7.55 (d, 1, *J* = 14 Hz, -CH=**CH**NO$_2$), 7.52 (m, 2, *J* = 8 Hz, phenyl Hs), 7.34 (m, 2, *J* = 8 Hz, phenyl H), 7.20 (m, 3, *J* = 9 Hz, styr-phenyl Hs, NH), 7.12 (m, 1, *J* = 8 Hz, phenyl H), 2.76 (t, 2, *J* = 7 Hz, -CH$_2$C=O), 2.54 (t, 2, *J* = 7 Hz, -

CH_2C=O), 2.20 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 355; Anal.Calcd. for $C_{19}H_{18}N_2O_5$: C, 64.40; H, 5.12; N, 7.91. Found: C, 64.36; H, 5.18; N, 7.85.

***m*-Methoxyphenyl ester 6d:** NMR ($CDCl_3$) δ 7.99 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.57 (m, 2, J = 9 Hz, styr phenyl Hs), 7.55 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.27 (t, 1, J = 7 Hz, CH_3O-phenyl H), 7.21 (m, 2, J = 8 Hz, styr phenyl H), 6.79 (m, 1, CH_3O-phenyl H), 6.68 (m, 1, CH_3O-phenyl H), 6.64 (m, 1, CH_3O-phenyl H), 3.79 (s, 3, CH_3O-), 2.76 (t, 2, J = 7 Hz, -CH_2C=O), 2.73 (t, 2, J = 7 Hz, -CH_2C=O), 2.20 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 386. Anal.Calcd. for $C_{20}H_{19}NO_7$: C, 62.33; H, 4.96; N, 3.63. Found: C, 62.23; H, 4.82; N, 3.69.

2-Phenylethyl ester 6e: NMR ($CDCl_3$) δ 7.99 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.57 (m, 2, J = 9 Hz, styr-phenyl Hs), 7.55 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.25 (m, 7, phenyl Hs, styr-phenyl Hs), 4.33 (t, 2, J = 5 Hz, -OCH_2), 3.95 (t, 2, J = 5 Hz, phenyl-**CH_2**), 2.62 (t, 2, J = 7 Hz, -CH_2C=O), 2.55 (t, 2, J = 7 Hz, -CH_2C=O), 2.04 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 384; Anal.Calcd. for $C_{21}H_{21}NO_6$: C, 65.78; H, 5.52; N, 3.65. Found: C, 65.81; H, 5.59; N, 3.61.

***o*-Methoxyphenyl ester 6f:** NMR ($CDCl_3$) δ 8.01 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.58 (m, 2, J = 9 Hz, styr phenyl Hs), 7.56 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.23 (m, 3, styr phenyl Hs, CH_3O-phenyl H), 7.05 (dd, 1, J = 8, 2 Hz, CH_3O-phenyl H), 6.97 (m, 2, CH_3O-phenyl Hs), 3.84 (s, 3, CH_3O-), 2.80 (t, 2, J = 7 Hz, -CH_2C=O), 2.76 (t, 2, J = 7 Hz, -CH_2C=O), 2.22 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 386. Anal.Calcd. for $C_{20}H_{19}NO_7$: C, 62.33; H, 4.96; N, 3.63. Found: C, 62.26; H, 4.95; N, 3.49.

Benzyl ester 6g: NMR ($CDCl_3$) δ 8.00 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.58 (m, 2, J = 9 Hz, styr-phenyl Hs), 7.56 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.37 (m, 5, phenyl Hs), 7.20 (m, 2, J = 9 Hz, styr-phenyl Hs), 5.15 (s, 2, phenyl-**CH_2**), 2.78 (t, 2, J = 7 Hz, -CH_2C=O), 2.53 (t, 2, J = 7 Hz, -CH_2C=O), 2.21 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 370; Anal.Calcd. for $C_{20}H_{19}NO_6$: C, 65.03; H, 5.18; N, 3.79. Found: C, 65.13; H, 5.27; N, 3.94.

Furfuryl ester 6h: NMR ($CDCl_3$) δ 7.99 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.57 (m, 2, J = 9 Hz, styr-phenyl Hs), 7.55 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.42 (m, 1, furfuryl H), 7.20 (m, 2, J = 9 Hz, styr-phenyl Hs), 6.41 (m, 1, furfuryl H), 6.37 (m, 1, furfuryl H), 5.10 (s, 2, OCH_2), 2.66 (t, 2, J = 7 Hz, -CH_2C=O), 2.50 (t, 2, J = 7 Hz, -CH_2C=O), 2.08 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 360; Anal.Calcd. for $C_{18}H_{17}NO_7$: C, 60.16; H, 4.77; N, 3.90. Found: C, 60.16; H, 4.82; N, 3.85.

Phenyl ester 6i: NMR ($CDCl_3$) δ 8.01 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.58 (m, 2, J = 9 Hz, styr-phenyl Hs), 7.56 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.40 (m, 2, J = 8 Hz, phenyl Hs), 7.26 (m, 1, J = 8 Hz, phenyl H), 7.22 (m, 2, J = 9 Hz, styr-phenyl Hs), 7.11 (m, 2, phenyl Hs), 7.09 (m, 1, phenyl H), 2.78 (t, 2, J = 7 Hz, -CH_2C=O), 2.75 (t, 2, J = 7 Hz, -CH_2C=O), 2.21 (quint, 2, J = 7 Hz, -**CH_2**CH_2C=O). FABMS $(M+H)^+$ 356; Anal.Calcd. for $C_{19}H_{17}NO_6$: C, 64.21; H, 4.82; N, 3.94. Found: C, 63.93; H, 4.88; N, 3.79.

***p*-Methoxyphenyl ester 6j:** NMR ($CDCl_3$) δ 7.98 (d, 1, J = 14 Hz, -**HC**=$CHNO_2$), 7.58 (m, 2, J = 9 Hz, styr phenyl Hs), 7.56 (d, 1, J = 14 Hz, -CH=**CH**NO_2), 7.22 (m, 2, styr phenyl Hs), 7.01 (m, 2, CH_3O-phenyl Hs), 6.89 (m, 2, CH_3O-phenyl Hs), 3.80 (s, 3, CH_3O-), 2.76 (t, 2, J = 7 Hz, -CH_2C=O), 2.72 (t, 2, J = 7 Hz, -CH_2C=O), 2.20 (m, 2,-**CH_2**CH_2C=O). FABMS $(M+H)^+$ 386. Anal.Calcd. for $C_{20}H_{19}NO_7$: C, 62.33; H, 4.96; N, 3.63. Found: C, 62.15; H, 4.90; N, 3.66.

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