Tyrphostins. 6. Dimeric Benzylidenemalononitrile Tyrphostins: Potent Inhibitors of EGF Receptor Tyrosine Kinase *in Vitro*

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Benzylidenemalononitrile (BMN) tyrphostins were previously found to be potent inhibitors of EGF receptor (EGFR) tyrosine kinase activity. Since these compounds were found to compete for the substrate and sometimes with the ATP site and since EGFR acts as a dimer, we prepared a series of dimeric tyrphostins. These dimeric tyrphostins were built from two BMN units linked by various spacers and designed to fit the dimeric cross-autophosphorylation signal transduction intermediate of the EGFR tyrosine kinases. Structure−activity relationship of these potent dimeric EGF receptor tyrosine kinase inhibitors is reported.

Introduction

Protein tyrosine kinases (PTKs) consist of a large family of enzymes which play a key role in cellular signal transduction leading to proliferation and differentiation.[1] Many of the oncoproteins discovered in the past 20 years are mutated or overexpressed PTKs, and their enhanced PTK activity is associated with cancer and other proliferative diseases (for reviews, see refs 1 and 2).

PTKs belong to two groups: receptor PTKs, which are transmembranal proteins and activated upon binding of a growth factor to their extracellular part, and nonreceptor PTKs, which reside in the cytosol and are activated indirectly after binding to activated signal transduction complexes.[2] Receptor PTKs function as dimers where the process of receptor activation occurs by the transautophosphorylation of one molecule by its neighbor.[2]

In order to inhibit the tyrosine phosphorylation activity of PTKs, we and others have developed small molecular weight inhibitors, including tyrphostins.[3−6] Tyrphostins are small organic molecules with molecular weights of 200−400 Da, which were designed as tyrosine mimetics that interact with the active site of the PTK. Relatively small chemical variations led to tyrphostins which can inhibit selectively different PTKs.[4−6]

We reasoned that by building tyrphostins with two pharmacophoric units and a suitable linker chain, provided there is no steric congestion, one can obtain bisubstrate inhibitors that would take advantage of the dimeric signal transduction mechanism of the receptor tyrosine kinases described above.[2] Such dimeric inhibitors are expected to have enhanced efficacy as compared to the monomeric inhibitors. A similar concept led to selective antagonists of the κ, μ, and δ opiate receptors.[7] In this communication we report our early attempts to design and test the concept of dimeric tyrphostins.

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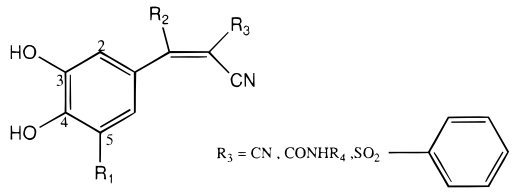
Chemistry

We have shown earlier that the pharmacophores of tyrphostins active against EGF receptor tyrosine kinase are hydroxylated aromatic rings with a *cis*-cyanovinyl group of the benzylidenemalononitriles (BMNs).[8−11]



I

Preparation of dimeric tyrphostins can be achieved by connecting two BMN molecules via R_1, R_2, or R_3 without interference with the essential pharmacophores. We reported previously[8,9] that substitution at R_3 did not impair the efficacy of tyrphostin inhibitors, while chemical variation of R_1 can lead to much improved selectivity even among the closely related EGFR and HER-2/c-erbB-2 PTKs.[10, 11] Substitution at R_2 decreased potency and would require use of the less reactive ketones instead of aldehydes in the dimer synthesis. By substitution at R_3 we obtained a large group of alkylaryl amides and ketones which were usually more potent.[9] We chose therefore the R_3 position as the most promising and also more facile because the required aldehydes are available and easy to prepare, and further variation at R_1 could lead to selective dimeric tyrphostins as was observed in the monomeric BMN tyrphostins.[10,11]

The synthesis of the dimeric tyrphostins was achieved by an analogous route to the preparation of BMN tyrphostins, but instead of substituted amidoacetonitrile we prepared the bifunctional synthon **III** from the diamine **II** and 2 mol of methyl cyanoacetate. Knoevenagel condensation of **III** with 2 mol of various aldehydes gave the desired dimeric tyrphosins, as shown in Scheme 1. Several dimeric tyrphostins (**VI**) were prepared from the commercially available sulfonyldiacetonitrile **V**.

Structure−Activity Relationship Studies

We have previously reported[8,9] that "monomeric" BMN tyrphostins inhibit the phosphorylation of the

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Scheme 1

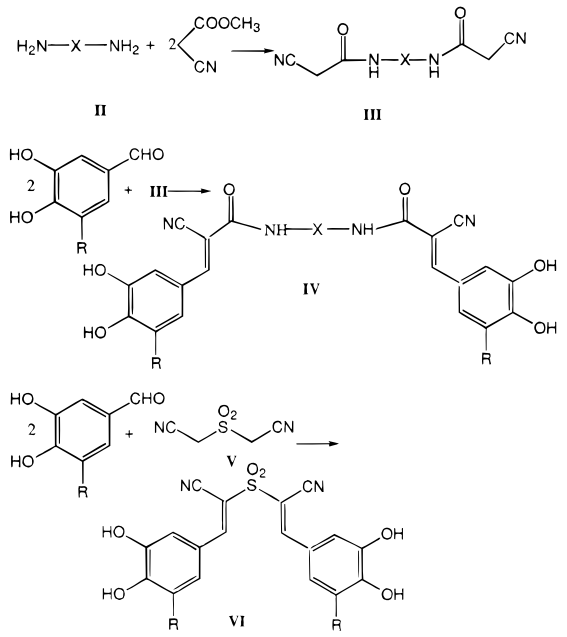
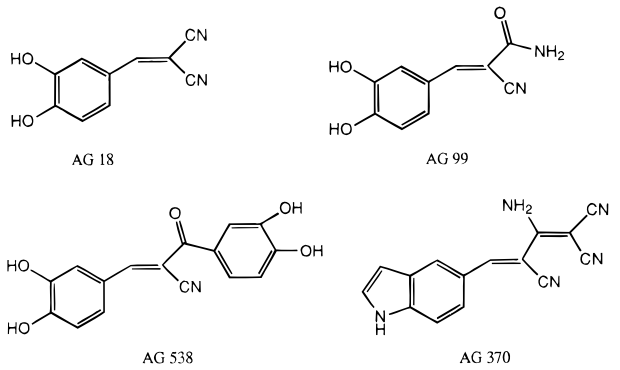


Chart 1



polyGAT (poly(Glu_6Ala_3Tyr)) substrate by the EGF receptor at the low micromolar range. The prototype tyrphostin AG **18** (Chart 1) has an IC_{50} of 35 μM, while its α-amide analog AG **99** (Chart 1) has an IC_{50} of 10 μM. On the basis of the rationale discussed above, we prepared a series of dimeric tryphostins, compounds **2**−**4**, **6**−**8**, and **13** in Table 1. In these compounds the amide groups of two AG **99** units were linked by a simple alkyl chain, $(CH_2)_n$, with n = 2−10. The IC_{50} values for this series range from 0.4 to 2.2 μM, which is a 5−25-fold improvement in potency compared to AG **99**.

Within the series the best inhibitor is compound **2**, with n = 3 and IC_{50} = 0.4 μM. With shorter or longer chain length, the potency decreases somewhat (IC_{50} = 0.5 μM for **3**, n = 2 and 0.6−0.7 μM for compounds **4**, **6**, and **7** with n = 4−6). However, compounds **2**−**7** fall within the same potency range where the potency falls appreciably for longer spacers (IC_{50} = 2.2 and 1.3 μM for compounds **13** and **8** with n = 8 and 10). When we compare the autophosphorylation inhibition of the dimeric tyrophosins to that of AG **99**, the pattern is similar. Compound **2** is the best inhibitor, with IC_{50} = 3.3 μM and about the same potency as the monomeric AG **99**, with IC_{50} = 4.5 μM, while a shorter or longer spacer decreases the potency in compounds **3**−**8** and **13**.

Changing the hydrogen at position 5 to Br or OH at both catecholic rings seems to make little difference. Thus compound **1** with R_2 = Br and n = 3 has IC_{50} = 0.34 μM compared to IC_{50} = 0.4 μM for compound **2** in the polyGAT assay, and compounds **4** and **5** (R_2 = OH, n = 4) have the same potency (IC_{50} = 0.6 μM). Compound **11** in which one of the essential dihydroxy groups is changed to methoxy is inactive (IC_{50} = 112 μM) but still better than its monomeric analog with IC_{50} = 160 μM in the polyGAT assay.[8] Attempts to improve potency by preparing more rigid spacers in the dimeric tyrphostins were not successful. Thus compounds **12** and **15** exhibit IC_{50} of 2.1 and 4.0 μM compared to IC_{50} = 0.5 μM for compound **3** (linker length n = 2), and compounds **9**, **10**, and **14** exhibit IC_{50} = 1.6, 1.8, and 2.2 μM compared to compound **6** with IC_{50} = 7.6 μM (linker length n = 5).

Three dimeric tyrphostins, compounds **22**−**24**, containing SO_2 linker (Table 2) were also prepared. These dimers possess only five atoms between the two catecholic rings while the distance between these two pharmacophores is much longer in compound **1**−**20** (eleven atoms in **2** up to 14 atoms in compound **7**). In spite of the difference in the length of the spacer, compound **22** with IC_{50} = 0.38 μM is equipotent to the best EGF receptor kinase blocker, compound **2** (IC_{50} = 0.4 μM) which has a much longer spacer. We have previously reported that AG **538** (Chart 1) is a potent EGF receptor blocker, with IC_{50} = 0.37 μM in the polyGAT assay and inhibits EGF receptor autophosphorylation with IC_{50} = 5.0 μM.[9]

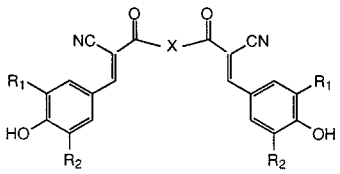
AG **538**, though not built from symmetrical units like the dimeric tyrphostins, possesses two catecholic rings connected with a 3-atom spacer and thus can be viewed as "dimer". It seems that compounds **22**−**24** belong with AG **538** to a different subclass than the long chain dimeric tyrphostins (see below for further discussion of this point).

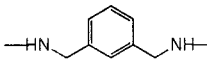
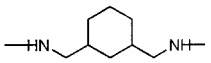
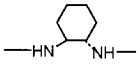
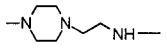
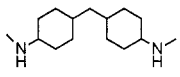
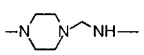
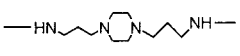
Within the "short" dimers **22**−**24**, substitution of the catecholic position 5 with Br or OH groups decreased potency about 2-fold in the polyGAT assay. In the autophosphorylation assay no clear pattern is observed. Compound **23** is 8 times more potent than **22**, while **24** is inactive.

We also prepared one example of a dimeric tyrphostin, compound **21**, composed of two 5-indole units instead of catecholic BMN units. **21** was inactive in both EGF receptor kinase assays, as might be expected because the monomeric analog AG **370** (Chart 1) was also inactive (IC_{50} = 820 μM).[9] However, it was also inactive against PDGF receptor kinase, unlike the monomeric AG **370** (data not shown).

In the cellular EGF dependent proliferation assay, all the dimeric tyrphostins except compounds **11**, **16**, and **21** showed good efficacy (Tables 1 and 2), with IC_{50} values in the range of 3−25 μM, and no clear correlation with their structure and spacer length. This is about the same range of potency shown by monomeric BMN analogs.[9] The dimeric tyrphostins are more polar than the monomeric tyrphostins due to the existence of four, instead of two, hydrophilic hydroxy groups, though this effect is compensated to various degrees by the lipophilic alkyl spacers. Therefore these compounds can be expected to penetrate into cells less efficiently. The

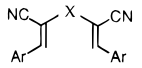
Table 1. Structures and Inhibitory and Antiproliferative Activities of Dimeric Tyrphostins

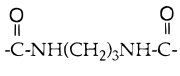


chemical data					$IC_{50}\mu M^a$ Biochemical data		$IC_{50}\mu M^a$ Biological data
		structure			phosphorylation of	autophosphorylation	EGF dependent proliferation of HER-14 cells
no.	AG	R_1	R_2	X	PolyGAT	EGFR autophosphorylation	
1	1075	OH	Br	$-NH(CH_2)_3NH-$	0.34±0..03	3.9±0.30	7.3±0.65
2	537	OH	H	$-NH(CH_2)_3NH-$	0.4±0.03	3.3±0.25	3±0.21
3	548	OH	H	$-NH(CH_2)_2NH-$	0.5±.0.04	113±10.5	10±1.05
4	550	OH	H	$-NH(CH_2)_4NH-$	0.6±0.05	4.3±0.40	5±0.45
5	575	OH	OH	$-NH(CH_2)_4NH-$	0.6±0.05	21.0±1.9	5±0.43
6	638	OH	H	$-NH(CH_2)_5NH-$	0.7±0.06	20.0±1.8	9±0.83
7	542	OH	H	$-NH(CH_2)_6NH-$	0.7±0.06	11.0±1.0	5±0.51
8	589	OH	H	$-NH(CH_2)_{10}NH-$	1.3±0.11	>100	25±1.98
9	590	OH	H		1.6±0.15	10.3±0.91	6±0.62
10	1136	OH	H		1.8±0.16	>100	12.5±1.30
11	574	OCH_3	NO_2	$-NH(CH_2)_4NH-$	112±9.51	19±1.71	>80
12	852	OH	H		2.1±0.22	13.5±1.41	4.0±0.35
13	588	OH	H	$-NH(CH_2)_8NH-$	2.2±0.22	21.0±2.10	24±1.82
14	591	OH	H		2.2±0.21	10±0.93	6.2±0.59
15	549	OH	H		4.0	25±1.95	10±0.95
16	1292	OH	NO_2	$-NH(CH_2)_3NH-$	N.D.	>100	35±2.92
17	573	OCH_3	Br	$-NH(CH_2)_4NH-$	N.D.	102±9.51	3±0.28
18	592	OH	H		N.D.	>83	20.3±2.11
19	593	OH	H		N.D.	>83	5.8±0.48
20	596	OH	H		N.D.	>167	7.0±0.73

[a] IC_{50} values based on three to four independent determinations.

Table 2. Structures and Inhibitory and Antiproliferative Activities of Dimeric Tyrphostins



chemical data				$IC_{50}\mu M$[a]		
				Biochemical data		Biological data
				phosphorylation of	autophosphorylation	EGF dependent proliferation of HER-14 cells
no.	AG	structure A_r	X	PolyGAT	EGFR	
21	662			>100	>100	>100
22	982		SO_2	0.38±0.04	12.0±1.10	2.0±0.21
23	1076		SO_2	0.85±0.09	1.4±1.21	11.0±1.11
24	1068		SO_2	0.94±0.09	>75	4.0±0.35

[a] IC_{50} values based on three to four independent determinations.

equipotency of these compounds in cellular inhibition may also reflect possibly their action against targets other than the EGFR kinase as was found for some of the monomeric tyrphostins.[11]

Discussion

In this study we have demonstrated that it is possible to prepare improved EGF receptor tyrosine kinase blockers based on first-generation BMN tyrphostins. These dimeric tyrphostins were obtained not by screening randomly substituted BMN tyrphostins but rather by trying to rationally design tyrphostins that would interact and inhibit the dimeric EGF receptor kinase at both protein chains instead of blocking the active site of the kinase at one protein chain, as the small molecular weight monomeric tyrphostins probably do. Since no X-ray or NMR structure of EGF receptor kinase is available, we prepared tyrphostin dimeric analogs with increasing spacer length in order to find the optimal inhibition of EGF receptor kinase *in vitro*. We found that they can be classified into two subclasses: the subclass with short spacers, characterized by a link of 3−5 atoms between the catecholic pharmacophores, and a subclass characterized by a long spacer group, ≥10 atoms.

We would like to suggest that the group with the short spacer interacts with only one EGF receptor protein chain within the receptor dimer. According to this model, one catecholic BMN unit inhibits the kinase active site, whereas the second BMN unit binds to a close hydrophilic site on the same EGF receptor chain. The second group of dimeric tyrphostins, with the long spacer, binds to two kinase chains within the EGFR dimer. We cannot of course exclude the possibility that the dimers with the long spacers, which posses rather flexible alkyl chains, can fold to a pincer-like configuration and thus interact with the sites within one of the polypeptide chains in the receptor dimer. This argument also holds for the more rigid compounds such as **9**, **10**, **12**, and **14** which can exist in an extended or folded conformation. This is not a likely possibility however for compound **15** for which the extended chairlike conformation is more energetically favored.

In this discussion we consider mainly the results on the phosphorylation of the activated EGF receptor on exogenous substrate since one is dealing with the active conformation of the receptor. Inhibition of autophosphorylation is mechanistically more complex since the active site (kinase domain) of the receptor is most probably blocked by a section of the receptor itself.

In a detailed kinetic study we reported[12] that monomeric BMN tyrphostins AG **18** and AG **99** are competitive inhibitors of both ATP and the substrate (polyGAT) sites, whereas the dimeric tyrphostins AG **538** and compound **2** were competitive inhibitors vis-a-vis the polyGAT substrate but noncompetitive against ATP. The fact that compound **2** and its analogs are more potent inhibitors than AG **99**, though they bind only to the substrate polyGAT site, may suggest that they indeed bind to the two substrate sites within the EGFR dimer.

In conclusion, whatever the binding mode of dimeric tyrphostins is, the improved efficacy of the first generation of dimeric tyrphostins should encourage rational design and development of more potent and selective tyrosine kinase blockers. Once the 3-dimensional structure of the tyrosine kinase receptor becomes available, this task would be more feasible.

Experimental Section

Materials and Methods. All starting materials were purchased from Aldrich or Sigma except sulfonyldiacetonitrile which was purchased from Lancaster. ^{1}H NMR spectra were recorded on a Bruker WP200 pulsed FT spectrometer. Chemical shifts are in ppm relative to TMS as internal standard. Combustion analyses for all new compounds were within 0.4% of the theoretical value. Mass spectra were recorded with a MAT 311 instrument. FAB spectra were recorded with a VG 705E spectrometer. For the dimeric tyrphostins only compounds **4**, **17**, **21**, and **23** recorded with a FAB spectrometer showed molecular peaks.

Synthetic Methods. The synthesis of the intermediate bis-acetonitriles **III** is described together with and preceding the synthesis of compounds **1**−**24**, under the title **III** and the number of the corresponding dimeric tyrphostin.

Compound 1. (a) III-1. 1,3-Propanediamine (2.2 g, 30 mM) and 6.4 g, 64 mM, of methyl cyanoacetate were stirred for 2 h at room temperature. After the exothermic reaction subsided a solid was formed. Trituration with ethanol, filtering, and recrystallization from ethanol gave 4.6 g, 74% yield, of a white solid: mp 148 °C; NMR (acetone-d_6) δ 3.58 (4H, s), 3.28 (4H, q, J = 6.7 Hz), 1.70 (2H, quin, J = 6.7 Hz); MS m/e 208 (M^+, 22), 140 (M − $NHCOCH_2CN$, 10), 125 (M − $NHCOCH_2CN$, 17), 124 (16), 112 (27), 111($C_2H_3NH_2COCH_2$-CN, 100), 98 (57), 97 (32), 72 (M − $2COCH_2CN$, 20).

(b) 3,4-Dihydroxy-5-bromobenzaldehyde (0.3 g, 1.4 mM), 0.15 g, 0.7 mM, of compound **III-1** from part a, and 15 mg of β-alanine in 25 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 0.24 g, 57% yield, of a green-yellow solid: mp 283 °C; NMR (DMSO-d_6) δ 8.26 (br t, NH), 7.92 (2H, s, vinyl), 7.59 (2H, d, J = 2.1 Hz), 7.54 (2H, d, J = 2.1 Hz), 4.0−3.1 (6H, m).

Compound 2. 3,4-Dihydroxybenzaldehyde (0.7 g, 5.1 mM), 0.5 g, 2.4 mM, of **III-1** (see compound **1** (a)), and 4 drops of piperidine in 25 mL of ethanol were refluxed to 4 h. Water and 5 drops of HCl were added, and the reaction mixtures was extracted with ethyl acetate. Evaporation, tritation with ethanol−CH_2Cl_2, and filtering gave 0.34 g, 32% yield, of a light green-yellow solid: mp 277 °C; NMR (DMSO-d_6) δ 7.94 (2H, s, vinyl), 7.54 (2H, d, J = 2.1 Hz, H_2), 7.28 (2H, dd, J = 8.2, 2.1 Hz, H_6), 6.87 (2H, d, J = 8.2 Hz, H_5), 3.22 (4H, t, J = 5.8 Hz), 1.72 (2H, quin, J = 5.8 Hz); MS m/e 328 (30), 387 (11), 261 (17), 203 (17), 201 (21), 188 (59), 164 (55), 161 (100), 159 (27), 137 (21), 123 (29), 114 (34), 111 (51), 110 (44), 105 (36), 98 (46).

Compound 3. (a) III-3. 1,2-Diaminoethane (1.8 g, 30 mM) and 6.6 g, 66 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. Trituration with ethanol, filtering, and recrystallization from ethanol gave 5 g, 86% yield, of a white solid, mp 183 °C; NMR (acetone-d_6) δ 3.55 (4H, s, CH_2CN), 3.36 (4H, br s).

(b) 3,4-Dihydroxybenzaldehyde (0.83 g, 6 mM), 0.58 g, 3 mM, of compound **III-3** from part a, and 3 drops of piperidine in 20 mL of ethanol were refluxed for 4 h. Cooling and filtering gave 1.12 g, 86%, of a yellow solid: mp 295 °C; NMR (DMSO-d_6) δ 7.94 (2H, s, vinyl), 7.54 (2H, d, J = 2.1 Hz, H_2), 7.27 (2H, dd, J = 8.2, 2.1 Hz, H_6), 6.87 (2H, d, J = 8.2 Hz, H_5), 3.36 (4H, br s); MS m/e 270 (7), 314 (20), 203 (29), 189 (30), 161 (66), 123 (70), 110 (100), 105 (25), 98 (21).

Compound 4. (a) III-4. 1,4-Diaminobutane (2.2 g, 25 mM) and 5.5 g, 55 mM, of methyl cyanoacetate were stirred for 0.5 h at room temperature. Trituration and recrystallization from ethanol gave 3.85 g, 69% yield, of a white solid: mp 145 °C; NMR (acetone-d_6) δ 3.55 (4H, s), 3.24 (4H, m), 1.56 (4H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.83 g, 6 mM), 0.67 g, 3 mM, of compound **III-4** from part a, and 3 drops of piperidine in 20 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 1.2 g, 86% yield, of a yellow solid: mp 283 °C; NMR (DMSO-d_6) δ 7.92 (2H, s, vinyl), 7.53 (2H, d, J = 2.0 Hz, H_2), 7.27 (2H, dd, J = 8.3, 2.0 Hz, H_6), 6.85 (2H, d, J = 8.3 Hz, H_5), 3.22 (4H, br s), 1.60 (4H, br s); MS m/e 463 (M^+, 100), 274 (60).

Compound 5. 3,4,5-Trihydroxybenzaldehyde (0.51 g, 3 mM,), 0.33 g, 1.5 mM, of compound **III-4** (a), and 3 drops of piperidine in 10 mL of ethanol were refluxed for 2 h. Cooling and filtering gave 0.7 g, 94% yield, of a deep yellow solid: mp >310 °C; NMR (DMSO-d_6) δ 7.80 (2H, s, vinyl), 7.01 (4H, s), 3.20 (4H, m), 1.50 (4H, m).

Compound 6. (a) III-6. 1,5-Diaminopentane (2.3 g, 25 mM) and 6.6 g, 64 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. Trituration from ethanol gave 4.1 g, 69% yield, of a white solid: mp 125 °C; NMR (acetone-d_6) δ 3.55 (4H, s), 3.21 (4H, q, J = 7.0 Hz), 1.53 (4H, m), 1.37 (2H, m).

(b) 3,4-Dihydroxybenzaldehyde (1.1 g, 8 mM), 0.95 g, 4 mM, of compound **III-6** from part a, and 3 drops of piperidine in 20 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 1 g, 53% yield, of a yellow solid: mp 248 °C; NMR (acetone-d_6) δ 8.05 (2H, s, vinyl), 7.66 (2H, d, J = 2.1 Hz, H_2), 7.36 (2H, dd, J = 8.3, 2.1 Hz, H_6), 6.94 (2H, d, J = 8.3 Hz, H_5), 3.40 (4H, m), 1.63 (4H, m), 1.44 (2H, m).

Compound 7. (a) III-7. 1,6-Diaminohexane (2.9 g, 25 mM) and 6.6 g, 66 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. The semisolid was triturated with ethanol, filtered, and recrystalized from ethanol to give 4 g, 64% yield, of a white solid: mp 140 °C; NMR (acetone-d_6) δ 3.55 (4H, s), 3.22 (4H, m), 1.30 (4H, m), 1.10 (4H, m).

(b) 3,4-Dihydroxybenzaldehyde (1.1 g, 8 mM), 1 g, 4 mM, of compound **III-7** from part a, and 20 mg of β-alanine in 15 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 2.1 g, 96% yield, of a yellow solid: mp 260 °C; NMR (DMSO-d_6) δ 7.90 (2H, s, vinyl), 7.53 (2H, d, J = 2.0 Hz, H_2), 7.26 (2H, dd, J = 8.1 Hz, H_6), 6.85 (2H, d, J = 8.1 Hz, H_5), 3.18 (4H, m), 1.50−1.25 (8H, m); MS m/e 250 (7), 210 (10), 165 (11), 153 (51), 123 (12), 114 (31), 111 (106), 110 (22), 98 (99).

Compound 8. (a) III-8. 1,10-Diaminodecane (3.5 g, 20 mM) and 5 g, 50 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. The solid was triturated with ethanol and filtered to give 4.9 g, 79% yield, of a white solid: mp 131 °C; NMR (acetone-d_6) δ 3.54 (4H, s), 3.21 (4H, t, J = 6.8 Hz), 1.50 (4H, m), 1.30 (12H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.61 g, 2 mM, of compound **III-8** from part a, and 3 drops of piperidine in 15 mL of ethanol were refluxed for 3 h. Cooling, filtering, and washing with CH_2Cl_2 gave 0.92 g, 77% yield, of a yellow solid: mp 218 °C; NMR (DMSO-d_6) δ 7.90 (2H, s, vinyl), 7.53 (2H, d, J = 2.2 Hz, H_2), 7.29 (2H, dd, J = 8.3, 2.2 Hz, H_6), 6.85 (2H, d, J = 8.3 Hz, H_5), 3.17 (4H, m), 1.50−1.30 (16H, m).

Compound 9. (a) III-9. *m*-Xylylenediamine (4.1 g, 30 mM) and 7.4 g, 75 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. Trituration with ethanol, filtering, and recrystallization from ethanol gave 5.8 g, 72% yield, of a white solid: mp 170 °C; NMR (acetone-d_6) δ 7.27 (4H, m), 3.65 (4H, s), 4.43 (4H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.54 g, 2 mM, of compound **III-9** from part a, and 3 drops of piperidine in 20 mL of ethanol were refluxed for 3 h. Concentration and trituration with CH_2Cl_2 gave 0.44 g, 43% yield, of a yellow-orange solid: mp 246 °C; NMR (DMSO-d_6) δ 7.97 (2H, s, vinyl), 7.54 (2H, d, J = 2.1 Hz, H_2), 7.30 (6H, m), 6.85 (2H, d, J = 8.2 Hz, H_5), 4.40 (4H, m).

Compound 10. (a) III-10. Cyclohexane-1,3-bis(methylamine) (3 g, 21 mM) and 6 g, 60 mM, of methyl cyanoacetate were stirred overnight at room temperature. Trituration with ethanol and filtering gave 2.17 g, 37% yield, of a white solid: mp 160 °C; NMR (acetone-d_6) δ 3.79 (4H, s, CH_2CN), 3.09 (4H, d, J = 7.0 Hz), 1.8−0.6 (10H, m); (DMSO-d_6) 8.25 (br s, NH), 3.61 (4H, s, CH_2CN), 2.92 (4H, br s), 1.70−0.6 (10H, m).

(b) Compound **III-10** (0.44 g, 1.6 mM) from part a, 0.45 g, 3.2 mM, of 3,4-dihydroxybenzaldehyde, and 20 mg of β-alanine in 20 mL of ethanol were refluxed for 7 h. Cooling and filtering gave 0.75 g, 91% yield, of a yellow solid: mp 225 °C; NMR (DMSO-d_6) δ 8.22 (br s, NH), 7.90 (2H, s, vinyl), 7.53 (2H, d, J = 2.0 Hz, H_2), 7.27 (2H, dd, J = 8.2, 2.0 Hz, H_6), 6.85 (2H, d, J = 8.2 Hz, H_6), 3.05 (4H, m, CH_2NH), 1.7−0.6 (10H, m).

Compound 11. 5-Nitrovanilline (0.78 g, 4 mM), 0.45 g, 2 mM, of compound **III-4** (see compound **4** (a)), and 3 drops of piperidine in 10 mL of ethanol were refluxed for 2 h. Cooling and filtering gave 0.68 g, 58% yield, of an orange solid: mp 168 °C; NMR (DMSO-d_6) δ 8.25 (2H, s, vinyl), 8.12 (2H, s, H_6), 7.85 (2H, s, H_2), 3.91, 3.70 (2 s, 6H, OCH_3), 3.08 (4H, m), 1.40 (4H, m); MS m/e 554 (M − CN, 100), 470 (22), 421 (36), 399 (48), 333 (43).

Compound 12. (a) III-12. 1,2-Diaminocyclohexane (2.3 g, 20 mM) and 4.5 g, 45 mM, of methyl cyanoacetate were stirred for 2 h at room temperature. Ethanol was added and the solid filtered and washed with ethanol to give 1.8 g, 36% yield, of a white waxy solid: mp 83 °C; NMR (acetone-d_6) δ 3.52 (4H, s), 3.30 (2H, m), 2.0−1.7 (8H, m).

(b) To 1.8 g, 7 mM, of compound **III-12** from part a and 1.9 g, 14 mM, of 3,4-dihydroxybenzaldehyde in 30 mL of ethanol was added 4 drops of piperidine. The reaction mixture was refluxed for 5 h, cooled, and filtered to give 1.5 g, 44% yield, of a yellow solid: mp 275 °C; NMR (DMSO-d_6) δ 7.94 (2H, s, vinyl), 7.54 (2H, d, J = 2.1 Hz), 7.30 (2H, dd, J = 8.3, 2.1 Hz), 6.88 (2H, d, J = 8.3 Hz), 3.30 (2H, m), 1.8 (8H, m).

Compound 13. (a) III-13. 1,8-Diaminooctane (2.9 g, 20 mM) and 5 g, 50 mM, of methyl cyanoacetate were heated for 0.5 h at 100 °C and stirred for another 1 h at room temperature. Ethanol was added and the solid filtered and washed with ethanol to give 4.3 g, 77% yield, of a white solid: mp 122 °C; NMR (acetone-d_6) δ 3.54 (4H, s), 3.21 (4H, br s), 1.50 (4H, m), 1.32 (8H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.55 g, 2 mM, of compound **III-13** from part a, and 3 drops of piperidine in 15 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 1 g, 96% yield, of a green-yellow solid: mp 273 °C; NMR (DMSO-d_6) δ 7.90 (2H, s, vinyl), 7.52 (2H, d, J = 2.2 Hz, H_2), 7.29 (2H, dd, J = 8.3, 2.2 Hz, H_6), 6.85 (2H, d, J = 8.3 Hz, H_5), 3.17 (4H, m), 1.50−1.30 (12H, m).

Compound 14. (a) III-14. 1-(2-Aminoethyl)piperazine (3.9 g, 30 mM) and 7.5 g, 75 mM, of methyl cyanoacetate were heated for 1 h at 100 °C. Chromatography on silica gel and elution with 3% CH_3OH in CH_2Cl_2 gave 3.5 g of a yellow oil, 44% yield: NMR (acetone-d_6) δ 3.87 (2H, s), 3.56 (2H, s), 3.50 (4H, m), 2.50 (8H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.83 g, 6 mM), 0.9 g, 3.4 mM, of **III-14** from part a, and 3 drops of piperidine in 15 mL of ethanol were refluxed for 2 h. Evaporation to dryness and trituration with EtAc−CH_2Cl_2 gave 1 g, 63% yield, of a orange-yellow solid: mp 95 °C; NMR (DMSO-d_6) δ 7.94 (2H, s, vinyl), 7.54 (2H, m), 7.24 (2H, m), 6.85 (2H, d, J = 8.2 Hz, H_5), 3.60 (8H, m), 3.30 (4H, m).

Compound 15. (a) III-15. Piperazine hydrate (4.7 g, 0.5 mM) and 6.6 g, 66 mM, of methyl cyanoacetate were heated for 1 h at 120 °C. Trituration with ethanol, filtering, and washing with ethanol gave 4 g, 73% yield, of a light yellow solid: mp 140 °C; NMR (acetone-d_6) δ 3.76 (4H, s, CH_2CN), 3.60 (8H, AA′BB′, m).

(b) 3,4-Dihydroxybenzaldehyde (0.83 g, 6 mM), 0.66 g, 3 mM, of compound **III-15** from part a, and 4 drops of piperidine in 20 mL of ethanol were refluxed for 3.5 h. Cooling and filtering gave 0.92 g, 70% yield, of a yellow solid: mp 177 °C; NMR (DMSO-d_6) δ 7.88 (2H, s, vinyl), 7.52 (2H, d, J = 2.0 Hz, H_2), 7.25 (2H, dd, J = 8.0, 2.0 Hz, H_6), 6.85 (2H, d, J = 8.0 Hz, H_5), 3.02 (8H, m); MS m/e 402 (33), 276 (23), 273 (24), 200 (30), 196 (76), 185 (50), 161 (100), 132 (50), 105 (90).

Compound 16. 3,4-Dihydroxy-5-nitrobenzaldehyde (**13**) (180 mg, 1.0 mM), 105 mg, 0.5 mM, of compound **III-1**, and 15 mg of β-alanine in 15 mL of ethanol were refluxed for 4.5 h. Cooling and filtering gave 250 mg, 92% yield, of a red solid: mp 237 °C; NMR (acetone-d_6) δ 8.16 (2H, d, J = 2.0 Hz), 8.11 (2H, s, vinyl), 7.84 (2H, d, J = 2.0 Hz), 3.3 (4H, m), 1.8 (2H, m).

Compound 17. 5-Bromovanilline (0.93 g, 4 mM), 0.45 g, 2 mM, of compound **III-4**, and 3 drops of piperidine in 10 mL of ethanol were refluxed for 2 h. Cooling and filtering gave 1.1 g, 85% yield, of a light yellow solid: mp 293 °C; NMR (DMSO-d_6) δ 8.03 (2H, s, vinyl), 7.80 (2H, s, H_6), 7.65 (2H, s, H_2), 3.87 (6H, s, OCH_3), 3.24 (4H, m), 1.52 (4H, m); MS m/e 646, 648, 650 (M^+, 10, 19, 10).

Compound 18. (a) III-18. 4,4′-Methylenebis(cyclohexylamine) (4.2 g, 20 mM) and 5 g, 20 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. Trituration with ethanol, filtering, and washing with ethanol gave 2.88 g, 42% yield, of a white solid: mp 248 °C; NMR (DMSO-d_6) δ 3.56 (4H, s), 3.42 (2H, m), 1.70−0.8 (20H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.69 g, 2 mM, of compound **III-18** from part a, and 3 drops of piperidine in 15 mL of ethanol were refluxed for 3 h. Cooling and filtering gave 0.95 g, 81% yield, of a yellow solid: mp >300 °C; NMR (DMSO-d_6) δ 7.86 (2H, s, vinyl), 7.53 (2H, d, J = 2.1 Hz, H_2), 7.27 (2H, dd, J = 8.2, 2.1 Hz, H_6), 6.85 (2H, d, J = 8.2 Hz, H_5), 3.61 (2H, m), 1.8−1.0 (20H, m).

Compound 19. (a) III-19. 4-(Aminomethyl)piperidine (2.3 g, 20 mM) and 5 g, 50 mM, of methyl cyanoacetate were heated for 1 h at 100 °C. The reaction mixture was cooled and triturated with benzene and the benzene discarded. The semisolid residue was triturated with ethanol and filtered to give 1.5 g, 30% yield, of a white solid: mp 144 °C; NMR (acetone-d_6) δ 3.85 (2H, s), 3.56 (2H, s), 3.16 (4H, m), 2.80 (6H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.5 g, 2 mM, of compound **III-19** from part a, and 3 drops of piperidine in 15 mL of ethanol were refluxed for 3 h. Evaporation gave a viscous oil which was recrystalized form acetone−CH_2Cl_2 to give 0.43 g, 44% yield, of a yellow solid: mp 248 °C; NMR (DMSO-d_6) δ 7.92, 7.90 (2H, s, vinyl), 7.53 (2H, d, J = 2.1 Hz, H_2), 7.26 (2H, m, H_6), 6.85 (2H, d, J = 8.2 Hz, H_5), 4.13 (2H, m), 3.10 (4H, m), 1.70 (5H, m).

Compound 20. (a) III-20. 1,4-Bis(3-aminopropyl)piperazine (4 g, 20 mM) and 5 g, 50 mM, of methyl cyanoacetate were stirred for 1 h at room temperature. Trituration, filtering, and washing with ethanol gave 4.3 g, 64% yield, of a white solid: mp 160 °C; NMR (acetone-d_6) δ 3.52 (4H, s), 3.30 (4H, m), 2.5−2.2 (12H, m), 1.57 (4H, m).

(b) 3,4-Dihydroxybenzaldehyde (0.48 g, 3.5 mM), 0.6 g, 1.8 mM, of compound **III-20** from part a, and 3 drops of piperidine in 10 mL of ethanol were refluxed for 1.5 h. Cooling and filtering gave 0.97 g, 89% yield, of an orange-yellow solid: mp 227 °C; NMR (DMSO-d_6) δ 7.92 (2H, s, vinyl), 7.53 (2H, d, J = 2.1 Hz, H_2), 7.20 (2H, dd, J = 8.3, 2.1 Hz, H_6), 6.84 (2H, d, J = 2.1 Hz, H_5), 3.24 (4H, m), 2.40 (12H, m), 1.64 (4H, m).

Compound 21. 5-Formylindole (**9**) (0.29 g, 2 mM), 0.22 g, 1 mM, of compound **III-1**, and 2 drops of piperidine in 15 mL of ethanol were refluxed for 1.5 h. Cooling and filtering gave 0.43 g, 90% yield, of a light pink-white solid: mp 275 °C; NMR (acetone-d_6) δ 8.90 (2H, s), 8.25 (2H, s, vinyl), 7.90 (2H, dd, J = 8.6, 2.0 Hz), 7.68 (2H, d, J = 2.0), 6.60 (4H, m), 3.2 (4H, m), 1.8 (2H, m); MS m/e 476 (M^+, 20), 387 (100), 289 (62).

Compound 22. 3,4-Dihydroxybenzaldehyde (0.55 g, 4 mM), 0.3 g, 2 mM, of sulfonyldiacetonitrile, and 30 mg of β-alanine in 30 mL of ethanol were refluxed for 6 h. Water and 1 mL of HCl were added, and the reaction mixture was extracted with EtAc. Evaporation and recrystallization from acetone−benzene gave 0.45 g, 58% yield, of a yellow solid: mp 264 °C; NMR (acetone-d_6) δ 8.15 (2H, s, vinyl), 7.77 (2H, d, J = 2.3 Hz), 7.59 (2H, dd, J = 8.4, 2.3 Hz), 7.07 (2H, d, J = 8.4 Hz); MS m/e 312 (M − 72, 28), 265 (24), 211 (100), 185 (15), 161 (44), 160 (80), 157 (35), 114 (43).

Compound 23. 3,4-Dihydroxy-5-bromo-benzaldehyde (230 mg, 1.06 mM), 76 mg, 0.53 mM, of diacetonitrile sulfone, and 10 mg of β-alanine in 10 mL of ethanol were refluxed for 5 h. Cooling and filtering gave 220 mg, 76% yield, of an orange solid: mp >300 °C; NMR (acetone-d_6) δ 8.18 (2H, s, vinyl), 7.90 (2H, d, J = 1.6 Hz), 7.78 (2H, d, J = 1.6 Hz). MS m/e 539, 541, 543 (M − 1, 45, 100, 53).

Compound 24. 3,4-Dihydroxy-5-methoxybenzaldehyde (150 mg, 0.9 mM), 65 mg, 0.45 mM, of diacetonitrile sulfone, and 15 mg of β-alanine in 20 mL of ethanol were refluxed for 4.5 h. Cooling and filtering gave 160 mg, 80% yield, of a yellow solid: mp 198 °C; NMR (acetone-d_6) δ 8.17 (H, s, vinyl), 7.49 (2H, s), 7.45 (2H, s), 3.93 (6H, s, OCH_3).

Biochemical and Cellular Assays. The efficacy of tyrphostins to inhibit EGFR autophosphorylation and inhibit the phosphorylation of the exogenous substrate poly(Glu_6Ala_3Tyr) (polyGAT) was measured by the same methodology published earlier, with no modifications.[8−11] Also the antiproliferative activity was determined by measuring the pathway of the tyrphostins to block [^{3}H]thymidine uptake in HER-14 cells stimulated by EGF as described.[8−11] The stability of BMN tyrphostins in tissue culture is good, and they possess $t_{1/2} \sim$ 16−30 h,[14] but the stability of the tyrphostins in this particular study was not examined. Therefore, the tyrphostin containing medium was replaced every 24 h in the assay for their antiproliferative activity.[8−11,14]

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