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PROTEIN-TYROSINE KINASE INHIBITION: MECHANISM-BASED DISCOVERY OF ANTITUMOR AGENTS[1]

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ABSTRACT.—Protein-tyrosine kinases (PTKs) have been shown to induce the cascade of altered cell parameters characteristic of transformed cells. This proposition provides an important rationale for the discovery of potential antitumor agents from natural sources on the basis of inhibition of PTK activity. Numerous naturally occurring and synthetic analogues of PTK inhibitors were systematically evaluated in this review based on their structure-activity relationships and potential antitumor efficacy.

All natural anticancer drugs have been discovered through direct isolation from natural sources or by synthesis based on natural lead compounds. The most critical step to the success of this discovery endeavor rests on the screening system for antitumor activity. Much of the screening effort in the United States has been stimulated and/or coordinated by the National Cancer Institute (NCI) through its comprehensive drug discovery program (1,2). The initial screening system developed by NCI was a murine antileukemic model. However, the clinical activities of the active compounds discovered through this screening system were predominantly in leukemia, lymphoma, and a few rare tumors. The low clinical efficacy of these drugs for the treatment of slowly growing solid cancers is probably due to the use of a single antileukemic screening system. Most of these drugs lack selectivity in damaging tumor cells and display minimal therapeutic indices. Therefore, it is evident that more specific screening approaches are essential for achieving greater selectivity in the chemotherapy of cancer. In this review, we will discuss in detail one of the biochemical mechanism-based approaches designed on the basis of inhibition of protein-tyrosine kinases (PTKs).

PROTEIN-TYROSINE KINASE

A tremendous advance in our understanding of the molecular mechanisms of malignant transformation has recently been achieved. One of the most exciting discoveries is the identification of oncogenes (3,4). More than 40 oncogenes have been identified, and the protein products of many of these have been characterized. These proteins include protein kinases, GTP-binding proteins, and nuclear transcription factors. This discovery provides a unique hypothesis that activation of transformation might act via protein phosphorylation. Protein-tyrosine kinases are a group of enzymes that catalyze the transfer of the γ-phosphate of ATP to the hydroxyl group of tyrosine on many key proteins, which, in turn, induce the cascade of altered cell parameters characteristic of transformed cells (5–9). This hypothesis has been supported by several recent findings:

1. Activated PTKs have been identified to be the products of approximately half of the known viral transforming genes (oncogenes) (Table 1).
2. The plasma-membrane receptors for several polypeptide growth factors, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), platelet-derived growth factor (PDGF), insulin-like growth factor 1 (IGF-1), macrophage colony stimulating factor (CSF-1), fibroblast growth factors (FGF-1 and FGF-2), nerve growth factor (NGF), and hepatocyte growth factor (HGF) are ligand-activated PTKs (Table 1).

[1]An invited review in the series on mechanism-based studies of natural products in drug discovery.

TABLE 1. Examples of Protein-Tyrosine Kinase Gene Families.[a]

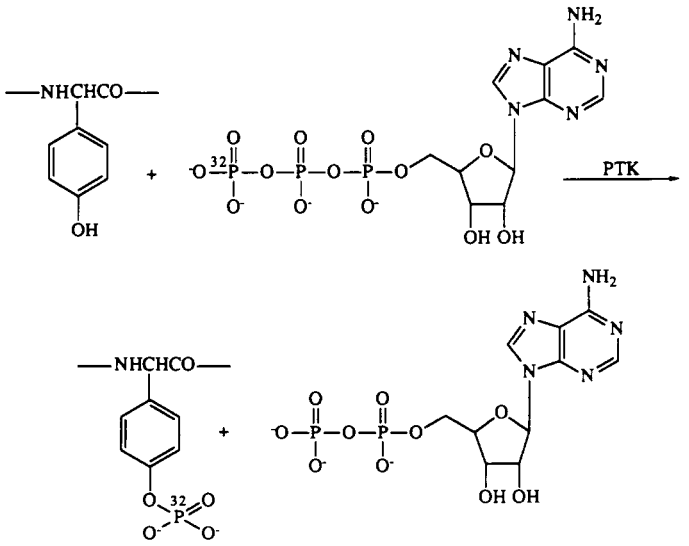
A.	Receptor Type Genes	
1.	EGF-R sub-family	EGF-R/TGFα-R, *HER-2/neu/erb* B-2
2.	I-R sub-family	I-R, IGF1-R, NGF-R/*trk*, HGF-R/*met*, *ltk/tyk*-1, *ros*
3.	PDGF-R sub-family	PDGF-R, CSF1-R/*fms*, *flt*, *KDR*, MGF-R/*kit*, *ret*
4.	FGF-R sub-family	FGF-R/*bek/flg*
B.	Cytoplasmic Type Genes	
1.	SRC sub-family	*blk*, *fgr*, *fyn*, *hck*, *lck*, *lyn*, *src*, *yes*
2.	FES sub-family	*fer/tyk-3*, *fes/fps*
3.	ABL sub-family	*abl*, *arg*

[a]EGF-R, epidermal growth factor receptor; TGFα-R, transforming growth factor-α receptor; I-R, insulin receptor; IGF1-R, insulin growth factor-1 receptor; NGF-R, nerve growth factor; HGF-R, hepatocyte growth factor receptor; PDGF-R, platelet-derived growth factor receptor; CSF1-R, colony stimulating factor-1 (macrophage colony stimulating factor) receptor; MGF-R, mast cell growth factor; FGF-R, fibroblast growth factor receptor.

3. Abnormal expression of growth factors, growth factor receptors, and proto-oncogenes can contribute to the transformed state of human cancers. Some recently documented examples are listed in Table 2.

SCREENS FOR PROTEIN-TYROSINE KINASE INHIBITORS

The search for inhibitors of PTKs requires a convenient assay. Most assays for the screening of inhibitors measure directly the ability of a compound of interest to decrease the phosphotransferase activity of a PTK present as a purified or partially purified enzyme, an immunocomplex, or a subcellular fraction prepared from a cell line that overexpresses that enzyme. The most common example of the latter approach is the use of membrane fractions prepared from A431 human epidermoid carcinoma cells. These cells contain an unusually high number of EGF receptors (85), and this receptor is a frequent target enzyme for the screening of PTK inhibitors (for examples, see 86–90). The T cell PTK, $p56^{lck}$, is another example of an enzyme that has been used extensively for the screening of both naturally occurring and synthetic inhibitors (91–97). This en-



SCHEME 1

TABLE 2. Protein-Tyrosine Kinases and Human Cancer.

Tyrosine kinase[a]	Cancer	Reference
EGF-R/TGFα-R	bladder	10
	brain	11, 12
	breast	13, 14
	colorectum	15
	esophagus	16
	kidney	17, 18
	lung	19–21
	nose	22
	ovary	23–25
	pancreas	26, 27
	squamous carcinoma	28
IGF1-R	breast	29, 30
	esophagus	31
	lung	32
	ovary	33
	pancreas	27
	uterus	34
NGF-R/*trk*	colon	35
	neuroblastoma	36
PDGF-R	midgut	37, 38
	pancreas	37, 38
	soft tissue	39, 40
CSF1-R/*fms*	breast	41
	leukemia	42
FGF-R/*bek*/*flg*	brain	43
	breast	44
	prostate	45
BCR/*ABL*	brain	46, 47
	leukemia	48–50
HER-/*neu*/*erb*B-2	breast	14, 51–68
	lung	69
	ovary	70
	stomach	71, 72
kit	lung	73, 74
lck	colon	75
	leukemia	76
ret	neuroblastoma	77
	thyroid	78, 79
ros	brain	80
src	bladder	81
	colon	82, 83
	kidney	84

[a]For abbreviations for various receptors (R), see the footnote to Table 1.

zyme is expressed abundantly in thymocytes and can be readily isolated from extracts of bovine thymus by a combination of simple chromatographic steps (98). Examples of other enzymes that have been employed include p40 (99,100), $p60^{v\text{-}src}$ (86, 101–104), $p60^{v\text{-}abl}$ (5,105,106), $p130^{v\text{-}fps}$ (86,107), and the insulin receptor (107–111).

The activity of PTKs is assessed by monitoring the rate of transfer of the γ-phosphate from [γ-^{32}P]ATP to a tyrosyl residue present either on the kinase itself (autophosphorylation) or on an exogenously added peptide or protein substrate (Scheme 1).

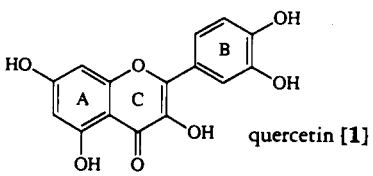
SUBSTRATE.—The use of an exogenous substrate has the advantage of allowing the kinetic mechanism of the inhibitor to be evaluated further. The use of peptide sub-

strates of defined sequences allows the activity of PTKs to be assayed unambiguously even though partially purified preparations of enzyme may be contaminated by the more abundant protein serine/threonine kinases (for review, see 8). Common substrates include peptides based on the sequence of angiotensins I (AspArgValTyrIleHisProPheHisLeu) or II (AspArgValTyrIleHisProPhe) (112), peptides based on the autophosphorylation site of $p60^{src}$ [e.g., the RR-SRC peptide, ArgArgLeuIleGluAspAlaGluTyrAlaAlaArgGly (113)], and synthetic polymers of glutamate and tyrosine (114).

BIOASSAY.—Typical assays contain a peptide or protein substrate, [γ-^{32}P]ATP, and a divalent cation, which, for PTKs, is either Mg^{++} or Mn^{++}. For the routine assay of the $p56^{lck}$ PTK, for example, reactions contain 1.2 mM angiotensin I, 50 μM [γ-^{32}P]ATP (1500-3000 cpm/pmol), 50 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate (a protein tyrosine phosphatase inhibitor), 25 mM Hepes (pH 7.4), and 2.0 M NaCl (98). For many PTKs high concentrations of NaCl increase the V_{max} of the enzymes for angiotensin analogues as substrates without altering their Km's for ATP or peptide and, at the same time, reduce nonspecific interactions that interfere with the screening of crude extracts for the presence of specific inhibitors.

NATURALLY OCCURRING INHIBITORS

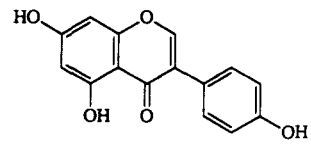
In spite of the important roles that protein-tyrosine kinases are thought to play in cellular growth regulation and transformation, relatively little work has appeared on systematic discovery of natural PTK inhibitors. In searching for the biochemical reason for the preferential inhibitory activity of quercetin [**1**] on malignant cells (115),



quercetin [**1**]

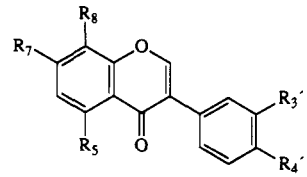
Graziani *et al.* (116) observed that quercetin inhibited partially purified, cyclic-AMP-independent protein kinase activities isolated from Ehrlich ascites tumor cells and did not affect cyclic-AMP-dependent protein kinase activity. This early observation has stimulated considerable interest in studying the potential inhibitory effect of many flavonoids on protein-tyrosine kinase activity.

FLAVONOIDS.—In order to further establish the specific inhibitory effect of quercetin, Graziani *et al.* (103) demonstrated for the first time that the tyrosine phosphorylation activity of the Rous sarcoma virus (RSV) *src*-gene product, $p60^{v\text{-}src}$, was inhibited both in vitro (K_i) and in vivo (IC_{50}) by quercetin in the range of 6–11 μM. The first report on the screening of protein-tyrosine kinase inhibition from microorganisms was published by H. Umezawa *et al.* (117). Using the epidermal growth factor receptor (EGF-R) of membranes from the human epidermoid carcinoma cell line, A-431, orobol [**2**] (in vitro $IC_{50} = 3$ μg/ml) was isolated from *Streptomyces neyagawaensis* var. *orobolere* (117). It inhibited the growth of RSV-transformed rat kidney cells (src^{ts}-NRK) ($IC_{50} = 4$ μg/ml at 33°). A structural isomer of orobol, genistein [**3**], was later isolated from *Pseudomonas* sp. using a similar enzyme preparation (118). In addition, psi-tectorigenin (8-methoxygenistein) [**4**] isolated from a culture filtrate of actinomycetes as an inhibitor of EGF-induced phosphatidylinositol turnover in A-431 cells was shown to be a strong EGF-R kinase inhibitor (119). A large number of natural or commercially available synthetic flavonoids have since been tested for their inhibitory activities.



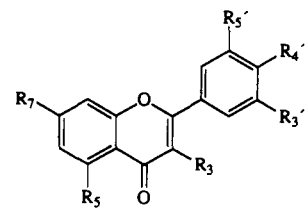
genistein [**3**]

TABLE 3. Inhibitory Effects of Isoflavonoids on Protein-Tyrosine Kinase Activity.



Compound	R_5	R_7	R_8	$R_{3'}$	$R_{4'}$	IC_{50} (μg/ml)	
						p40 (99)	EGF-R
Orobol [**2**]	OH	OH	H	OH	H		3×10^0 (117)
							1×10^{-1} (119)
Genistein [**3**]	OH	OH	H	H	OH	$>5 \times 10^2$	7×10^{-1} (120)
							1×10^{-1} (119)
psi-Tectorigenin [**4** . . .	OH	OH	OMe	H	OH		1×10^{-1} (119)
Prunetin [**5**]	OH	OMe	H	H	OH		4×10^0 (120)
Biochanin A [**6**]	OH	OH	H	H	OMe	$>5 \times 10^2$	3×10^1 (120)
Genistin [**7**]	OH	Glu	H	H	OH		$>10^2$ (120)
Diadzein [**8**]	H	OH	H	H	OH		$>10^2$ (120)
9	H	H	H	H	OH	$>5 \times 10^2$	

TABLE 4. Inhibitory Effects of Flavonols on Protein-Tyrosine Kinase Activity.

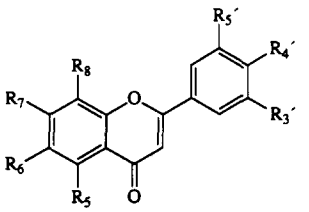


Compound	R_5	R_7	R_3	$R_{3'}$	$R_{4'}$	$R_{5'}$	IC_{50} (μg/ml)		
							p40 (99)	$p56^{lck}$ (94)	EGF-R (120)
Myricetin [**10**] . . .	OH	OH	OH	OH	OH	OH	1×10^1		
Myricitrin [**11**] . . .	OH	OH	Rha	OH	OH	OH	$>5 \times 10^2$		
Syringetin [**12**] . . .	OH	OH	OH	OMe	OH	OMe		2×10^1	
Quercetin [**1**]	OH	OH	OH	OH	OH	H	1×10^1	4×10^0	5×10^0
13	OH	OH	OH	OH	H	OH		3×10^1	
Robinetin [**14**] . . .	H	OH	OH	OH	OH	OH	7×10^0		
Fisetin [**15**]	H	OH	OH	OH	OH	H	4×10^1	4×10^0	
Kaemferol [**16**] . . .	OH	OH	OH	H	OH	H			3×10^0
17	OH	OH	OMe	H	OMe	H	$>5 \times 10^2$		
18	OH	OMe	OMe	H	OMe	H	$>5 \times 10^2$		
Galangin [**19**] . . .	OH	OH	OH	H	H	H		8×10^1	

Isoflavonoids and Flavonols (Tables 3 and 4).—A hydroxyl group at C-5 or C-4′ appears to be essential. Replacement of the hydroxyl group by the methoxyl group considerably reduces activity. Glycosylation (**3** vs. **7** and **10** vs. **11**) may completely abolish activity. Genistein was the most potent isoflavonoid inhibitor against the EGF-R PTK (102,119,120). It was active against $p60^{v\text{-}src}$ and $p110^{gag\text{-}fes}$ (99). Interestingly, it was completely inactive as an inhibitor of p40 (99) or $p56^{lck}$ (91,94). Myricetin [**10**] was highly active against $pp130^{fps}$ ($K_i = 1.8\ \mu M$) and insulin receptor ($K_i = 2.6\ \mu M$) PTKs (107). Quercetin [**1**] was almost as active as myricetin (107).

Flavones (Table 5).—An extensive evaluation of 32 natural and synthetic flavones as $p56^{lck}$ PTK inhibitors was recently reported by Cushman and co-workers (91,94). It

TABLE 5. Inhibitory Effects of Flavones on Protein-Tyrosine Kinase Activity.



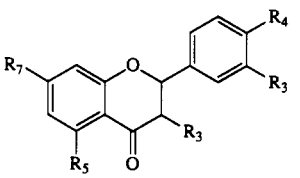
Compound	R_5	R_6	R_7	R_8	$R_{3'}$	$R_{4'}$	$R_{5'}$	IC_{50} (μg/ml)		
								p40 (99)	$p56^{lck}$ (91,94)	EGF-R (120)
20	H	H	OH	OH	OMe	OH	OMe		4×10^1	
21	H	H	OH	OH	OMe	OMe	OMe		1×10^2	
22	H	H	OMe	OMe	OMe	OMe	OMe		$>8 \times 10^2$	
23	H	H	OH	OH	OMe	OR[a]	OMe		$>8 \times 10^2$	
24	H	H	OAc	OAc	OMe	OR[a]	OMe		$>8 \times 10^2$	
Nevadensin [**26**]	OH	OMe	OH	OMe	H	OMe	H	5×10^1		
Luteolin [**27**]	OH	H	OH	H	H	OH	OH		1×10^1	
28	H	OH	H	H	OMe	OH	OMe		4×10^1	
29	H	OH	H	H	OMe	OMe	OMe		2×10^1	
30	H	OH	H	H	OMe	OR[a]	OMe		2×10^2	
31	H	OMe	H	H	OMe	OMe	OMe		$>8 \times 10^2$	
32	H	H	OH	H	OMe	OH	OMe		4×10^2	
33	H	H	OH	H	OMe	OR[a]	OMe		7×10^1	
34	H	H	OH	H	OMe	OMe	OMe		$>8 \times 10^2$	
35	H	H	OAc	H	OMe	OH	OMe		$>8 \times 10^2$	
36	H	H	OAc	H	OMe	OR[a]	OMe		$>8 \times 10^2$	
37	H	H	OMe	H	OMe	OMe	OMe		$>8 \times 10^2$	
Apigenin [**38**]	OH	H	OH	H	H	OH	H	9×10^1	4×10^0	3×10^1
Acacetin [**39**]	OH	H	OH	H	H	OMe	H			4×10^1
Gonkwanin [**40**]	OH	H	OMe	H	H	OH	H		8×10^1	
41	OH	H	OMe	H	H	OMe	H	$>5 \times 10^2$		
42	H	H	OH	OH	OH	H	H		5×10^1	
43	H	H	H	H	OMe	OH	OMe		8×10^0	
44	H	H	H	H	OMe	OMe	OMe		$>8 \times 10^2$	
Chrysin [**45**]	OH	H	OH	H	H	H	H		5×10^0	
46	OH	H	H	H	OH	H	H		8×10^1	
47	OH	H	H	H	H	OH	H		4×10^0	
48	OH	H	H	H	H	OBn	H		$>8 \times 10^2$	
49	H	H	OH	OH	H	H	H		8×10^1	
50	H	OH	H	H	OH	H	H		4×10^0	
51	H	OH	H	H	H	OH	H		3×10^1	
52	H	OH	H	H	H	OR[a]	H		4×10^1	
53	H	H	OH	H	OH	H	H		1×10^1	
54	H	H	OH	H	H	OH	H		4×10^1	
55	H	H	OH	H	H	OBn	H		7×10^2	
56	H	H	OH	H	H	OR[a]	H		4×10^2	
57	H	H	OH	H	H	H	H		8×10^1	
58	H	H	H	H	H	OH	H		1×10^2	
Flavone [**59**]	H	H	H	H	H	H	H			5×10^1

[a]R = Si(*t*-Bu)Me_2.

appears that two or three hydroxyl groups at appropriate positions result in most potent inhibition (**45**, 5,7-dihydroxyflavone; **47**, 5,4′-dihydroxyflavone; **50**, 6,3′-dihydroxyflavone; **38**, 5,7,4′-trihydroxyflavone). Limited structure-activity studies also showed that the inhibitory potencies of flavones for tyrosine kinase activities of pp130^{fps} and insulin receptor partially correlate with the numbers of hydroxyl groups (107). With the exception of compounds **29** and **41**, addition of methoxyl groups or replacement of hydroxyl groups with methoxyl groups generally weakens the activity. Apigenin [**38**] displays a moderate selectivity against p56^{lck} kinase.

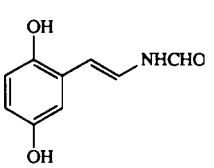
Flavanones (Table 6).—A hydroxyl group at C-4′ seems important. Overall, this class of compounds is much less active than the corresponding flavonoids, suggesting that the planarity of the ring C is critical to the inhibitory activity. A similar requirement was also reported for the inhibition of cyclic AMP phosphodiesterase or pancreatic ribonuclease by other heterocyclic compounds (121).

TABLE 6. Inhibitory Effects of Flavanoids on p40 Tyrosine Kinase Activity (121).

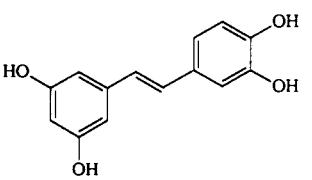


Compound	R_3	R_5	R_7	$R_{3'}$	$R_{4'}$	IC_{50} (μg/ml)
Fustin [**60**]	OH	H	OH	OH	H	$>5 \times 10^2$
Taxifolin [**61**]	OH	OH	OH	OH	OH	3×10^2
Pinocembrin [**62**]	H	OH	OH	H	H	$>5 \times 10^2$
Naringenin [**63**]	H	OH	OH	H	OH	8×10^1
64	H	OH	OH	H	Me	3×10^1
65	H	H	OH	H	H	$>5 \times 10^2$

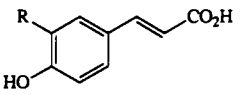
STYRENES.—Through the screening of culture filtrates of actinomycetes, a novel styreneamine analogue, erbstatin [**66**] was isolated from *Streptomyces* sp. as a potent EGF-R kinase inhibitor ($IC_{50} = 0.55$ μg/ml) (117). This discovery has stimulated enormous interest in the synthesis of better analogues (tyrphostins) in recent years (see later section). We recently reported (100) that piceatannol [**67**], previously isolated from seeds of *Euphorbia lagascae* as a stilbene antileukemic agent (122), inhibited p40 ($IC_{50} = 4$ μg/ml), as well as p56^{lck} ($IC_{50} = 20$ μg/ml) kinase activities. In addition, two lignans (**68**, **69**) were also examined for their inhibitory activity against p40 kinase. None of them was active enough for further evaluation ($IC_{50} > 300$ μg/ml).



erbstatin [**66**]



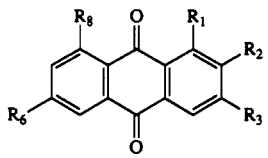
piceatannol [**67**]



coumaric acid [**68**] R=H
ferulic acid [**69**] R=OMe

QUINONES.—We have recently isolated an anthraquinone derivative, emodin [**70**] from the root of *Polygonum cuspidatum* (Polygonaceae), guided by a $p56^{lck}$ kinase inhibition assay (95). A large number of anthraquinones and naphthoquinones have been studied regarding their structural requirements for tyrosine kinase inhibitory activity. The inhibitory activities of several representative analogues are listed in Table 7. It appears that a non-intramolecular hydrogen-bonded hydroxyl group at the β position (**73** vs. **74**) is essential for the activity. Blockage of this hydroxyl group with a methyl (**70** vs. **71**), glucosyl (**70** vs. **72**), or acetyl (**74** vs. **75**) group results in total loss of inhibition.

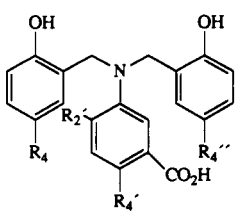
TABLE 7. Inhibitory Effects of Anthraquinones on $p56^{lck}$ Tyrosine Kinase Activity.



Compound	R_1	R_2	R_3	R_6	R_8	IC_{50} (μg/ml)
Emodin [**70**]	OH	H	Me	OH	OH	5×10^{0} (95)
71	OH	H	Me	OMe	OH	$>8\times10^{2}$ (95)
72	OH	H	Me	Glu	OH	$>8\times10^{2}$ (95)
73	OH	H	H	H	H	$>8\times10^{2}$
74	H	OH	H	H	H	2×10^{1}
75	H	OAc	H	H	H	$>8\times10^{2}$

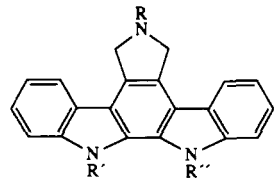
MISCELLANEOUS COMPOUNDS.—*Lavendustins*.—Two novel tyrosine kinase inhibitors, lavendustins A [**76**] and B [**77**], were isolated from *Streptomyces grieseolavendus* by screening about 1000 *Streptomyces* culture filtrates using the EGF-R receptor of the membrane fraction of A431 cells as the enzyme (123). Other lavendustin derivatives and synthetic intermediates were also analyzed (Table 8). Lavendustin A is one of the

TABLE 8. Inhibitory Effects of Lavendustins on EGF-Receptor Tyrosine Kinase Activity (123).



Compound	R_4	$R_{2'}$	$R_{4'}$	$R_{4''}$	IC_{50} (μg/ml)
Lavendustin A [**76**]	OH	H	OH	H	4×10^{-3}
Lavendustin B [**77**]	H	H	OH	H	5×10^{-1}
78	OH	H	OH	OH	1×10^{-2}
79	OH	OH	H	H	2×10^{-1}

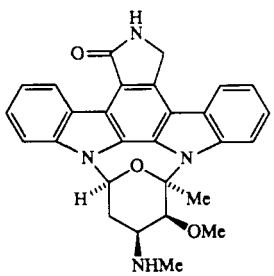
most potent PTK inhibitors that has thus far been isolated from natural sources or chemically synthesized. Preliminary structure-activity studies suggest that the 2,5-dihydroxybenzylamine is an essential fragment for the inhibitory action (123).



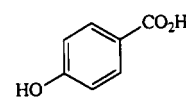
Bisindole alkaloids

Staurosporines.—A series of microbial bisindole alkaloids were recently isolated as antitumor agents (124–130). Staurosporin [**80**] (AM-2282) was the first member of this class of compounds isolated from *Streptomyces staurosporeus* as an antimicrobial agent effective against fungi and yeasts (131). In the course of screening for protein kinase C (PKC) inhibitors from microorganisms (132,133), staurosporin was identified as a potent PKC inhibitor [IC_{50} = 1.3 ng/ml (134); 0.47 ng/ml (135)]. It has also been shown to inhibit $p60^{v\text{-}src}$ (IC_{50} = 3.1 ng/ml) (136) and human neutrophile PTK (IC_{50} = 2.4 ng/ml) (135). Other closely related bisindole alkaloids could be potent PTK inhibitors as well.

Hydroxybenzoic Acid.—In addition to genistein, *p*-hydroxybenzoic acid [**81**] was also isolated as an EGF-R tyrosine kinase inhibitor (IC_{50} = 10 μg/ml) from the fermentation broth of *Pseudomonas* sp. (5). However, Gazit *et al.* (137) later found that it was inactive against the same kinase.



staurosporin [**80**]



p-hydroxybenzoic acid [**81**]

SYNTHETIC INHIBITORS

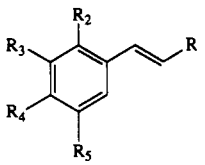
It has been estimated that approximately 50% of the known proteins either bind to or process compounds possessing a phosphoryl group (138). Naturally, it is important to design inhibitors that can selectively block the biological transfer of phosphoryl group catalyzed by specific phosphotransferases (kinases). These inhibitors could not only provide molecular tools through which the biological roles of various kinases may be specifically defined, but also provide a potential therapeutic approach to the diagnosis or treatment of certain diseases. In this review, we will not discuss all known synthetic kinase inhibitors. Many of these have been reviewed by Kenyon and Garcia (139). We will focus on synthetic inhibitors based on the naturally occurring inhibitors aforementioned.

STYRENES.—The discovery of erbstatin [**66**] as a potent EGF-R kinase inhibitor (117) led to the development of a two-step total synthesis from the corresponding benzaldehydes and diethyl(isocyanomethyl)phosphonate by a modified Schollkopf's proce-

dure (140). Several closely related analogues (**86**, **87**, and **90**) showed a comparable inhibitory activity (Table 9) (141), suggesting that a para or ortho dihydroxyphenyl group is essential. It is noted that the corresponding analogue **92** of cinnamic acid is almost as active as erbstatin. Meanwhile, Shiraishi *et al.* (142) showed that several *p*-hydroxycinnamamides **93–96** were also strong tyrosine kinase inhibitors (Table 10).

A series of α-cyano-*p*-hydroxycinnamamides were then prepared for specific inhibition of EGF-R tyrosine kinase (143). The structure-activity studies revealed that the presence of the *p*-hydroxy group and the double bond was required for potent inhibitory activity (Table 10). The strongest inhibitor was α-cyano-3-ethoxy-4-hydroxy-5-phenylthiomethylcinnamamide (ST-638) [**96**] ($IC_{50} = 4 \times 10^{-1}$ μM). The dihydrocinnamamide derivatives were much less active than the corresponding cinnamamide compounds.

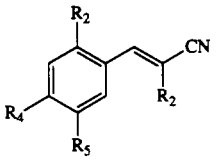
TABLE 9. Inhibitory Effects of Erbstatin Analogues on EGF-Receptor Tyrosine Kinase Activity (141).

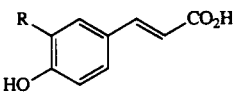


Compound	R	R_2	R_3	R_4	R_5	IC_{50} (μg/ml)
Erbstatin [**66**]	NHCHO	OH	H	H	OH	6×10^{-1}
82	NHCHO	OH	H	H	H	$>1 \times 10^{2}$
83	NHCHO	H	OH	H	H	$>1 \times 10^{1}$
84	NHCHO	H	H	OH	H	$>1 \times 10^{1}$
85	NHCHO	OH	H	OH	H	$>3 \times 10^{1}$
86	NHCHO	OH	OH	H	H	3×10^{-1}
87	NHCHO	H	OH	OH	H	1×10^{0}
88	NHCHO	OH	H	H	OMe	$>6 \times 10^{0}$
89	NHCHO	OH	H	H	Br	$>6 \times 10^{0}$
90	NHCHO	OH	OH	OH	H	8×10^{-1}
91	NHCOMe	OH	H	H	OH	3×10^{0}
92	CO_2H	OH	H	H	OH	8×10^{-1}

The success of these synthetic approaches has prompted further interest in the synthesis of other styrene analogues, termed "tyrphostins," based on the benzylidenemalononitrile nucleus (137,87). Some of the more active inhibitors are listed in Table 11. The *trans*-cinnamonitrile compound **128** and *trans*-hydroxycinnamic acids **68** and **129** are much weaker inhibitors (137). 2,5-Dihydroxy-α-cyano-*cis*-cinnamic acid [**130**] was the only *trans*-cinnamonitrile showing moderate activity ($IC_{50} = 2 \times 10^{1}$ μM) (87). A significant increase (10–15-fold) in potency is observed when two or three phenolic groups are present (compounds **107**, **108**, **120–122**, **124**, and **125**) with the exception of compound **115**. Overall, when a trans substituent ($R_{2'}$, Table 11) is added to the *cis*-cinnamonitrile moiety, a significant increase of inhibitory activity is displayed:

$$C(NH_2)=C(CN)_2 > CSNH_2 > CONH_2 > CN > CO_2H$$



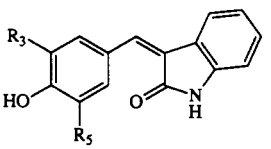


128 $R_{2'}=R_2=R_5=H$, $R_4=OH$ coumaric acid [**68**] R=H
130 $R_{2'}=CO_2H$, $R_2=R_5=OH$, $R_4=H$ caffeic acid [**129**] R=OH

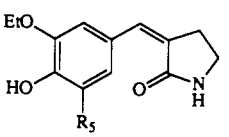
The hydrophobic substituents on the phenyl ring appear to reduce the activity, whereas the hydrophobic substituents of the cinnamamide compounds synthesized by Shiraishi *et al.* (143) enhance the potency.

In order to further evaluate the structural requirements for the aromatic fragment of the benzylidenemalonondinitriles, the hydroxy/methoxyphenyl ring was replaced by another heterocyclic ring or substituted phenyl ring (144). However, none of these analogues ($IC_{50} > 2 \times 10^2$ μM) were as active as the original compounds. In addition, several conformationally constrained benzylidenemalonondinitriles were synthesized to

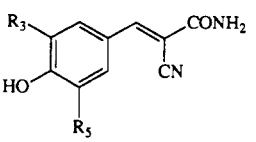
TABLE 10. Inhibitory Effects of Cinnamamides on FGF-Receptor Tyrosine Kinase Activity (142,143).



Compound	R_3	R_5	IC_{50} (μM)
ST-280 [**93**]	*iso*-Pr	*iso*-Pr	$4 \times 10^{-}$
ST-458 [**94**]	CH_3SCH_2	CH_3SCH_2	4×10^{-1}

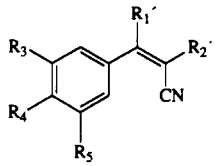


ST-642 [**95**]		$C_6H_5SCH_2$	9×10^{-1}



ST-638 [**96**]	EtO	$C_6H_5SCH_2$	4×10^{-1}
97	EtO	*p*-Me-$C_6H_5SCH_2$	2×10^{1}
98	EtO	*m*-MeO-$C_6H_5SCH_2$	4×10^{0}
99	EtO	$C_6F_5SCH_2$	3×10^{0}
100	EtO	*p*-HO-$C_6H_5SCH_2$	3×10^{0}
101	MeO	$C_6H_5SCH_2$	5×10^{-1}
102	$C_6H_5CH_2O$	$C_6H_5SCH_2$	8×10^{-1}
103	$C_6H_5CH_2$	$C_6H_5CH_2$	6×10^{-1}
104	*tert*-Bu	*tert*-Bu	5×10^{1}
105	*iso*-Pr	*iso*-Pr	1×10^{0}
106	C_6H_5	C_6H_5	4×10^{-1}

TABLE 11. Inhibitory Effects of Benzlidenemalononitriles on EGF-Receptor Tyrosine Kinase Activity (87,137).



Compound	$R_{1'}$	$R_{2'}$	R_3	R_4	R_5	IC_{50} (μM)
107	CN	CN	OH	OH	OH	3×10^0
108	CN	CN	OH	OH	OMe	6×10^0
109	CN	CN	OMe	OH	OMe	9×10^2
RG50810 [**110**] (AG-18)	CN	CN	OH	OH	H	1×10^1
111	CN	CN	OH	H	OH	1×10^1
112	CN	CN	OH	H	H	4×10^2
113	CN	CN	H	OH	H	6×10^2
114	CN	CN	H	CHO	H	6×10^2
115	H	CO_2H	OH	OH	H	3×10^1
116	H	CO_2H	H	OH	H	2×10^2
117	H	CO_2	H	CHO	H	5×10^1
118	OH	CN	OH	OH	OH	1×10^1
119	OH	CN	H	OH	H	5×10^2
120	H	$C(NH_2)=C(CN)_2$	OH	OH	OH	8×10^{-1}
121	H	$C(NH_2)=C(CN)_2$)	OH	OH	OMe	1×10^0
122	H	$C(NH_2)=C(CN)_2$)	OH	OH	Br	5×10^{-1}
123	H	$C(NH_2)=C(CN)_2$)	OH	OH	H	3×10^0
124	H	$C(NH_2)=C(CN)_2$)	H	OH	H	1×10^{-1}
RG50864 [**125**] (AG-213)	H	$CSNH_2$	OH	OH	H	3×10^0
126	H	$CONH_2$	OH	OH	H	1×10^1
127	H	$CONH_2$	H	OH	H	8×10^2

determine the conformational regiospecificity (Table 12). The relative inhibitory activities revealed that the syn compound **131** is more active than the anti compound **132**, and the corresponding isatin derivatives were less potent (144).

Conversion of caffeic acid [**129**] ($IC_{50} = 1 \times 10^3$ μM) into its cyanoamide analogue **126** ($IC_{50} = 1 \times 10^1$ μM) strongly enhanced its inhibitory activity (137). Other amide and keto analogues of *trans*-3,4-dihydroxycinnamonitrile were therefore synthesized to evaluate their relative potencies (Table 13) (137). Overall, these analogues were more active than the parent compound **125**. However, structural modification on the aromatic ring did not bring about much variation in the inhibitory activity, except that compound **150** was totally inactive.

In the course of a search for erbstatin analogues, Traxler *et al.* (89) found that nitrostyrenes were moderately potent inhibitors (Table 14). This pharmacophore was utilized in the design of more potent bisubstrate inhibitors.

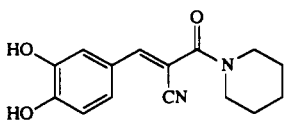


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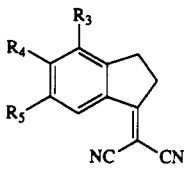
TABLE 12. Inhibitory Effects of Benzylidenemalonondinitriles on EGF-Receptor Tyrosine Kinase Activity (144).



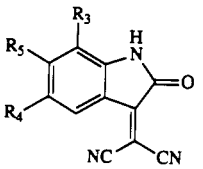
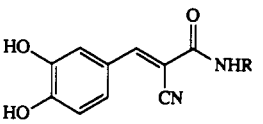
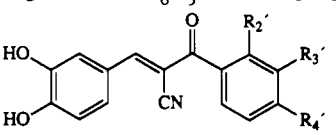
Compound	R_3	R_4	R_5	IC_{50} (μM)
131	H	OH	OH	5×10^{-1}
132	OH	OH	H	7×10^{0}
				
133		OH	OH	3×10^{0}
134		OMe	OH	6×10^{1}
135		OH	H	1×10^{1}
136		H	H	$>3 \times 10^{2}$

TABLE 13. Inhibitory Effects of *trans*-3,4-Dihydroxycinnamonitriles on EGF-Receptor Tyrosine Kinase Activity (137).

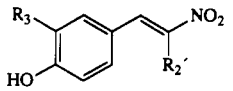


Compound	R	IC_{50} (μM)
126	H	1×10^{1}
137	C_6H_5	7×10^{-1}
AG-490 [**138**]	C_6H_5–CH_2	2×10^{0}
139	C_6H_5–$(CH_2)_2$	9×10^{-1}
140	C_6H_5–$(CH_2)_3$	7×10^{-1}
141	C_6H_5–$(CH_2)_4$	1×10^{-1}
142	C_6H_{11}	2×10^{0}
143	*o*-Cl-C_6H_5	1×10^{1}



	$R_{2'}$	$R_{3'}$	$R_{4'}$	
144	H	H	H	1×10^{0}
145	Cl	H	H	2×10^{0}
146	H	H	NO_2	2×10^{0}
147	'H	H	Me	9×10^{-1}
148	H	H	F	6×10^{-1}
149	H	OH	OH	4×10^{-1}

TABLE 14. Inhibitory Effects of Nitrostyrenes on EGF-Receptor Tyrosine Kinase Activity (89).



Compound	$R_{2'}$	R_3	IC_{50} (μM)
151	H	H	1×10^1
152	H	OH	9×10^0
153	H	OMe	8×10^0
154	Me	OH	9×10^0

A series of pyridine-containing stilbene and amide derivatives based on the structure of piceatannol [**67**] were recently prepared and tested for inhibition of $p56^{lck}$ tyrosine kinase (92) (Figure 1). None of these compounds was highly active. The strongest inhibitor (**151**, IC_{50} 2×10^2 μM) was a competitive binder with respect to ATP. Thirty-three phenylhydrazone derivatives of polyhydroxylated benzaldehydes were also synthesized and tested as $p56^{lck}$ inhibitors (93) (Figure 2). None of these compounds was as active as the parent compound, piceatannol. The most active compound (**152**, IC_{50} 7×10^1 μM) was a competitive inhibitor of $p56^{lck}$ with respect to the peptide substrate.

FLAVONOIDS.—A series of 23 isoflavonoids based on the structure of genistein [**3**] were prepared by modifications at the C-2 position and tested for the inhibition of EGF-R tyrosine kinase (115). However, none of these synthetic compounds was more potent

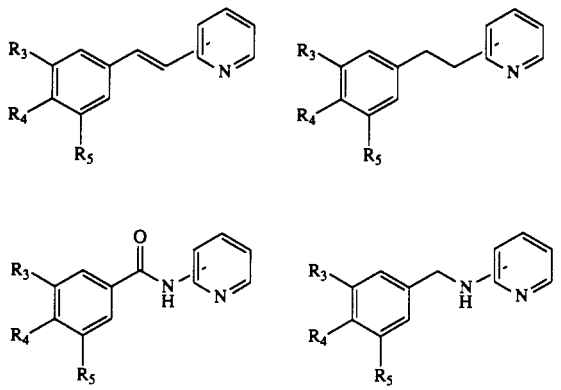
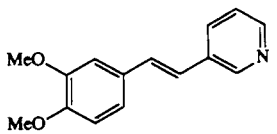


FIGURE 1. Potential protein-tyrosine kinase inhibitors designed on the basis of piceatannol [**67**] (92), a naturally occurring protein-tyrosine kinase inhibitor. R_3, R_4, R_5 = OH, OMe, or H.



151

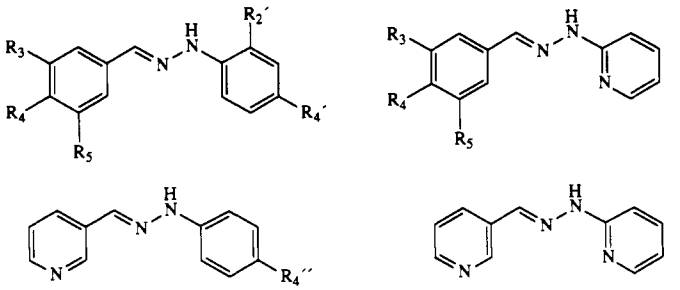


FIGURE 2. Phenylhydrazone derivatives of piceatannol [**67**] prepared for inhibition of protein-tyrosine kinases (93). R_3,R_4,R_5 = OH, OMe, NO_2, Br, or H; $R_{2'},R_{4'}$ = OMe, NO_2, Br, or H; $R_{4''}$ = Br or H.

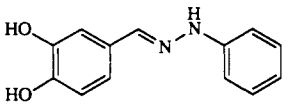


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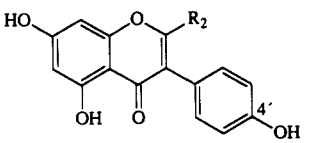
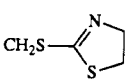
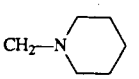
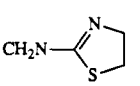
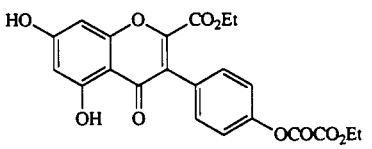
TABLE 15. Inhibitory Effects of Synthetic Isoflavonoids on EGF-Receptor Tyrosine Kinase Activity (145).



Compound	R_2	IC_{50} (μg/ml)
Genistein [**3**]	H	7×10^{-1}
153	Me	2×10^{0}
154	CO_2H	2×10^{0}
155	CO_2Me	2×10^{-0}
156	CO_2Et	2×10^{0}
157	CO_2*iso*-Pr	1×10^{1}
158	$CONH_2$	1×10^{1}
159	CH_2SMe	5×10^{0}
160		5×10^{0}
161	$CH_2SCH_2CO_2Et$	4×10^{0}
162		1×10^{1}
163		2×10^{1}

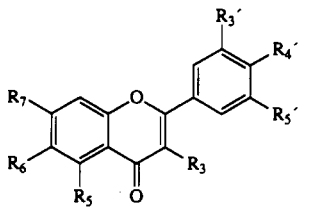
than genistein. It appears that variations of the C-2 substituent do not produce much change in their relative potencies (Table 15). Removal of the acidic hydrogen of the C-4′ phenolic group drastically reduced the activity with the exception of compound **164** (IC_{50} = 1 μg/ml).



164

Various alkyl-(2-hydroxyaryl)-3-oxopropanoates were transformed into a series of 3-(alkoxy-carbonyl)-2-arylflavones, which were used to prepare a variety of flavonoids (91, Figure 3). They were tested for inhibition of $p56^{lck}$ tyrosine kinase (91). It is interesting to notice that replacement of a hydroxyl at C-4′ with an amino group enhanced the inhibitory activity (Table 16). The most active inhibitor (**167**) was approximately 10-fold more potent than the natural flavonol, quercetin [**1**].

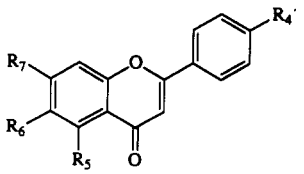
PEPTIDES.—*Irreversible inhibitors.*—In certain cases, protease inhibitors are capable of selectively inhibiting the growth of transformed cells or restoring them to the



$R_{4'}$ = OH, OMe, NO_2, NH_2, NHAc or Br
$R_{3'}$,$R_{5'}$ = H or OMe
R_3 = H, CO_2H or CO_2Me
R_5,R_6,R_7 = H, OH or OMe

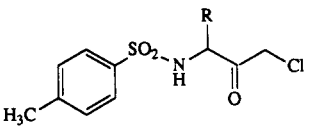
FIGURE 3. Synthetic flavonoid analogues tested for the inhibition of protein-tyrosine kinase inhibitors (91).

TABLE 16. Inhibitory Effects of Synthetic Flavones on $p56^{lck}$ Tyrosine Kinase Activity (91).



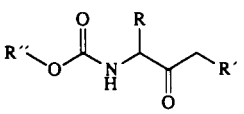
Compound	R_5	R_6	R_7	$R_{4'}$	IC_{50} (μM)
165	H	H	H	NH_2	2×10^2
58	H	H	H	OH	5×10^2
166	H	H	OH	NH_2	1×10^2
54	H	H	OH	OH	2×10^2
167	H	OH	H	NH_2	1×10^0
51	H	OH	H	OH	1×10^2
168	OH	H	OH	NH_2	7×10^0
Apigenin [**38**]	OH	H	OH	OH	2×10^1

phenotypic states of normal cells (146,147). Richert *et al.* (148) found that the protease inhibitor, *N*-α-tosyl-L-lysyl chloromethyl ketone (TLCK) **[169]** inhibited the transformation-specific kinase activity associated with $p60^{v\text{-}src}$ and induced the phenotypic reversion of avian sarcoma virus-transformed cells to normal. The phenylalanine analogue (TPCK) **[170]** was less active at a low concentration (0.03 mM), but was too toxic at high concentration. Other serine protease inhibitors had no effect on kinase activity.



TLCK **[169]** R=$(CH_2)_4\overset{+}{N}H_3$
TPCK **[170]** R=$CH_2C_6H_5$

A series of halomethyl ketone derivatives of amino acids and peptides were then selected to specifically inhibit tyrosine kinase activity in membranes from A431 epidermoid carcinoma cells (149). *N*-α-*tert*-butoxycarbonylleucyl bromomethyl ketone (Boc-Leu-CH_2Br) **[171]** and *N*-α-benzyloxycarbonylalanine chloromethyl ketone (Z_1-Ala-CH_2Cl) **[172]** were the most effective inhibitors (Table 17). Boc-Leu-CH_2Br was found to inhibit the EGF-R, $p60^{src}$ and $p130^{fps}$ kinases.



	R	R′	R″
Boc-Leu-CH_2Br **[171]**	*i*-Bu	Br	*t*-Bu
Z-Ala-CH_2Cl **[172]**	Me	Cl	Bn

TABLE 17. Inhibitory Effects of Halomethyl Ketone Derivatives (400 nmole/mg protein) on Protein Phosphorylation in Triton Extracts of Plasma Membranes of A431 Cells (149).

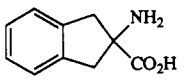
Compound[a]	Inhibition (%)	
	without EGF	with EGF
Boc-Leu-CH_2Br **[171]**	69	65
Z-Ala-CH_2Cl **[172]**	44	71
Ac-Phe-Gly-Ala-Leu-CH_2Cl **[173]**	29	59
Z-Leu-CHN_2 **[174]**	18	15
Z-Phe-CH_2Cl **[175]**	15	21
Z-Trp-CH_2Cl **[176]**	10	6
Ac-Ala-Ala-Phe-Thr-CH_2Cl **[177]**	10	4
Ac-Gly-Gly-Ala-Phe-CH_2Cl **[178]**	0	5
Leu-CH_2Cl **[180]**	0	16

[a]Boc, *t*-butyloxy; Z, benzyloxy.

Reversible inhibitors.—Autophosphorylation on tyrosine of $p60^{v\text{-}src}$ is important for its transforming activity. Numerous peptide fragments with sequences based on this site of autophosphorylation have been used as simple substrates for a variety of protein-tyrosine kinases. These have been reviewed recently by Geahlen and Harrison (8).

Wong and Goldberg found that [Val^5]angiotensin II ([Val^5]AT-II) **[181]** was phosphorylated by $p60^{src}$, whereas the dehydrophenylanine analogue, [ΔPhe^4]AT-II **[184]**, inhibited the phosphorylation of [Val^5]AT-II (K_i = approximately 0.7 mM) (101). The nitrotyrosine **[182]** and 2-aminoindanecarboxylic acid (Ain) **[183]** analogues were inactive as inhibitors.

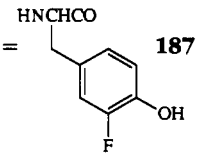
AspArgValTyrValHisProPhe [Val^5]AT-II [**181**]
AspArgVal(NO_2-Tyr)ValHisProPhe [**182**]
AspArgValAinValHisProPhe [**183**]
AspArgVal(ΔPhe)ValHisProPhe[ΔPhe^4]AT-II [**184**]

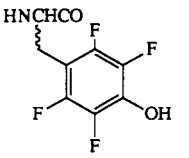


Ain

Baldwin *et al.* (150) utilized human gastrin undecapeptide analogue of residues 22–30 [**185**] as a substrate of the EGF receptor tyrosine kinase. This peptide was later found to be a good substrate of the insulin receptor tyrosine kinase as well (111). The tyrosine-containing peptide was phosphorylated with higher V max and K_m values than the 3-fluorotyrosine-containing peptide **186**, suggesting that ionization of the tyrosyl hydroxyl group is a critical event in the phosphorylation reaction. Based on this discovery, two analogues **187D** and **187L** containing a D- and L-tetrafluorotyrosyl residue, respectively, were then prepared and were found to be inhibitors with K_i values in the μM range [K_i = 20 (**187D**) and 4 (**187L**) μM] (151).

ArgArgLeuGluGluGluGluGluAla-X-Gly

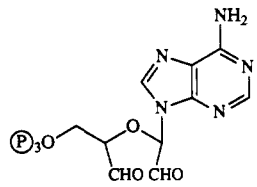
185 X=Tyr **186** X= 

187 X= 

In addition to angiotensin and gastrin peptides, several synthetic random polypeptides containing tyrosine, glutamic acid, alanine, and lysine (such as Glu^{80}, Tyr^{20}) also serve as inexpensive substrates (114). Some of these synthetic polypeptides with ordered sequences were found to be moderate inhibitors (114).

The kinase activity of $p60^{v\text{-}src}$ also appears to be regulated by a region located within the noncatalytic SH2 (*src* homology 2, residues 140–250) domain. A 21-residue synthetic peptide **188**, corresponding to residues 137–157, was found to selectively inhibit the tyrosine kinase activity of $p60^{v\text{-}src}$ (IC_{50} = 7.5 μM) (152). The major site of tyrosine phosphorylation of $p60^{c\text{-}src}$ is located near its carboxyl terminus. Phosphorylation of $p60^{c\text{-}src}$ at tyrosine-527 suppresses its tyrosine kinase activity and transforming potential. A highly transforming mutant, $p60^{c\text{-}src(F527)}$, was established by replacing tyrosine-527 with phenylanine (153). A tridecapeptide corresponding to the C terminus containing phosphorylated tyrosine (**189**) completely inhibited the phosphorylation of $p60^{c\text{-}src(F257)}$ in detergent-insoluble, cytoskeletal matrix preparation, and immune complexes (154).

ADENOSINE 5′-TRIPHOSPHATE ANALOGUES.—*Irreversible inhibitors.*—Adenosine nucleotides with reactive *p*-fluorosulfonylbenzoyl, azido, aldehyde, or halomethylketone groups have often been used to irreversibly modify nucleotide binding proteins (155). Van Obberghen *et al.* (156) determined the ATP-binding and autophosphorylation site of the hepatic insulin receptor induced by insulin using the dialdehyde analogue **190** of ATP. The resulting Schiff's base was further stabilized by treatment with $NaCNBH_3$. A photoaffinity analogue of ATP, 3′-*O*-{3-[*N*-(4-azido-2-nitrophenyl)-amino]propionyl} 5′-triphosphate [**191**] was capable of complete inactivation of the EGF-R tyrosine kinase from A431 cells (157). In addition, 5′-[4-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) [**192**] was shown to react irreversibly with lysine residue at the active site of $p60^{v\text{-}src}$ (158) and the EGF-R (159).



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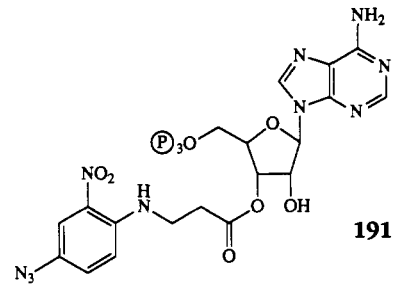

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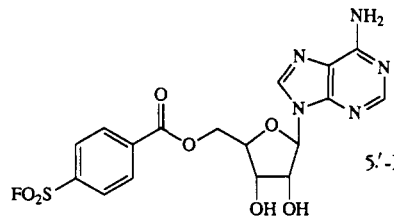
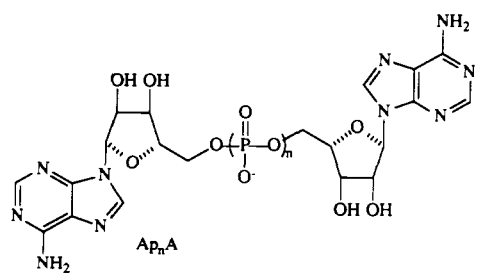


5′-FSBA [**192**]

BISUBSTRATE INHIBITORS.—*Reversible inhibitors.*—Highly coherent interactions between two co-substrates, protein and ATP, are essential for the optimal catalytic function of tyrosine kinases. Bisnucleoside oligophosphate analogues have been shown to enhance remarkably the binding potentials in early studies of ATP-binding proteins, such as adenylate kinase (160,161) and thymidylate kinase (162). Diadenosine 5′-oligophosphates, Ap_nA ($n = 2$–6), inhibited phosphorylation of IgG from tumor-bearing rabbits by $p60^{v\text{-}src}$ purified from Rous sarcoma virus-transformed rat tumor cells (104). The concentration of Ap_4A [**193**] required for inhibition of the viral $p60^{v\text{-}src}$ kinase ($IC_{50} = 1$ μM) was found to be significantly lower than that required for inhibition of the cellular $p60^{c\text{-}src}$ kinase ($IC_{50} = 46$ μM), indicating that the viral and cellular $p60^{src}$ kinases are functionally distinguishable, which is an essential observation from the point of view of drug design (163). Viral and cellular $p60^{src}$ kinases differed to a lesser extent with respect to inhibition by other nucleotides (Table 18) (163). One of the adenosines presumably binds to the phosphodonor site for ATP. The second adenosine binding site has not been clearly defined.

A true bisubstrate tyrosine kinase inhibitor should consist of ATP and a tyrosine analogue. Structural elements required for recognition of tyrosine for the endogenous substrates of tyrosine kinase are not known. The first phosphotyrosine mimics used in the design of bisubstrate inhibitors were based on the aminophenyl functionality. The linkage between adenosine and the tyrosine mimics is formed via an ester functionality, mimicking the phosphate group. Kruse *et al.* (105) prepared a series of analogues of 5′-



Ap_nA

	n	Ki (μM)
Ap_4A [**193**]	4	15
Ap_6A [**194**]	6	9

TABLE 18. Inhibitory Effects of Nucleotides on $p60^{src}$ Tyrosine Kinase Activity (163).

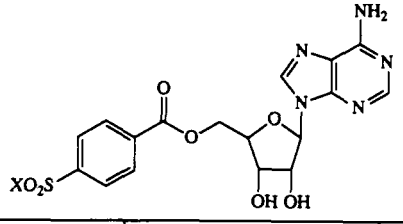
Kinase[b]	IC_{50} (μM)[a]			
	Ap_4A [193]	Gp_4G [195]	Ap_4 [196]	ADP
$p60^{v\text{-}src}$	1×10^0	$>10^2$	3×10^0	3×10^{-1}
$p60^{c\text{-}src}$	5×10^1	$>10^2$	9×10^0	1×10^{-1}

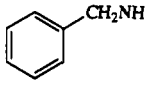
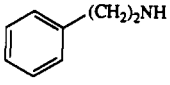
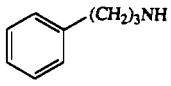
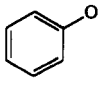
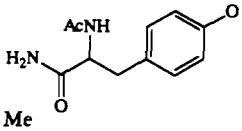
[a]Ap_4A, di(adenosine-5′)teraphosphate; Gp_4G, di(guanosine-5′)tetraphosphate; Ap_4, adenosine 5′-tetraphosphate; ADP, adenosine 5′-diphosphate.

[b]$p60^{v\text{-}src}$, viral $p60^{src}$; $p60^{c\text{-}src}$, cellular $p60^{src}$.

FSBA and tested for their inhibitory effect on $p60^{v\text{-}abl}$, the tyrosine kinase encoded by the transforming gene (v-*abl*) of the Abelson murine leukemia virus (Table 19). This series of inhibitors displayed moderate activity and did not behave as bisubstrate inhibitors. They probably only bound to the ATP-binding site regardless of structural

TABLE 19. Inhibitory Effects of Sulfonylbenzoyl Adenosine Analogues on $p60^{v\text{-}abl}$ Tyrosine Kinase Activity (105).

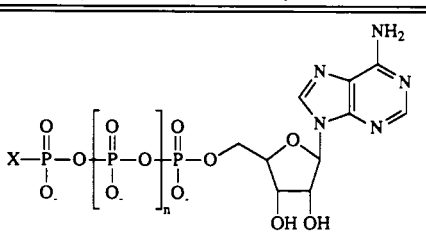


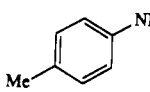
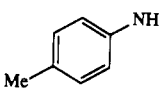
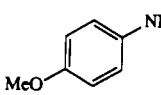
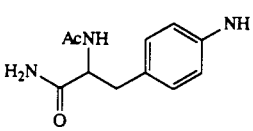
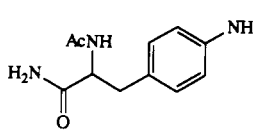
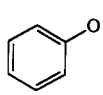
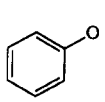
Compound	X	IC_{50} (μM)
197	F	2×10^2
198	NH_2	1×10^2
199		2×10^1
200		5×10^1
201		5×10^1
202		6×10^1
203		2×10^2
204		1×10^2
205	Me	6×10^1

differences in the aminophenyl groups. Therefore, several different tyrosine mimics were attached to 5′-adenosine through either a phosphoramidate or phosphate linkage using a triphosphate or tetraphosphate linker as shown in Table 20 (106). However, structural modifications of the tyrosine mimic had little effect on potency, suggesting that further search of the structural features required for recognition of the tyrosine-containing substrate is needed.

Erbstatin [**66**] has been demonstrated to be a strong inhibitor of protein-tyrosine kinases. It is competitive with the peptide substrate and non-competitive with ATP. In the course of a search for erbstatin analogues, nitrostyrenes were found to be moderately potent inhibitors of the EGF-R tyrosine kinases **151–154** (Table 14) (89). This suggested that nitrostyrenes may serve as tyrosine mimics. In combination with sul-

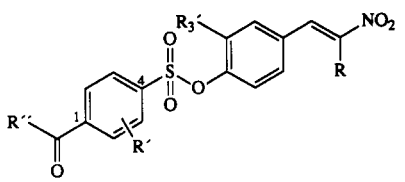
TABLE 20. Inhibitory Effects of Adenosine Oligophosphates on $p60^{v\text{-}abl}$ Tyrosine Kinase Activity (106).



Compound	X	n	IC_{50} (μM)
206		1	2×10^2
207		2	2×10^1
208		1	9×10^1
209		1	4×10^1
210		2	1×10^2
211	$AcNHCH_2CH_2NH$	1	3×10^1
212		1	1×10^1
213		2	4×10^1

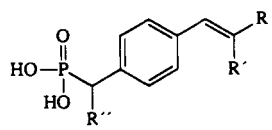
fonylbenzoyl (ATP mimic) functionality, a series of bisubstrate inhibitors were prepared and tested for their inhibitory activities against EGF-R and $p60^{v\text{-}abl}$ tyrosine kinases. The most potent compound (**216**) selectively inhibited the EGF-R tyrosine kinase with an IC_{50} value of 50 nM (Table 21). Its 3′-hydroxyl analogue **217** was a strong inhibitor ($IC_{50} = 300$ nM) as well. Computer-assisted molecular modeling studies based on the transition state of the γ-phosphoryl transfer from ATP to a tyrosine moiety and molecular fitting analysis for the inhibitor **216** supported the hypothesis that the sulfonylbenzoyl group mimics a diphosphate fragment in the transition state. The adenosine adducts **221** and **222** also show highly selective inhibitory activity against EGF-R tyrosine kinase, with IC_{50} values around 500 nM compared to the activity against $p60^{v\text{-}abl}$ with IC_{50} values ≥ 100 μM (Table 21).

TABLE 21. Inhibitory Effects of Nitrostyrenes on Tyrosine Kinase Activity (89).

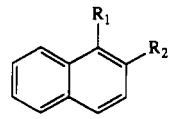


Compound	R	$R_{3'}$	R′	R″	IC_{50} (μM)	
					EGF-R	v-*abl*
214 . . .	H	H	H	OH	1×10^{0}	10^{2}
215 . . .	H	OMe	H	OH	3×10^{0}	4×10^{1}
216 . . .	H	H	2-OH	OH	5×10^{-2}	3×10^{1}
217 . . .	H	H	3-OH	OH	3×10^{-1}	10^{2}
218 . . .	Me	OMe	H	OH	3×10^{0}	
219 . . .	H	H	H	OMe	2×10^{0}	$>10^{2}$
220 . . .	H	H	2-OH	OMe	2×10^{0}	$>10^{2}$
221 . . .	H	H	H	Adenosine-5′	6×10^{-1}	$>10^{2}$
222 . . .	H	OMe	H	Adenosine-5′	5×10^{-1}	10^{2}
223 . . .	H	H	H	NHMe	4×10^{-1}	4×10^{1}
224 . . .	H	H	H	NH-*n*-Bu	5×10^{0}	$>10^{2}$
225 . . .	H	H	H	NH-*i*-Pro	1×10^{0}	$>10^{2}$
226 . . .	Me	OMe	H	NHMe	4×10^{0}	
227 . . .	H	H	H	$NH(CH_2)_2NHCOO$-*t*-Bu	1×10^{0}	$>10^{2}$
228 . . .	H	H	H	$NH(CH_2)_2NH_2 \cdot HCl$	5×10^{0}	5×10^{0}
229 . . .	H	H	H	L-Ala-Ala-O-*t*-Bu	4×10^{1}	$>10^{2}$
230 . . .	H	H	H	L-Ala-Ala-OH	7×10^{0}	4×10^{1}
231 . . .	H	H	H	$L\text{-Ala-Ala-NH}(CH_2)_2NH\text{-BOC}$	$>5 \times 10^{1}$	
232 . . .	H	H	H	$L\text{-Ala-Ala-NH}(CH_2)_2NH_2 \cdot HCl$	2×10^{1}	2×10^{1}

PHOSPHONATE INHIBITORS.—Various styrene derivatives have been proposed as conformationally constrained analogues of tyrosine. In order to further enhance their binding affinity, several methylphosphate (**233–235**) and (hydroxymethyl)phosphonate (**236–238**) derivatives were prepared as hydrolytically stable mimics of phosphotyrosine (97). However, none of these compounds were active as kinase inhibitors. A series of phosphonate and sulfonate analogues of naphthalene (**239–244**) were also synthesized. Only (2-naphthalenylhydroxymethyl)phosphonic acid showed marginal activity against EGF-R tyrosine kinase (**240**, $IC_{50} = 3 \times 10^{2}$ μM) (97).



	R	R′	R″
233	CN	CN	H
234	$CONH_2$	CN	H
235	$CONH_2$	$CONH_2$	H
236	CN	CN	OH
237	$CONH_2$	CN	OH
238	$CONH_2$	$CONH_2$	OH

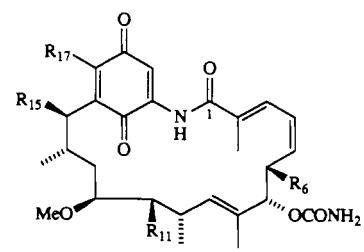


	R_1	R_2
239	$CH(OH)PO(OH)_2$	H
240	H	$CH(OH)PO(OH)_2$
241	$PO(OH)_2$	H
242	H	$PO(OH)_2$
243	$SO(OH)_2$	H
244	H	$SO(OH)_2$

ANTITUMOR ACTIVITIES

It has been recognized that the use of a single antileukemic screening system could bias the end results and lead to the isolation of compounds active mainly in the chemotherapy of fast growing tumors. NCI has developed a "disease-oriented" approach based on antitumor cytotoxicity against human tumor cell line panels (Table 22) consisting of approximately 60 cell lines of major human tumors (lung, colon, CNS, skin, ovary, and kidney tumors, in addition to leukemia) (1,164). Compounds showing differential cytotoxicity for specific tumor types will be followed up with in vivo xenograft testing using the same sensitive cell lines. However, this cytotoxicity-based model may not be suitable for the evaluation of antitumor efficacy of the mechanism-based, non-cytotoxic compounds, such as emodin. Emodin [**70**] was a strong $p56^{lck}$ tyrosine-kinase inhibitor isolated from *Polygonum cuspidatum* (95). However, its human tumor cytotoxicity profiles (Table 22) showed only moderate cytostatic activity based on the 50% growth inhibition values (GI_{50}), and non-cytotoxicity based on the 50% lethal concentration values (LC_{50}). It appears that a new approach must, therefore, be envisioned to determine the antitumor potential of protein-tyrosine kinase inhibitors with weak cytotoxicity.

REVERSION OF TRANSFORMATION.—Richert *et al.* (148) first found that the protease inhibitor *N*-α-tosyl-L-lysyl chloromethylketone (TLCK) [**169**] inhibited $p60^{v\text{-}src}$ and induced the reversion of the cell morphology of avian sarcoma virus-transformed fibroblasts to normal. During the course of searching for natural products converting the morphology of Rous sarcoma virus-infected rat kidney cells to normal, an active constituent produced by *Streptomyces* sp. (MH237-CF8) was identified as herbimycin A [**245**] (165), a benzoquinoid anasmycin which had previously been isolated as a herbicide (166). Two other benzoquinoid ansamycins, macbecin [**246**] and geldanamycin [**247**], were also found to induce the phenotypic change from *src*-transformed to normal



	R_6	R_{11}	R_{15}	R_{17}
herbimycin A [**245**]	OMe	OMe	OMe	H
macbecin I [**246**]	Me	OMe	OMe	H
geldanamycin [**247**]	OMe	OH	H	OMe

TABLE 22. Cytotoxicity Profiles of Emodin Against Human Tumor Cell Panels.[a]

Panel/Cell Line	$Log_{10} GI_{50}$[b]	$Log_{10} LC_{50}$[b]
Leukemia		
CCRF-CEM	−4.42	>−4.00
HL-60 (TB)	−4.57	>−4.00
MOLT-4	−4.49	>−4.00
RPMI-8226	−4.61	>−4.00
SR	−4.63	>−4.00
Non-Small-Cell Lung Cancer		
A549/ATCC	−4.57	>−4.00
HOP-18	−5.40	>−4.00
HOP-62	−5.09	>−4.00
HOP-92	−4.94	>−4.00
NCI-H226	−4.48	>−4.00
NCI-H23	−4.84	>−4.00
HCI-H322M	−4.45	>−4.00
NCI-H460	−4.70	>−4.00
NCI-H522	−4.84	>−4.00
LXFL-529L	−4.61	>−4.00
Small Cell Lung Cancer		
DMS 114	−4.39	>−4.00
DMS 273	−4.70	>−4.00
Colon Cancer		
COLO 205	−4.75	−4.00
DLD-1	−4.63	>−4.00
HCC-2998	−4.49	>−4.00
HCT-116	−4.44	>−4.00
HCT-15	−4.57	>−4.00
HT29	−4.50	>−4.00
KM12	−4.58	>−4.00
KM20L2	−4.39	>−4.00
SW-620	−4.63	>−4.00
CNS Cancer		
SF-268	−4.59	>−4.00
SF-295	−4.55	>−4.00
SNB-19	−4.52	>−4.00
SNB-75	−4.59	>−4.00
SNB-78	−4.31	>−4.00
U251	−4.56	>−4.00
XF 498	−4.58	>−4.00
Melanoma		
LOX IMVI	−4.77	>−4.00
MALME-3M	−4.93	>−4.00
M14	−4.60	>−4.00
SK-MEL-2	−4.69	>−4.00
SK-MEL-28	−4.44	>−4.00
SK-MEL-5	−5.58	−4.29
UACC-257	−4.55	>−4.00
UACC-62		
Ovarian Cancer		
IGROV1		
OVCAR-4	−4.52	>−4.00
OVCAR-5	−4.42	>−4.00
OVCAR-8		
SK-OV-3	−4.63	>−4.00

TABLE 22. Continued.

Panel/Cell Line	$Log_{10} GI_{50}$[b]	$Log_{10} LC_{50}$[b]
Renal Cancer		
786-0		
A498	−4.76	>−4.00
ACHN	−4.78	>−4.00
CAKI-1	−4.72	>−4.00
RXF-393	−4.34	>−4.00
RXF-631	−4.41	>−4.00
SN12C	−4.65	>−4.00
TK-10	−4.17	>−4.00
MG MID	−4.63	−4.01
Delta	0.95	0.28
Range	1.41	0.29

[a]Provided by the National Cancer Institute Developmental Therapeutics Program through Dr. Matthew Suffness.

[b]GI_{50} and LC_{50} values are defined by M.R. Boyd, K.D. Paull, and L. Rubinstein in "Antitumor Drug Discovery and Development," ed. by F.A. Valeriote, T. Corbett, and L. Baker, Kluwer Academic Publishers, Amsterdam, Netherlands, pp. 11–34 (1992).

morphologies, and to reduce the intracellular phosphorylation of $p60^{v\text{-}src}$ (167). Other non-benzoquinoid ansamycins, ansamitocin P-3, streptovaricin, and rifamycin did not produce these effects. The immune complex formed by mixing the herbimycin-A-treated cell extracts with monoclonal antibody against $p60^{v\text{-}src}$ was inactive in vitro as measured by autophosphorylation. However, the immune complex produced from untreated cell extracts was active in vitro in the presence of herbimycin A, suggesting that the benzoquinoid ansamycins might not directly act on the $p60^{src}$ tyrosine kinase in situ (167).

In order to further probe the inhibitory specificity of tyrosine kinase oncogenes by herbimycin A, the effectiveness of herbimycin A to reverse the morphology of chicken and mammalian cells transformed by various oncogenes was investigated (Table 23) (168,169). It was demonstrated that this antibiotic was effective for cells transformed by the PTK oncogenes *src, yes, fps, ros, abl, erb*B, and was unable to reverse the transformed morphologies induced by the non-PTK oncogenes *ras, raf,* and *myc*. Herbimycin A also caused *src*-expressed cells to become sensitive to contact inhibition but did not affect *ras*-expressing cells (170). Yamaki *et al.* (171) found that geldanamycin and herbimycin A inhibited the expression of c-*myc* and stimulated the expression of the p53 tumor suppressor gene. Treatment of *src*-transformed cells with herbimycin A resulted in a reduction in the phosphotyrosine content of total cellular proteins, 36K protein phosphorylation, and autophosphorylation of the tyrosine-416 of $p60^{v\text{-}src}$ (168,172). Measurement of the rate of $p60^{src}$ synthesis and degradation showed that herbimycin A accelerated the degradation of $p60^{src}$ (172). In addition, herbimycin A was recently shown to inhibit $p60^{v\text{-}src}$ irreversibly in an in vitro immune complex kinase assay ($IC_{50} = 7$ μg/ml). Addition of a sulfhydryl compound abolished its inhibitory activity (173). On the contrary, recent studies using the HT-29 human colon adenocarcinoma cell line showed that growth and $p60^{c\text{-}src}$ inhibition were reversible following removal of herbimycin A (174).

Recently, erbstatin [**66**], IC_{50} (EGF-R tyrosine kinase) $= 2 \times 10^{-1}$ μg/ml (175), and its synthetic analogue methyl 2,5-dihydroxycinnamate [**248**], IC_{50} (EGF-R tyrosine kinase) $= 2 \times 10^{-1}$ μg/ml (176), were also shown to induce morphological changes in temperature-sensitive Rous sarcoma virus-transformed rat kidney cells to

TABLE 23. Effects of Herbimycin A on the Morphology of Mammalian Cells Transformed by Various Oncogenes (168).

Oncogene	Cell Transformed[a]	Morphological Reversion
src	NRK	++
	NIH/3T3	++
	3Y1	++
	Field vole	++
	DDD	+
fps	3Y1	++
abl	NIH/3T3	+
	Balb/c	++
raf	NIH/3T3	−
K-*ras*	NRK	±
H-*ras*	NIH/3T3	−
	3Y1	−
myc	3Y1	−
	C3H10T 1/2	±
	A431	+

[a]NRK, normal rat kidney; NIH/3T3, Swiss mouse fibroblast; 3Y1, Fischer rat fibroblast; DDD, mouse fibroblast ascites tumor; Balb/c and C3H10T 1/2, mouse embryonic fibroblast; A431, human epidermoid carcinoma.

the morphology of normal cells (175). Erbstatin did not change the morphology of either normal or K-*ras*-transformed cells (175).

INDUCTION OF DIFFERENTIATION.—The human chronic myelogenous leukemic (CML) cell line K562 and leukemic cells of patients with CML with an accompanying chromosomal translocation (t9; 22) express a structurally altered c-*abl* protein ($p120^{c\text{-}abl}$) with elevated tyrosine kinase activity (177,178). A non-cytotoxic concentration of herbimycin A [**245**] was found to induce erythroid differentiation of K562 concomitant with a rapid reduction in the tyrosine phosphorylation of $p120^{c\text{-}abl}$ but not of other human myeloid leukemia cell lines (HL-60, THP-1, and U937). In addition, K562 cells were the most sensitive to the cytotoxic effect of herbimycin A ($IC_{50} = 9.5 \times 10^{-2}$ μM) among the human leukemia cell lines tested ($IC_{50} > 1$ μM) (179). Kondo *et al.* (180) also found that herbimycin A could induce endoderm differentiation of murine embryonal carcinoma (F9) cells and terminal erythroid differentiation of murine erythroleukemia (MEL) cells. These results suggest that tyrosine phosphorylation may play an important role in the regulation of tumor cell differentiation.

Recently, another natural protein-tyrosine kinase inhibitor, genistein [**3**], was shown to induce differentiation of MEL cells, and it was found that it differs from the differentiation induced by other agents, such as DMSO and hexamethylenebisacetamide (HMBA) (181). Morphological studies showed that genistein induced differentiation of myeloblastic ML-1 cells into promyelocytes and of HL-60 cells into mature granulocytes (182). Psi-tectorigenin [**4**] (8-methoxygenistein) was more effective than genistein (182). On the contrary, methyl 2,5-dihydroxycinnamate [**248**] was only a weak inducer (182).

COMBINATION WITH OTHER AGENTS.—Erbstatin [**66**] is weakly cytotoxic toward A 431 human epidermal carcinoma, Rous sarcoma virus-infected rat kidney, and L1210 murine leukemic cells (GI_{50} = approximately 3 μg/ml) (183). It showed no in vivo antitumor effect on L1210 mouse leukemia when it was injected alone. Adminis-

tration of erbstatin with foroxymithine (a potent chelator for ferric ion) showed moderate activity against L1210 leukemia (T/C% = 155) (183). In order to investigate the induction of tumor cell differentiation by protein-tyrosine kinase inhibitors, Watanabe *et al.* (184) showed that genistein [**3**] and ST-638 [**96**] induced differentiation of murine erythroleukemia cells in a synergistic manner with an agent that blocks DNA synthesis, such as mitomycin C. A non-toxic concentration of herbimycin A [**245**] also enhanced the cytotoxicity of adriamycin or 1-β-D-arabinofuranosylcytosine against K562 cells (179).

Quercetin [**1**] and staurosporin [**80**] are potent inhibitors of both protein-tyrosine kinases and protein kinase C. A synergistic effect was observed on the inhibition of cell proliferation by a combination of *cis*-diaminedichloroplatinum (II) (cisplatin) with quercetin or staurosporin (185). Grunicke *et al.* (185) further demonstrated the enhancement of the antitumor effect of cisplatin by quercetin against human large cell lung carcinoma (LXFG 529) in athymic mice.

CONCLUSION

A specific screening approach based on the inhibition of protein-tyrosine kinases has led to the discovery of several structurally distinct classes of inhibitors, including quinones, styrenes, flavonoids, and indole alkaloids, from natural sources. These natural inhibitors have served as valuable leads for further design of better analogues. Many of these inhibitors have also been used in probing the molecular and cellular mechanisms involved in the protein-tyrosine kinase mediated signal transduction. While it is evident that biochemical mechanism-based screenings will undoubtedly uncover many structurally unique and biologically interesting compounds from natural sources, it remains a challenging problem to design an appropriate in vivo model for evaluating the antitumor efficacy of many non-cytotoxic PTK inhibitors. Thus far, the discovery of benzoquinoid ansamycins as *src* PTK inhibitors and their selective induction of the phenotypic change from *src*-transformed to normal-like morphologies have prompted NCI to initiate preclinical studies on geldanamycin [**247**] and macbecin II (dihydromacbecin I) [**246**].

An important challenge for the future will be the design or discovery of inhibitors that exhibit a high degree of selectivity. Such agents would function not only as general PTK inhibitors but would discriminate between specific types or classes of PTKs. The relative potencies of various inhibitors reported in the literature are affected by assay conditions including type of substrate, concentration of substrate and cofactors, and presence of contaminating kinase or phosphatase activities in the enzyme preparation. Since there is currently no generally accepted standard method for the purification and assay of PTKs, a special caution must be taken to avoid over-interpretation of tabular data comparing activity of the same inhibitors against different enzymes.

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