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INTEGRATED SYSTEM FOR THE SCREENING OF THE SPECIFICITY OF PROTEIN KINASE INHIBITORS

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Abstract—Tyrosine protein kinases (TPKs) play a major role in the transformation of cells. They are currently used as molecular targets for new generations of anticancer compounds. Numerous TPKs have been described from various tissues using either classical molecular biochemical techniques or cloning strategies. As a natural extension of these discoveries, a large number of "specific" inhibitors have been described in the literature. The major problem with these inhibitors is that there is no simple way to compare their specificity and/or selectivity from one report to another. We have set up a simple, straightforward technique to compare the inhibitory potency of 14 classical inhibitors towards six well-described and at least partially purified protein kinases. This technique is based on a new assay, easy to carry out and non-restrictive in terms of the type of protein substrate used. It permits direct comparisons between the results obtained from various sources. Data obtained showed that, when assessed in this integrated system, specificity and selectivity of many "classical" inhibitors are often weak, thus demonstrating that a universal technique such as ours is essential for the molecular screening of new protein kinase inhibitors. Compounds showing specificity for this panel of protein kinases will be more easily targeted to some defined types of oncogene and of transformed cells.

Since the discovery of the role of tyrosine protein kinase (TPK‡) activities in growth and cancer development, an enormous amount of work has been published dealing with: (i) the nature and characterization of TPKs, and (ii) their inhibition by a large number of chemicals [see 1–3 for reviews]. Indeed, TPK overexpression is associated with various types of disease such as cancer, psoriasis, atherosclerosis, pulmonary fibrosis, myelofibrosis and chronic myeloid leukemias [4].

None of the TPKs have the same endogenous substrate(s), and thus the same specificity. Therefore, they do not have the same implication—and regulation—leading to the same role in the growth signal transduction [5–7]. The recent findings by Levitzki's group [8–10] of a family of compounds (tyrphostins) with specific inhibitory potency towards the epidermal growth factor (EGF) receptor- or the platelet-derived growth factor receptor-associated TPKs emphasize the possibility of discriminating between inhibitors on the basis of their specificity (serine/threonine versus TPK) as well as their selectivity (among TPKs themselves). Nevertheless, the specificity of an inhibitor towards a given oncogene product might indeed be in some cases extremely high. As an example, Li *et al.* [11] explored the capacity of a styryl-based inhibitor (67B-83-A) to inhibit the phosphorylation of enolase in an immune-complex assay by six members of the src family, namely *lck*, *src*, *fyn*, *yes*, *lyn* and *blk*. The inhibitory capacity of this particular compound, in terms of IC_{50}, turns out to range from less than 1 μM for *lck* to more than 1.2 mM for *lyn*. Based on the available literature data, it is nowadays impossible to compare the inhibitory potency of a given chemical toward a member of the kinase family.

The present work deals with this problem and describes a simple technique to check easily the capacity of a given chemical to inhibit the activity of a panel of protein kinases. The method rests on the use of a simple assay of protein kinase activities on the same substrate, poly(E,Y)1-4, a polymeric mixture of peptide chains containing glutamic acid and tyrosine in a 4 to 1 ratio and with chain lengths ranging from 20,000 to 50,000 Da [12]. At the molecular level, our studies provide a solid background for further characterization of the cellular pharmacology of the newly discovered inhibitors and of their potential as therapeutic agents. Obviously, because many TPKs have been described in the literature, it is impossible to check a given inhibitor against "all" the TPKs. Therefore, we restrict ourselves in this study to 14 inhibitors on six protein kinase activities: protein kinases A and C (PKA and PKC), pp56*LCK*, pp60*C-SRC*, EGF receptor-associated tyrosine protein kinase and a tyrosine protein kinase partially purified from HL-60 (HPK40) [13]. p56*LCK* has been extensively used as a molecular target for different series of compounds: erbstatin derivatives [12, 14], flavonoids [15]. For all kinases, except PKC, a unique method

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‡ Abbreviations: cA-DPKI, cAMP-dependent protein kinase inhibitor; EGF, epidermal growth factor; HPK40, HL-60 cell cytosolic tyrosine protein kinase; PKA, protein kinase A cAMP-dependent protein kinase; PKC, protein kinase C; TPK, tyrosine protein kinase.

of activity measurement was used, derived from the method described by Damuni *et al.* [16] which we assessed and standardized for this purpose. The inhibitors included staurosporine, suramin, erbstatin, genistein, quercetin, H-7, H-8, H-9, H-1004, cAMP-dependent protein kinase inhibitor (cA-DPKI), Cibacron blue and three different tyrphostins. Under the standard conditions used, reliably different values for the inhibitory potency of these compounds could be obtained.

MATERIALS AND METHODS

The inhibitors erbstatin, suramin and tyrphostins 46, 50 and 9 [8–10] were synthesized in our Institute. Genistein was purchased from Extrasynthèse (Genay, France), quercetin and Cibacron blue from the Sigma Chemical Co. (St Louis, MO, U.S.A.), H-7, H-8, H-9 and HA-1004 from the Seikagaku Kogyo Co. (Tokyo, Japan), cA-DPKI and staurosporine from Novabiochem (Switzerland).

Protein kinase A (catalytic subunit) was from Sigma; while protein kinase C was partially purified from rat brain according to Uchida and Filburn [17]; HPK40 from HL-60 was partially purified according to Ernould *et al.* [13] and Boutin *et al.* [18]; LSTRA cells were grown as ascites on BALB/c mice (Iffa-Credo, France) and p56*LCK* was partially purified from this material according to Reuter *et al.* [19]; pp60*C-SRC* was partially purified from minipig platelets (Large White-INRA, France) [19]; EGF receptor TPK was extracted according to Lin *et al.* [20] from solid tumours on A431-grafted nude mice (Iffa-Credo, France).

All enzymatic preparations were free of other protein kinase activities, as judged by one of the following criteria: no catalytic activity towards serine/threonine protein kinase, no TPK (for PKA and PKC). Since the preparation of EGF receptor-associated TPK was far from reaching purity, the activity was calculated after substraction of the non-EGF-treated controls.

The assay for PKC was done with the Amersham (U.K.) test kit, according the manufacturer's instructions. The assay for all TPK was carried out with Poly(Y,E)1-4 (Sigma) as substrate and for PKA with histone VIIS (Sigma) as substrate. Both assays were otherwise identical and were derived from the assay of protamine kinase as described by Damuni *et al.* [16]. The enzyme preparation (10 μL) was incubated in 70 μL of a buffer (HMMBG) comprising 20 mM Hepes/NaOH pH 7.4, 5 mM $MgCCl_2$, 5 mM $MnCl_2$, 2.5 mg/mL bovine serum albumin and 10% (v/v) glycerol in the presence of the inhibitor for 5 min at room temperature. The substrate (5 mg/mL, final) was then added and the mixture allowed to equilibrate for another 5 min. The reaction was started by the addition of [γ-^{32}P]ATP (130 μM, 67 μCi/mL). The incubation ran for 15–30 min, depending on the enzymatic source, at 30°, and was stopped by 1.4 mL of TCASP solution (10% trichloroacetic acid containing 10 mM of sodium phosphate). The incubation was kept at 4° for 5 min and centrifuged at 10,000 rpm/min for 2 min (Microfuge 12, Beckman). The supernatant was discarded and the pellet washed four times by 2 mL of TCASP solution. The final pellet was then dissolved in 1 mL of scintillation fluid (Picofluor, Packard) and the remaining β radioactivity counted in a Beckman LS1800 spectrophotometer. For the measurement of EGF receptor-associated TPK activity, the enzymatic preparation was preincubated with 10 ng/mL of EGF (Sigma). When inhibitors were included in the system, the amount of HMMBG was reduced to 60 μL and 10 μL of the inhibitor were included in the assay.

RESULTS

The purification of the various kinases tested in the present work led to partially purified enzymatic preparations. Their respective specific activities are compared with the literature in Table 1.

The assay was assessed and optimized individually for each enzyme source as a function of concentration and incubation time (data not shown). We have determined K_m values (ATP) for the TPK used in this work: 2 μM for HPK40, 6 μM for p60*C-SRC*, 10 μM for p56*LCK* and 3 μM for the EGF receptor TPK. These data compare well with those published by others [see references 13, 22, 23, 25, respectively].

Assuming a bell-shape distribution of the chain lengths in poly(E,Y)1-4, we chose 35,000 Da as a mean molecular mass for all calculations. K_m values

Table 1. Specific activities of the various partially purified protein kinases

	This work		Literature	
Protein kinase	Source	Sp. act. (pmol/min/mg protein)	Source	Sp. act. (pmol/min/mg protein)
HPK40	HL-60 cytosol	7100[a]	HL-60	2040[b] [13]
EGF receptor TPK	A431 membrane	7.0[a]	A431 membrane	3.3[c] [21]
p60*C-SRC*	Minipig platelet	0.61[a]	Human platelet	0.15[d] [22]
p56*LCK*	LSTRA membrane	20[a]	LSTRA membrane	70[e] [23]
PKA (catalytic subunit, product P2645, Sigma)	Bovine heart	110[f]	Bovine heart	45[g] [Sigma, according to 24]
PKC	Rat brain	230[h]	Rat brain	5410[i] [17]

Substrate is: [a]poly(Y-E)1-4, [b]angiotensin II, [c]histone H2B, [d]IgG, [e]purified protein p58, [f]histone VIIS, [g]casein, [h]Amersham protein kinase C kit peptide, [i]histone H1.

Table 2. IC_{50} of 14 inhibitors on six partially purified protein kinase activities

	HPK40[4]	EGF receptor[5]	pp60*C-SRC*[6]	p56*LCK*[7]	PKA[8]	PKC[9]
Staurosporine	0.020	0.010	0.090	0.200	0.080	0.008
Suramin	400	70	200	200	10	60
Erbstatin	3	>100	70	900	>1000	>1000
Genistein	>100	300	>1000	>1000	300	>1000
Quercetin	200	50	600	>1000	>1000	>1000
H-7	>1000	>1000	>1000	>1000	>1000	80
H-8	>1000	>1000	200	800	>1000	100
H-9	>1000	>1000	>1000	>1000	>1000	200
HA.1004	>1000	>1000	>1000	>1000	>1000	10
cA-DPKI[1]	>10	>10	>10	>10	0.001	>10
Cibacron blue	2.5	100	300	200	6	100
Tyrphostin 46[2]	>100	20	1000	>1000	>1000	>1000
Tyrphostin 50[2]	>100	>100	>1000	>1000	>1000	>1000
Tyrphostin 9[3]	>100	>100	>1000	>1000	>1000	>1000

All IC_{50} are expressed in μM.

Substrate for enzymes 4–7 was poly(Y,E)1-4, for enzyme 8 was histone VIIS and for enzyme 9 the Amersham kit peptide.

[1]c-AMP-dependent protein kinase inhibitor.

[2]As described by Gazit *et al.* [8].

[3]As described by Yaish *et al.* [10].

[4]HPK40 is 2800 times purified from HL60 according to Ernould *et al.* [13].

[5]EGF receptor-associated TPK was extracted from A431 cell membrane according to Lin *et al.* [20].

[6]pp60*C-SRC* was purified from minipig platelets according to Reuter *et al.* [19].

[7]p56*LCK* was purified from LSTRA according to Reuter *et al.* [19].

[8]PKA (catalytic subunit) was from Sigma.

[9]PKC was partially purified from rat brain according to Uchida and Filburn [17].

were determined with poly(E,Y)1-4 for HPK40, pp60*C-SRC* and p56*LCK* and found to lie in the micromolar range (from 2 to 20). These values were in the same range as those found by Anafi *et al.* [26] for the p140*C-ABL* TPK (10 μM) and by Gazit *et al.* [8] for the EGF receptor TPK (70 μM).

The inhibitor potencies of the 14 compounds toward the six protein kinase activities are summarized in Table 2. PKA shows sensitivity to its peptidic, specific inhibitor, cA-DPKI, with an IC_{50} of about 1 nM. This value validates both the assay, the enzyme preparation and the inhibition conditions. The main result for this particular enzyme is the lack of response to H-7 and H-8 (>1 mM), a major discrepancy when compared to the results of Hidaka *et al.* [27] (3 and 1.2 μM, respectively). The observed inhibition of PKA by staurosporine, IC_{50} = 80 nM, compares fairly well with the value reported by Tamaoki [28], 8 nM, considering the differences in experimental conditions.

Staurosporine was found to be the most potent inhibitor of PKC, with an IC_{50} of 8 nM. In the same assay, the IC_{50} of suramin was 60 μM while the isoquinoline derivatives (H-7 and H-8) only weakly inhibited this activity with an IC_{50} of 80 and 100 μM, respectively. These repeatedly found values in our test compared badly with the data reported by Hidaka *et al.* [27], K_i values of 6 (H-7) and 15 (H-8) μM, respectively, and only slightly better with the K_i (20 μM) for H-7 found by Badwey *et al.* [29].

The only study on TPK p56lck inhibition was carried out with erbstatin for which Li *et al.* [11] reported as we do a weak inhibition.

p60*C-SRC* was inhibited by staurosporine (IC_{50} = 90 nM, compared with 6 nM obtained by Tamaoki [28]), by erbstatin (70 μM) and weakly by H-8 (200 μM) and Cibacron blue (300 μM). p60*C-SRC* was not inhibited under our conditions by genistein and quercetin in contrast to the report of Akiyama and Ogawara [30].

HPK40, the enzyme originally described by our group [18, 31, 32], was sensitive to erbstatin (IC_{50} = 3 μM). It was also responsive to staurosporine (IC_{50} 20 μM, comparable to the value obtained with cytosolic TPK [29]), to Cibacron blue (2.5 μM), and poorly to quercetin (200 μM) and suramin (400 μM).

The action of the inhibitors was also measured on EGF receptor-associated TPK activity. Staurosporine was the most potent inhibitor with an IC_{50} of 10 nM (25 nM reported by Meyer *et al.* [33]). The IC_{50} of tyrphostin 46 was 20 μM, of suramin, 70 μM, of Cibacron blue, 100 μM, of quercetin, 50 μM and of genistein, 300 μM.

As a specific PKC inhibitor, staurosporine is certainly the most potent compound of this series, as largely described in the literature. In addition, it is almost equally potent, under our experimental conditions, towards the EGF receptor-associated TPK and HPK40 activities, a result which has not been reported so far, to our knowledge.

DISCUSSION

One of the major shifts, in recent years, in cancer research, has been from cytotoxic, DNA synthesis-oriented compounds to selective inhibitors for a given enzymatic target present in cancer cells but not in non-proliferative cells. The novel anti-oncoprotein therapies [56] include not only TPK, the core of the present study, but also, for example, *N*-

myristoyltransferases [57, 58] and farnesyltransferase [59]. Despite encouraging initial results, it is obvious that there is a long way to go before the new enzyme inhibitors will replace or complete the current clinical pharmacopae of cytotoxic drugs.

TPKs are probably the most extensively explored field in this anti-oncoprotein therapy. Because of the increasing number of described TPKs, there is an urgent need to find highly specific compounds as future anticancer drugs or molecular tools. At least three aspects have to be considered, as far as specific TPK inhibitors are concerned: (i) recognition of the ATP binding site versus the substrate binding site [60], (ii) specificity towards TPK versus serine/

Table 3. *In vitro* potency and selectivity of TPK inhibitors

Inhibitor	Kinase	IC_{50}	Substrate	Reference
Staurosporine	EGF receptor	0.6	src peptide	34
		0.025	AII	33
		0.01	PolyEY	This work
	Insulin receptor	0.06	src peptide	34
	v-src	0.006	Autophosphorylation	35
		0.11	Enolase	36
		0.006	Immunocomplex	28
	c-src	0.01	PolyEY	37
		0.09	PolyEY	This work
	v-abl	0.08	[V5]AII	37
	HPK40	0.02	PolyEY	This work
	PKA	0.015	Kemptide	33
		0.2	Histone VS	38
		0.008	Histone IIS	28
		0.08	Histone IIS	33
		0.1	Histone IIS	36
		0.08	Histone VIIS	This work
	PKC	0.006	Histone H1	33
		0.01	Histone IIIS	38
		0.05	Amersham kit peptide	36
		0.003	Histone IIS	28
		0.008	Amersham kit peptide	This work
Suramin	PKC	30	Histone IIIS	39
		60	Amersham kit peptide	This work
Erbstatin[a]	EGF receptor	14	Autophosporylation	40
		6.0 (K_i)	src peptide	41
		3	src peptide	42
		<5	Autophosphorylation	11
		12.7	AII	37, 43
		>100	PolyEY	This work
	c-src	70	PolyEY	This work
	v-abl	>100	[V5]AII	37, 43
	p56*LCK*	⩾1200	Immunocomplex	11
		900	PolyEY	This work
	HPK40	50	Autophosphorylation	31
		4	AII	13
		3	PolyEY	This work
	PKA	>100	Kemptide	37
		>1000	Histone VIIS	This work
	PKC	>100	Histone H1	43
		>1000	Amersham kit peptide	This work
Genistein	EGF receptor	2.6	Autophosphorylation	21
		22	Histone 2B	21, 30
		1.0	AII	37, 43
		300	PolyEY	This work
	v-src	26	Casein	21, 30
		30	Immunocomplex	30
	c-src	>50	PolyEY	37
		>1000	PolyEY	This work
	v-abl	39	[V5]AII	37, 43
	p40[b]	>500	AI	44
	HPK40	100	AII	13
		>100	PolyEY	This work
	PKA	>100	Kemptide	37
		>370	?	30
		300	Histone VIIS	This work
	PKC	15	Histone H1	37, 43
		>370	?	30
		>100	Histone H1	21
		>1000	Amersham kit peptide	This work

Table 3 (cont'd). *In vitro* potency and selectivity of TPK inhibitors

Inhibitor	Kinase	IC_{50}	Substrate	Reference
Quercetin	EGF receptor	50	PolyEY	This work
		26.5	Histone 2B	30
		13	Autophosphorylation	45
		0.4	Autophosphorylation	46, 47
	v-src	63	Immunocomplex	47
	c-src	600	PolyEY	This work
		1.2	Immunocomplex	47
		26.5	Casein	30
	p56*LCK*	40	AI	15
		>1000	PolyEY	This work
	TPK-I	80	AII	48
	TPK-IIA	>100	AII	48
	TPK-IIB	80	AII	48
	TPK-III	15	AII	48
	TPK[c]	30	AII	49
	TPK[d]	10	PolyEY	50
	p40	40	AI	44
	HPK40	200	PolyEY	This work
	PKA	10	Tubulin	45
		>300	Kemptide	15
		>331	?	30
		>100	Histone 2A	46
		>1000	Histone VIIS	This work
	PKC	8	Histone IIIS	45
		20	pNPP	15
		82.8	?	30
		22	Histone H1	46
		>1000	Amersham kit peptide	This work
H-7	PKA	3 (K_i)	Histone H2B	27
	PKA[e]	>1000	Histone VIIS	This work
	PKC	6 (K_i)	Histone H1	27
		20	Histone IIIS	29
		80	Amersham kit peptide	This work
H-8	PKA	1.2 (K_i)	Histone H2B	27
	PKA[e]	>1000	Histone VIIS	This work
	PKC	15 (K_i)	Histone H1	27
		100	Amersham kit peptide	This work
Cibacron blue	PKA	100 (K_i)	Histone IIA	51
		6	Histone VIIS	This work
Tyrphostins				
RG50864[f]	EGF receptor	2.3	PolyEAY	10
	Insulin receptor	640 (K_i)	PolyEY	10
	p210*ABL*	6	PolyEY	26
RG50810[g]	EGF receptor	10	PolyEAY	10
		42	AII	37
	Insulin receptor	1200 (K_i)	PolyEY	
	PKA	>1000	?	10
	PKC	>1000	?	10
Serial number 9[h]	EGF receptor	2	PolyEAY	10
		>100	PolyEY	This work
	c-src	>1000	PolyEY	This work
	HPK40	>100	AII	13
		>100	PolyEY	This work
	PKA	>1000	Histone VIIS	This work
	PKC	>1000	Amersham kit peptide	This work
Serial number 46[i,j]	EGF receptor	10	PolyEAY	8
		>1200	Autophosphorylation	11, 14
		20	PolyEY	This work
	c-src	850	Immunocomplex	11
		1000	PolyEY	This work
	p56*LCK*	7	Immunocomplex	11, 14
		>1000	PolyEY	This work
	fyn	750	Immunocomplex	11
	yes	75	Immunocomplex	11
	lyn	⩾1200	Immunocomplex	11
	blk	250	Immunocomplex	11
	HPK40	>100	AII	13
		>100	PolyEY	This work
	PKA	>1000	Histone VIIS	This work
	PKC	>1000	Amersham kit peptide	This work

Table 3 (cont'd). *In vitro* potency and selectivity of TPK inhibitors

Inhibitor	Kinase	IC_{50}	Substrate	Reference
Serial number 50[i]	EGF receptor	2.5	PolyEAY	8
		>100	PolyEY	This work
	c-src	>1000	PolyEY	This work
	HPK40	>100	AII	13
		>100	PolyEY	This work
	PKA	>1000	Histone VIIS	This work
	PKC	>1000	Amersham kit peptide	This work
Hydroxy-cinnamamide (ST638)	EGF receptor	1	Autophosphorylation	47
		0.4	Autophosphorylation	46, 52
		9	AII	37
	c-src	18	Immunocomplex	47
	v-src	87	Immunocomplex	47
	v-abl	39	[V5]AII	37
	PKA	>100	Histone IIA	46
	PKC	>100	Histone H1	46
Thiazolidine-diones				
Compound 1	EGF receptor	6	AII	37
	c-src	4	PolyEY	37
	v-abl	>100	[V5]AII	37
	PKA	>500	Kemptide	37
	PKC	>500	Histone H1	37
Compound 2	EGF receptor	1	AII	37
	c-src	3	PolyEY	37
	v-abl	100	[V5]AII	37
	PKA	>500	Kemptide	37
	PKC	>100	Histone H1	37
Compound 3	EGF receptor	2	AII	37
	c-src	7	PolyEY	37
	v-abl	>100	[V5]AII	37
	PKA	>500	Kemptide	37
	PKC	350	Histone H1	37
Radicicol	*v-src*	0.3	Enolase	36
	PKA	250	Histone IIS	36
	PKC	270	Amersham kit peptide	36
Piceatannol	p56*LCK*	340	AI	53
	p40[k]	23	AI	53
	PKA	>1800	Kemptide	53
Sulfobenzoyl-nitrostyroles				
Compound 8	EGF receptor	0.4	AII	43
	v-abl	40	[V5]AII	43
	PKC	290	Histone H1	43
RDIFETDFFRK[l]	EGF receptor	2	RDIYETDYYRK[l]	54
	Insulin receptor	2.5	RDIYETDYYRK[l]	54
	p60*V-SRC*	5	RDIYETDYYRK[l]	54
Myricetin	Insulin receptor	2.6 (K_i)	PolyEY	55
	p130*FPS*	1.8 (K_i)	Myosin light chain	55
	p40	11	AI	44
	PKA	12.1 (K_i)	Histone H1	55
	PKC	27.5 (K_i)	Histone H2B	55

Many of the data presented in this table have been gathered by Geissler *et al.* [37].
? substrate not specified.
[a]Termed 67B-113-A in Ref. 11.
[b]p40 is a TPK purified to homogeneity from bovine thymus [see reference in 44].
[c]This TPK is associated with membranes of 7,12-dimethylbenz[*a*]anthracene-induced rat mammary tumours [see reference in 49].
[d]This TPK is partially purified from rat lung [see reference in 50].
[e]cAMP-dependent protein kinase catalytic unit.
[f]Also termed AG213.
[g]Also termed AG18.
[h]From Ref. 9 only.
[i]From Ref. 8 only.
[j]Termed 67B-83-A in Ref. 11.
[k]p40 is a TPK isolated from bovine thymus [see reference in 53].
[l]Amino acid single letter code.

threonine protein kinase, and (iii) specificity towards the target TPK, be it receptor-associated or not [see 2 for review]. The data widespread in the literature on potential inhibitors of TPKs form an enormous amount of information, not yet gathered in a single review, although information on major structural features has been collected, especially by Burke [3] and Powis [61].

For ease of understanding of the discrepancies between published data, we gather the main results of the literature in Table 3, as an extension of a table published by Geissler *et al.* [37]. Our table concerns the inhibitors tested in the present work, and some other promising "new" compounds. Some studies on new protein kinase inhibitors were excluded *de facto* from Table 3, either because their inhibition specificities were not studied, or because the compounds were not easy to obtain (herbimycin A, aeroplysinin, epiderstatin . . . [3]). Furthermore, very complete studies of series of inhibitors have been published, such as on flavonoids [15, 44, 45], pseudonucleotide bisubstrates [62, 63] and tyrphostins [8–10]. These studies clearly showed that even in the early screening stage of new compounds, a large panel of protein kinases should be tested, as reported, for example, for thiazolidine-diones [37].

For compounds such as staurosporine, it can be seen that IC_{50} values range from 3 nM (for PKC) up to 0.6 μM for EGF receptor-associated TPK. As an example of important discrepancies between measured IC_{50} values, even when obtained via the same approach, the values found for staurosporine on PKA varied from 8 [28] to 80 [33] nM. Particularly instructive were also the comparisons for tyrphostin 46. When the results for p56*LCK* autophosphorylation and kinase activity in the presence of this compound were compared, tyrphostin 46 was efficient in inhibiting the incorporation of radioactivity in the p56*LCK* immunocomplex, but totally inefficient in inhibiting the catalytic activity of p56*LCK*. This kind of discrepancy causes major problems for further studies of such compounds. In contrast, tyrphostin 46 showed a great deal of specificity among oncogene product TPKs (from 7 μM on p56*LCK* to more than 1200 μM on *lyn* products [11]).

Differences between some of our data and those gathered in Table 3 could be due to: (i) technical differences in assays (note for example, our observation [13] that proteins and amino acid copolymers are better kinase substrates than peptides, in terms of K_m values, at least by a factor of one hundred), (ii) differences in the degree of purity of the enzyme preparation [a possible explanation for the differences in the PKA inhibitory potency of H-7 is that although this substance binds to the PKA catalytic subunit [64], it may require the dimeric PKA (regulatory plus catalytic subunits) in order to inhibit the enzyme activity], (iii) differences in the biological source of the enzymes (p60*C-SRC* comes from minipig platelets in our studies, from human platelets in other studies) and (iv) differences in cosubstrate concentrations. In this last case, whether the inhibitor is competitive toward the ATP binding site or not is of major importance. Indeed, at high concentrations, ATP may compete at the

Table 4. Cellular effects of TPK inhibitors (some examples)

Inhibitor	Cell line	Parameter	Reference
Suramin	NB2A cells (mouse)	Antiproliferative	39
Erbstatin	L1210 cells (mouse)	Antineoplasic (*in vivo*)	75
	Rous sarcoma virus-transformed kidney cells (rat)	Morphological changes	74
	C1 cells (mouse)	Growth inhibition and differentiation	65
Genistein	Erythroleukemia (mouse)	Differentiation	76
	3Y1 cells (rat)	Growth inhibition	30
	NIH-3T3 cells (mouse)	Growth inhibition	77
	C1 cells (mouse)	Growth inhibition and differentiation	21
Quercetin	3MC-transformed BALB 3T3 cells	Inhibition of transformation	78
Tyrphostin	HER-14 cells	EGF-stimulated growth inhibition	79
	K721A cells	EGF-stimulated growth inhibition	79
	A431/clone 15 cells	EGF-stimulated growth inhibition	9
	Squamous cell carcinoma (human)	Antiproliferative effect	80
	A431 cells (human)	EGF-stimulated growth inhibition	81
Aeroplysinin	MCF-7 cells (human)	EGF-dependent growth inhibition	82
Radicicol	Friend leukemia cells	Differentiation	36
	v-src-transformed 3Y1	Morphological reversion	36
Herbimycin A	K562 leukemia (human)	Differentiation	83
	Oncogene-transformed cells (circa 10), various origins	Morphological changes	84
	src-transformed fibroblasts (rat)	Phenotype reversion	85, 86
	Rous sarcoma virus-transformed kidney cells (rat)	Fibronectin expression	87
	C1 cells (mouse)	Growth inhibition and differentiation	82
SBNS	MK cells	Antiproliferative	43
Thiazolidine-diones	BALB/MK cells	Antiproliferative	37

SBNS, sulfonylbenzoylnitrostyroles.

binding site with the inhibitor and lead to apparent lack of inhibition, while at low ATP concentrations, the compound would show inhibitory potency.

Many different chemicals have been described as "specific" inhibitors of a given TPK [see 2, 3 for reviews]. Reported specificities of TPK inhibitors [see Table 2 in 65] appear now to be low, many of them inhibiting other types of protein kinase if not other types of enzyme. For instance, genistein, described as a "specific TPK inhibitor" in 1987 [21], was later reported to be active as a topoisomerase II inhibitor [66], a feature also attributed to erbstatin [61]. Furthermore, tyrphostin 23 and genistein have also been described as potent calcium channel current blockers [67]. The case of staurosporine is particularly interesting. Although it is a potent inhibitor of PKC, this compound has a high capacity for inhibiting almost any type of protein kinase (see Tables 2 and 3) including the widely distributed p60*C-SRC*, in all cases at concentrations below 1 μM (see also Rüegg and Burgess [68]). Studies in which staurosporine is often used as a molecular tool to analyse a given cellular pathway [e.g. 69–71] should therefore be evaluated cautiously.

These observations, made available by using our standarized test system, stress the necessity to start inhibition studies on the actual molecular targets as, for instance, blind screening of microorganism broths or systematic chemical screening. Then, the number of screened targets will be increased using an integrated system such as the present one, where analytical and biochemical variations are kept to a minimum. Once compounds have been revealed by molecular testing, cellular pharmacology will be the last step before going to animal models. Some of the supposedly specific TPK inhibitors are capable of differentiating cancer cells, in association with other compounds [74, 75] or not [65, 74], as has also been reviewed for tyrphostins [4]. In order to depict some of the current trends in cellular pharmacology research on TPK inhibitors, we summarize some data from the literature in Table 4. The cellular pharmacology tools seem to be still very close to those used in research on cytotoxic compounds. This particular feature has probably slowed down discoveries of new leads during the last few years.

Finally, for the clinical application of such inhibitors, an early assessment of the target cells in terms of oncogene product(s) overexpression(s) [see for instance 88 and 89] will be essential to provide clues as to the treatment strategy, especially when compounds like tyrphostin 46 shows such a narrow target specificity [11].

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