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TX-1123: An Antitumor 2-Hydroxyarylidene-4-cyclopentene-1,3-Dione as a Protein Tyrosine Kinase Inhibitor Having Low Mitochondrial Toxicity

Hitoshi Hori,[a,*] Hideko Nagasawa,[a] Masaki Ishibashi,[a] Yoshihiro Uto,[a] Akihiko Hirata,[a] Kouichi Saijo,[a] Kazuto Ohkura,[a] Kenneth L. Kirk[b] and Yoshimasa Uehara[c]

[a]*Department of Biological Science & Technology, Faculty of Engineering, The University of Tokushima, Tokushima 770-8506, Japan*

[b]*Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute of Health, Bethesda, MD 20892, USA*

[c]*Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Shinjuku-ku, Tokyo 162-8640, Japan*

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Abstract—A series of 2-hydroxyarylidene-4-cyclopentene-1,3-diones were designed, synthesized, and evaluated with respect to protein tyrosine kinase (PTK) inhibition, mitochondrial toxicity, and antitumor activity. Our results show that the cyclopentene-dione-derived TX-1123 is a more potent antitumor tyrphostin and also shows lower mitochondrial toxicity than the malononitrile-derived AG17, a potent antitumor tyrphostin. The O-methylation product of TX-1123 (TX-1925) retained its tyrphostin-like properties, including mitochondrial toxicity and antitumor activities. However, the methylation product of AG17 (TX-1927) retained its tyrphostin-like antitumor activities, but lost its mitochondrial toxicity. Our comprehensive evaluation of these agents with respect to protein tyrosine kinase inhibition, mitochondrial inhibition, antitumor activity, and hepatotoxicity demonstrates that PTK inhibitors TX-1123 and TX-1925 are more promising candidates for antitumor agents than tyrphostin AG17.

Introduction

The protein tyrosine kinases (PTKs) are members of a large family of oncoproteins and proto-oncoproteins that play major roles in signal transduction during normal cell division, terminal cell differentiation, and apoptosis.[1–5] Because enhanced PTK activity is associated with proliferative disorders such as cancers, PTK inhibitors have been developed as potential therapeutic agents for cancer treatment.[6–8] Indeed, PTK inhibitors such as tyrphostins have been shown to inhibit growth of tumor cell lines[9] as well as tumor growth in vivo.[10,11] Tyrphostins were originally described as a group of low-molecular-weight protein tyrosine kinase inhibitors designed to compete with substrate rather than ATP.[12,13] Tyrphostins include three structurally diverse classes of compounds: the dihydroxy-*cis*-benzylidene-malononitrile group, lavendustin derived-blockers, and bis-substrate (protein substrate and ATP) quinoline analogues (Fig. 1).[3]

In general, criteria for therapeutic potential include target selectivity, cell permeability, bioavailability, appropriate pharmacokinetic properties, and absence of host toxicity. In a screening study AG17 (Fig. 2) was found to be the most potent tumor-cell-growth inhibitor among a series of tyrphostins examined as inhibitors of breast carcinoma cell growth.[14] Prior studies with AG17 had demonstrated that it could function as an inhibitor of the epidermal growth factor receptor tyrosine kinase (EGFR-K).[15] Studies in intact cells have revealed that AG17 is a potent inhibitor of platelet-derived growth factor (PDGF)-stimulated growth of rabbit vascular smooth muscle cells as well as PDGF-mediated tyrosine phosphorylation of several intracellular substrates.[16] Other investigators have further characterized AG17 as a potent inhibitor of epidermal growth factor-stimulated pancreatic carcinoma cell growth[17] and interleukin-2-stimulated human peripheral mononuclear cell proliferation.[18]

*Corresponding author. Tel.: +81-88-656-7514; fax: +81-88-656-9164; e-mail: hori@bio.tokushima-u.ac.jp

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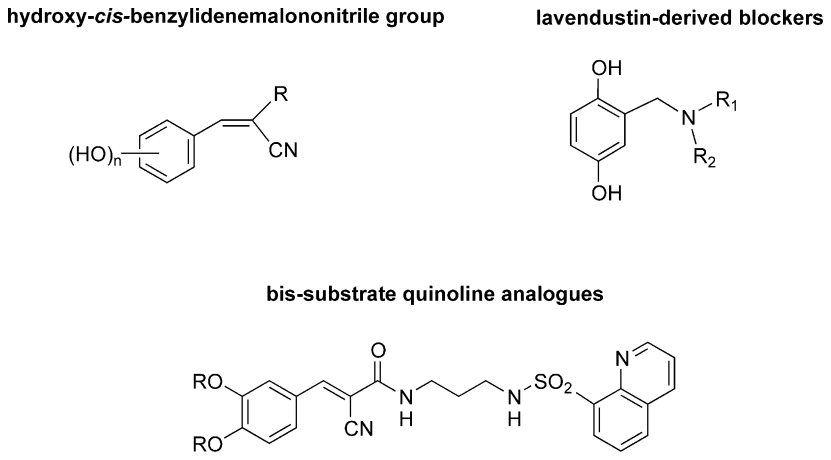


Figure 1. Structures of three classes of tyrphostins.

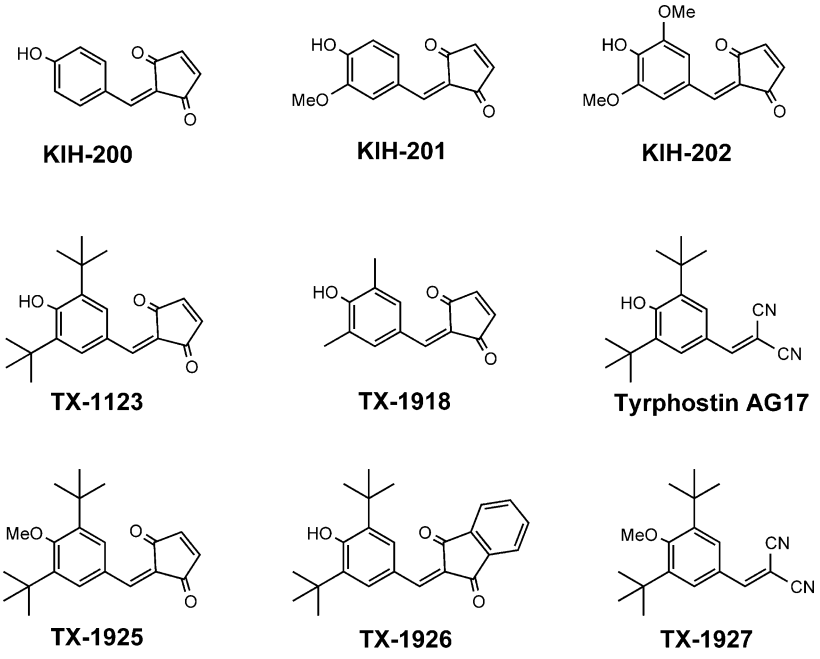


Figure 2. Structures of KIH-200–202, TX-1123, tyrphostin AG17, and their derivatives and analogues.

Recently, we found that AG17 suppressed insulin action in rat white adipocytes.[19] Sausville and co-authors found that AG17 was the most potent tumor cell growth inhibitor among a series of tyrphostins examined as inhibitors of breast carcinoma cell growth.[20] They also noted the possibility that AG17 may act in part by altering mitochondrial function and/or structure, and that impairment of mitochondrial function (mitochondrial toxicity[21]) may be exploitable as a potentially useful mechanism to modulate tumor cell proliferation.[20] Two years later, Levitzki reported that AG17 induces apoptosis and inhibition of cdk2 activity through a mechanism that purportedly does not involve reduction of cellular ATP levels. Shortly thereafter Sausville cautioned against Levitzki's use[22] of AG17 as a pure protein tyrosine kinase inhibitor.[23,24]

In considering potential mechanisms of the anti-proliferative action of AG17, as Sausville cautioned, we should note that SF6847 (an alternate name for AG17) was shown to act as an uncoupler of oxidative phosphorylation in isolated rat liver and heart mitochondria.[25] In this study, we describe a new tyrphostin, TX-1123, as a tyrphostin analogue of AG17 that has lower mitochondrial toxicity.

Results and Discussion

Design and synthesis

2-Arylidine-4-cyclopentene-1,3-diones were previously reported to be antitumor agents.[26] This unique soft-acid-type electrophilic cyclopentene-1,3-dione moiety was chosen as our lead structure for drug design. We

then developed some 2-hydroxyarylidene-4-cyclopentene-1,3-diones such as KIH-200, KIH-201, and KIH-202 as non-nitro radiosensitizers and hypoxic cytotoxins,[27] and as potent inhibitors of phosphate transport in mitochondria (Fig. 2).[28] We also developed hydroxybenzylidene-cyclopentenedione as a new phenolic enhancer of chemiluminescence and found KIH-201 to be the most potent enhancer in the luminol–hydrogen–horseradish peroxidase peroxide reaction.[29] TX-1123 was also tested in this regard, but was found to have no activity. We here describe the use of TX-1123 and its analogues such as TX-1925, TX-1926, and TX-1918 (Fig. 2) as tyrphostin AG17 analogues. The presence of cyclopentenedione moiety instead of the malononitrile moiety found in AG17 was expected to result in reduced mitochondrial toxicity.

As shown in Table 1, TX-1123 and its analogues have hydrophobicity parameters, such as dGW and calcd or obsd log*P*, similar to those of AG17, presumably reflecting the presence of two *tert*-butyl groups in each structure. From the results of studies with of KIH-200, KIH-201, and KIH-202, which have the same cyclopentenedione moieties, relative to SF6847 (AG17), TX-1123 and analogues could be expected to be more potent inhibitors of phosphate transport but should have reduced uncoupling activities in mitochondria. In general, target kinases appear to be very flexible, and their adenine pockets are dominated by hydrophobic interactions. These factors may conspire to produce a permissive binding site which accepts a range of planar heterocyclic systems, many of which have been uncovered by high-throughput screening.[30] These TX-1123 analogues having sterically demanding *o*,*o*′-di-*tert*-butyl groups and cyclopentenedione moieties are expected to act as hydrophobic and reactive Michael reaction acceptors.

In order to correlate their electron affinities (EAs) as a measure of their tyrosine kinase inhibitory activities, we calculated the orbital energies of the lowest unoccupied molecular orbital (LUMO) of TX-1123 analogues. The PM3 MO calculations show that TX-1123 ($E_{LUMO} = -0.82$ eV) has electron density regions with high coefficients localized only at the cyclopentenedione moiety, while AG17 has broad LUMO ($E_{LUMO} = -1.24$ eV) electron density regions at the methylene-malononitrile moiety (Fig. 3). The methylene-cyclopentenedione moiety in TX-1123 is less sterically hindered than the methylene-malononitrile moiety in AG17 (Fig. 4). Based on these LUMO coefficients and steric arguments we expected that the more electrophilic (lower E_{LUMO}) cyclopentenedione moiety in TX-1123 would be more subject to nucleophilic attack than the less electron-deficient AG17. We calculated hydrophobicity of the compounds using parameters such as log*P* (calculated and observed) and dGW. In general, higher hydrophobicities coorelate with more favorable pharmacological activities. dGW, a measure of solvation free energies, is a new hydrophobicity parameter that includes steric factors that are not included in the calculation of partition coefficients (log*P*). dGW values correlate better with obsd log*P* than with calculated log*P*. Thus, hydrophobicities and biological activities of cyclopentenedione-type inhibitors such as TX-1123 analogues and methylene-malononitrile-type inhibitors such as tyrophostin AG17 and TX-1927 correlated with each other within each series.

We synthesized TX-1123 and related compounds using a modified Knoevenagel reaction of the appropriate *o*,*o*′-disubstituted hydroxybenzaldehyde with 4-cyclopentene-1,3-dione under acidic conditions, as shown in Scheme 1. We found that direct methylation reactions of TX-1123 and tyrphostin AG17 did not afford the corresponding O-methyl derivatives, TX-1925 and TX-1927. These reaction conditions were not optimized. Their physical data including electron affinity ($-E_{LUMO}$), hydrophobicity and UV spectra are shown in Table 1.

Biological activities

We examined kinase inhibitory activities, mitochondrial toxicities, antitumor activities, and hepatotoxicities of TX-1123 and related compounds. The data are presented in Tables 2–4, respectively. TX-1918 was the most potent eukaryotic elongation factor 2 kinase (eEF2-K) inhibitor ($IC_{50} = 0.44\ \mu M$) among the compounds tested. The inhibitory activity of TX-1123 against Src tyrosine kinase (Src-K), ($IC_{50} = 2.2\ \mu M$) was stronger than that of AG17 ($IC_{50} > 350\ \mu M$). The dose–response curves of inhibitory activity of TX-1123 and AG17 against Src-K are shown in Figure 5. TX-1123 showed weaker uncoupling ($C_{max} = 2\ \mu M$ with $V_{max} = 260$ natomO/mg/min) and ATP-synthesis inhibitory activities ($IC_{50} = 5\ \mu M$) than did AG17 ($C_{max} = 0.02\ \mu M$ with $V_{max} = 270$ natomO/mg/min, $IC_{50} = 3.5$ nM), as shown in Table 3. We propose that the more potent kinase inhibitory activities of TX-1123 compared to that of AG17 is due to the presence of the methylene-cyclopentene-1,3-dione moiety as a more reactive Michael reaction acceptor in comparison with that of the methylene-malononitrile moiety because of

Table 1. Physical data of TX-1123, tyrphostin AG17, and their derivatives and analogues

Compounds	M_r	Mp (°C)	*EA* [$-E_{LUMO}$ (eV)]	dGW (kJ)	log*P* (calcd)	pK_a	λ_{max} (nm) (ε_{max})
TX-1123	312.4	155–157	0.82	−60.4	2.33 (4.93)	7.40	473 (6181) 373 (12,230)
TX-1925	326.4	110–112	0.88	−58.7	2.40 (6.44)	—	339 (9592)
TX-1918	228.3	186–188	0.83	−69.7	2.12 (3.35)	7.30	374 (13,700)
TX-1926	362.5	154–156	0.84	−59.4	2.39 (6.30)	7.85	508 (15,157)
Tyrphostin AG17	282.4	131–134	1.24	−62.4	2.32 (5.02)	6.87	464 (10,031) 364 (11,101)
TX-1927	296.4	104–105	1.32	−60.6	2.83 (5.52)	—	336 (9979) 278 (24,107)

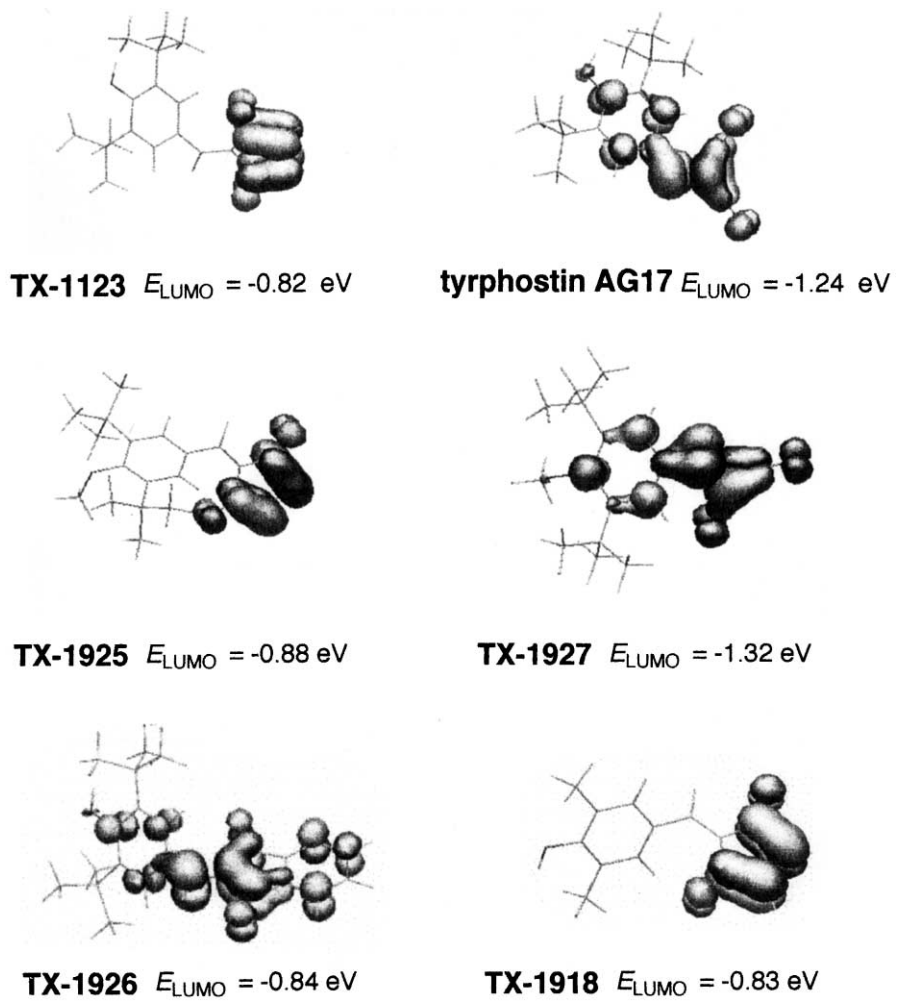


Figure 3. LUMOs of TX-1123, tyrphostin AG17, TX-1925, TX-1927, TX-1926, and TX-1918.

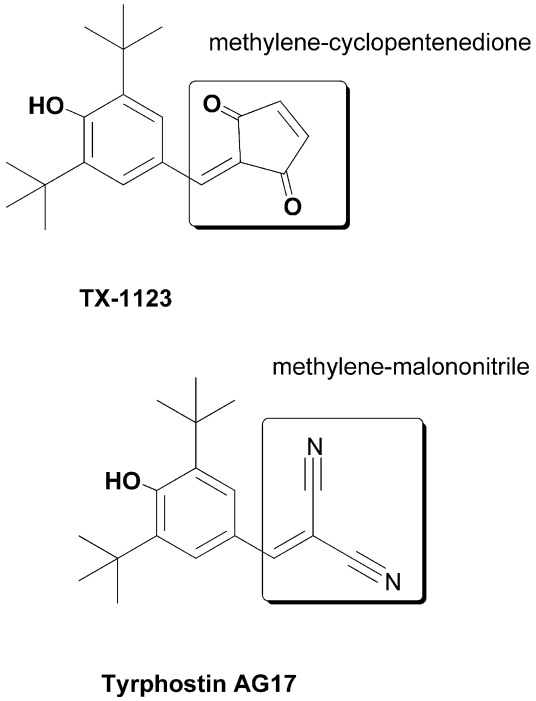
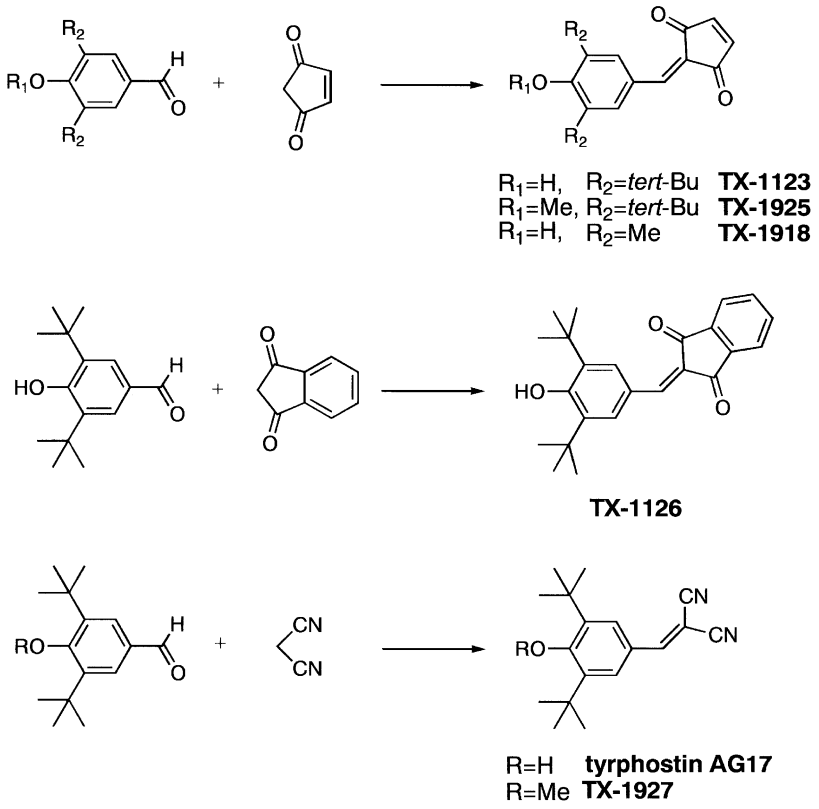


Figure 4. Methylene-cyclopentenedione moiety in TX-1123 and methylene-malononitrile moiety in tyrphostin AG17.

localized LUMO coefficients of the methylene-cyclopentene-1,3-dione moiety.

The antitumor activity of TX-1123 for HepG2 cells ($EC_{50} = 3.66\,\mu M$) was stronger than that of AG17 ($EC_{50} = 34.4\,\mu M$ for HepG2 cells), while its antitumor activity against HCT116 cells ($EC_{50} = 39\,\mu M$) was weaker than that of AG17 ($EC_{50} = 0.20\,\mu M$), as shown in Table 4. These data show good correlation between the cytotoxicity and kinase inhibitory activity in HepG2 cells, as well as a good correlation between cytotoxicity and uncoupling activity in HCT 116 cells. However, the precise mechanisms of action remain to be delineated. AG17 ($IC_{50} = 0.97\,\mu M$) also has much stronger hepatotoxicity than TX-1123 ($IC_{50} = 57\,\mu M$) as shown in Table 4. The difference of hepatotoxicities of AG17 and TX-1123 are probably related to the potency of their uncoupling activities. These data suggest that TX-1123 may be a more promising candidate than AG17 as an antitumor PTK inhibitor agent, especially because of the absence of mitochondrial- and hepato-toxicity.

With the exception of AG17, the common pharmacophore for most of the known tyrphostins is an unhindered phenolic styrene that can be viewed as a 'dehydrogenated' tyrosine-mimic.[6] The molecular structure



Scheme 1. Synthesis of TX-1123, tyrphostin AG17, and their derivatives and analogues.

of AG17 shows that its sterically hindered phenolic hydroxyl group is expected to act as a protonophore, but is not able to take part in hydrogen bonding. To confirm the non-essential nature of the hydroxyl groups in tyrphostins for their PTK inhibitory activities, we compared kinase inhibitory activities of the *O*-methylated TX-1123 (TX-1925) and *O*-methylated AG17 (TX-1927, MeO-SF[31]) with those of TX-1123 and AG17. As expected TX-1925 showed Src-K inhibitory activity ($IC_{50} = 3.1\,\mu M$) and antitumor activity ($IC_{50} = 89\,\mu M$ for HCT116 cells; $IC_{50} = 6.36\,\mu M$ for HepG2 cells) comparable to TX-1123.

Table 2. Protein kinase inhibitory activities (IC_{50}, μM) of TX-1123, tyrphotin AG17, and their derivatives and analogues

	Kinases				
Drugs	PKA	PKC	Src-K	eEF2-K	EGFR-K
TX-1123	9.6	320	2.2	3.2	320
TX-1925	31	31	3.1	3.1	31
TX-1918	44	44	4.4	0.44	440
TX-1926	>27	>27	>27	>27	>27
Tyrphostin AG17	>350	>350	>350 (75%)	>350	>350 (14%)
TX-1927	>340	>340	>340	>340	>340

Values in parentheses indicate the percent inhibition at the maximum concentration when the inhibitory activities were measured.

It is reasonable to expect that these *O*-methylated phenolic compounds, TX-1925 and TX-1927, should be weaker uncouplers because of the absence of a protonophoric moiety in the molecules. Indeed, TX-1925 showed a weaker mitochondrial toxicity, with lower uncoupling activity ($C_{max} = 40\,\mu M$; $V_{max} = 290$ natomO/mg/min) and ATP-synthesis inhibitory activity ($IC_{50} = 26\,\mu M$) as compared to TX-1123. Significantly, TX-1927 was completely devoid of properties associated mitochondrial toxicity, as shown by examination of uncoupling activity ($C_{max} = 200\,\mu M$; $V_{max} = 130$ natomO/mg/min) and ATP-synthesis inhibitory activities ($IC_{50} = 700\,\mu M$), and also showed no hepatotoxicity ($IC_{50} = 105\,\mu M$). Interestingly, it possessed comparable antitumor activity for HepG2 cells ($IC_{50} = 34.1\,\mu M$), but much lower activity for HCT116 cells ($IC_{50} = 22\,\mu M$) in comparison with AG17 ($IC_{50} = 34.4\,\mu M$ for HepG2 and $IC_{50} = 0.20\,\mu M$ for HCT116 cells).

Table 3. Mitochondrial cytotoxicity of TX-1123 & tyrphostin AG17 and their derivatives and analogues

Mitochondrial cytotoxicity		TX-1123	TX-1925	TX-1918	TX-1926	Tyrphostin AG17	TX-1927
Uncoupling activity	C_{max} (μM)	2	40	70	0.9	0.02	200
	V_{max} (natomO/mg/min)	260	290	150	310	270	130
ATP synthesis inhibition:	IC_{50} (μM)	5	26	19.5	0.05	0.0035	700

Table 4. Cytotoxicities of TX-1123 and tyrphostin AG17 and their derivatives and analogues for rat hepatocytes and tumor cells

Compounds	IC_{50} (μM)		
	Rat hepatocytes	HCT116	HepG2
TX-1123	57	39	3.66
TX-1925	230	89	6.36
TX-1918	90	230	2.07
TX-1926	120	4.2	8.05
Tyrphostin AG17	0.97	0.20	34.4
TX-1927	105	22	34.1

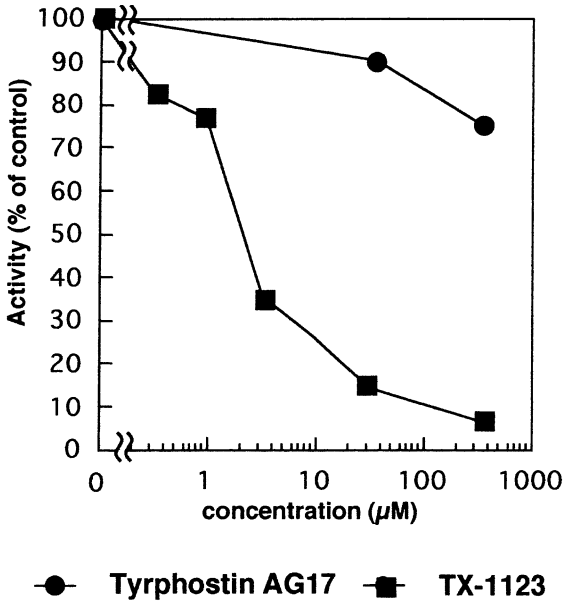


Figure 5. Dose–response curves of tyrphostin AG17 and TX-1123 on Src-K activity.

Conclusion

We designed, synthesized, and evaluated the antitumor activities of 2-hydroxyarylidene-4-cyclopentene-1,3-diones as PTK inhibitors having low mitochondrial toxicity. TX-1918 was the most potent eEF2-K inhibitor ($IC_{50}=0.44\,\mu M$) among the compounds tested. TX-1123 was a more potent Src-K inhibitor ($IC_{50}=2.2\,\mu M$) than tyrphostin AG17 ($IC_{50}>350\,\mu M$). The *O*-methyl derivative of TX-1123 (TX-1925) showed a weaker mitochondrial toxicity, such as uncoupling activity ($C_{max}=40\,\mu M$; $V_{max}=290$ natomO/mg/min) and ATP-synthesis inhibitory activity ($IC_{50}=26\,\mu M$) than did TX-1123. Significantly, the *O*-methyl derivative of AG17 (TX-1927) was completely devoid of properties associated mitochondrial toxicity, such as uncoupling activity ($C_{max}=200\,\mu M$; $V_{max}=130$ natomO/mg/min) and ATP-synthesis inhibitory activities ($IC_{50}=700\,\mu M$). The antitumor activity of TX-1123 ($IC_{50}=3.66\,\mu M$ for HepG2 cells) was stronger than that of AG17 ($IC_{50}=34.4\,\mu M$ for HepG2 cells), while its antitumor activity ($IC_{50}=39\,\mu M$ for HCT116) was weaker than that of AG17 ($IC_{50}=0.20\,\mu M$). AG17 ($IC_{50}=0.97\,\mu M$) also has much higher hepatotoxicity than TX-1123 ($IC_{50}=57\,\mu M$). TX-1925 has PTK inhibitory activity ($IC_{50}=3.1\,\mu M$ for Src-K) and antitumor activity ($IC_{50}=89\,\mu M$ for HCT116 cells; $IC_{50}=6.36\,\mu M$ for HepG2 cells) similar to TX-1123. As expected, TX-1925 has lower mitochondrial toxicity [uncoupling activity ($C_{max}=40\,\mu M$; $V_{max}=290$ natomO/mg/min), ATP-synthesis inhibitory activities ($IC_{50}=26\,\mu M$)], and hepatotoxicity ($IC_{50}=230\,\mu M$) than does TX-1123. These data suggest that TX-1123 and TX-1925 are more useful candidates of PTK-targeted antitumor agent having low mitochondrial toxicity than tyrphostin AG17. We conclude from these results that PTK inhibitors TX-1123 and TX-1925 are more promising candidates for antitumor agents than tyrophsotin AG17.

Experimental

General procedures

All chemicals and drugs were of high purity grade and commercially available, purchased from either Wako Pure Chemical Industries, Ltd. (Osaka, Japan) or Sigma-Aldrich Japan (Tokyo, Japan). Oxygen consumption and pH were monitored with a YTS multi-ion monitor (Yamashita-Giken Ltd., Tokushima, Japan), that included a Clark oxygen electrode and a pH electrode. The *n*-octanol–water partition coefficient (logP) was measured according to the method of Fujita et al.[31] The calculated hydrophobic parameters such as dGW (solvation free energy)[32] and log*P*(calcd) of the compounds were estimated using MOPAC (COSMO method)[33] and PrologP 5.1 (log*P* Prediction Module of Pallas 3.0, CompuDrug International Inc., CA, USA), respectively. All melting points were determined by Yanagimoto micro melting point apparatus without correction. IR spectra were recorded in KBr pellets on a Perkin-Elmer 1600 spectrometer. Ultraviolet–visible (UV–vis) absorption spectra were determined in ethanol on a Hitachi U-2000 spectrophotometer. The acid dissociation constant (pK_a) was measured spectrophotometrically at 25 °C from the values of absorbance at a specified wavelength measured at different pH values. Proton nuclear magnetic resonance (^1H NMR) spectra were recorded on a JEOL JNM-EX400 (400 MHz) spectrometer with tetramethylsilane as the internal standard, and chemical shifts (ppm) are given in δ values. Fast atom bombardment mass spectra (FABMS) and FAB high resolution mass spectra (FABHRMS) were measured on a JEOL JMS-SX 102A instrument. Elemental analyses were performed with a Yanaco CHN Corder (MT-5). Chromatographic separations were performed on silica gel columns (Kieselgel 60, 230–400 mesh, Merck). To obtain LUMO energy levels (E_{LUMO}) and coefficients, the semi-empirical molecular orbital (MO) calculations of compounds were applied by the PM3 method of J. J. P. Stewart[34] with his program MOPAC 97(Fujitsu WinMOPAC V.3, Fujitsu, Tokyo, Japan).

Syntheses of drugs

Tyrphostin AG17 (SF6847). A solution of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde (244 mg, 1.0 mmol) and malononitrile (80 mg, 1.2 mmol) in anhydrous DMF (3 mL) was refluxed for 10 h. The solvent was distilled

off under reduced pressure and the residue was purified by column chromatography on silica gel (hexanes/Et_2O, 1:1) to give tyrphostin AG17 (SF6847) (233 mg, 83%) as a yellow solid: mp 131–134 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 7.81 (s, 2H), 7.65 (s, 1H), 6.06 (s, 1H), 1.47 (s, 18H). EI–MS: *m*/*z* 282 (M^+). Anal. calcd for $C_{18}H_{22}N_2O$(M_r 282.4): C, 76.56; H, 7.85; N, 9.92. Found: C, 76.34; H, 7.66; N, 9.63. log*P*: 5.02 (calcd), 2.32 (obsd). dGW: −62.4 KJ. pK_a: 6.87 (ref[36], 6.80) UV–vis max: 464 nm (10,031), 364 nm (11,101).

Synthesis of 2-{(3,5-di-*tert*-butyl-4-hydroxyphenyl)-methylene}-4-cyclopentene-1,3-dione (TX-1123). A solution of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde hemihydrate (239.34 mg, 0.98 mmol), 4-cyclopentene-1,3-dione (123.81 mg, 1.29 mmol), *p*-toluenesulphonic acid (124.35 mg, 0.72 mmol) and magnesium sulfate (400 mg) in anhydrous $CHCl_3$ (12 mL) was refluxed for 19.5 h. The reaction mixture was filtrated and washed with water. The filtrate was chromatographed on silica gel with CH_2Cl_2 to afford TX-1123 (218.4 mg, 71%) as a yellow solid: mp 155–157 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.50 (bs, 18H), 5.93 (s, 1H), 7.18 and 7.22 (AB type, each 1H, $J = 6.4$ Hz), 7.58 (s, 1H), 8.34 (s, 2H). IR (KBr) ν cm^{-1} : 3629, 3581, 3431, 2959, 1683, 1623, 1587, 1435, 1176, 1135, 1046, 846. Anal. calcd for $C_{20}H_{24}O_3$ (M_r 312.4): C,76.89; H, 7.74. Found: C, 76.60; H, 7.50. log*P*: 4.93(calcd), 2.33 (obsd). dGW: −60.4 KJ. pK_a: 7.40. UV–vis max 473 nm (6181), 373 nm (12,230).

Synthesis of 2-{(3,5-dimethyl-4-hydroxyphenyl)methylene}-4-cyclopentene-1,3-dione (TX-1918). A solution of 3,5-dimethyl-4-hydroxybenzaldehyde (324.5 mg, 2.16 mmol), 4-cyclopentene-1,3-dione (210.3 mg, 2.19 mmol) and *p*-toluenesulphonic acid (64.82 mg, 0.34 mmol) in anhydrous $CHCl_3$ (25 mL) was refluxed for 24 h. The reaction mixture was washed with water and chromatographed on silica gel with CH_2Cl_2/AcOEt/*n*-hexane (1:1:2) to afford TX-1918 (166.9 mg, 33.8%) as a yellow solid: mp 186–188 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 2.32 (bs, 6H), 5.32 (s, 1H), 7.19 and 7.23 (AB type, each 1H, $J = 5.6$ and 6.4 Hz), 7.51 (s, 1H), 8.10 (s, 2H). IR (KBr) ν cm^{-1}: 3237, 2955, 2840, 1664, 1595, 1489, 1435, 1388, 1347, 1320, 1253, 1209, 1136, 1029, 970, 947, 870, 791, 689. Anal. calcd for $C_{14}H_{12}O_3$(M_r 228.3): C,73.67; H, 5.30. Found: C, 73.44; H, 5.60. log*P*: 3.35 (calcd), 2.12 (obsd). dGW: −69.7 KJ. pK_a: 7.30. UV–vis max: 374 nm (13,700).

Synthesis of 3,5-di-*tert*-butyl-4-methoxybenzaldehyde. To a suspension of 3,5-di-*tert*-butyl-4-hydroxybenzaldehyde hemihydrate (500 mg, 2.06 mmol) and potassium carbonate (560 mg, 4.05 mmol) in anhydrous acetone (20 mL), was added methyliodide (2.28 g, 16.1 mmol). After being refluxed for 15 h, the reaction mixture was filtered and washed with water. The filtrate was chromatographed on silica gel with CH_2Cl_2 to afford 3,5-di-*tert*-butyl-4-methoxybenzaldehyde (164.3 mg, 32.1%) as a white solid: mp 59 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.46 (s, 18H), 3.74 (s, 3H), 7.79 (s, 2H), 9.91 (s, 1H). IR (KBr) ν cm^{-1}: 3386, 2967, 2871, 2719, 2557, 1994, 1697, 1594, 1466, 1380, 1286, 1226, 1196, 1113, 1008, 964, 888, 802, 677, 584. Anal. calcd for $C_{16}H_{24}O_2$(M_r 236.2): C, 77.38H, 9.74. Found: C, 77.19; H, 9.54.

Synthesis of 2-{(3,5-di-*tert*-butyl-4-methoxyphenyl) methylene}-4-cyclopentene-1,3-dione (TX-1925). A solution of 3,5-di-*tert*-butyl-4-methoxybenzaldehyde (1.24 g, 5.00 mmol), 4-cyclopentene-1,3-dione (603 mg, 6.27 mmol) and *p*-toluenesulphonic acid (311 mg, 1.63 mmol) in anhydrous $CHCl_3$ (30 mL) was refluxed for 48 h. The reaction mixture was chromatographed on silica gel with CH_2Cl_2 to afford TX-1925 (442 mg, 27%) as a yellow solid: mp 110–112 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.48 (bs, 18H), 3.74 (s, 3H), 7.23 and 7.27 (AB type, each 1H, $J = 6$ Hz), 7.60 (s, 1H), 8.34 (s, 2H). IR (KBr) ν cm^{-1}: 3432, 2963, 1707, 1683, 1610, 1585, 1413, 1228, 1172, 1117, 1047, 1007, 889, 854. Anal. calcd for $C_{21}H_{26}O_3$ (M_r 326.4): C, 77.27; H, 8.03. Found: C, 77.05; H, 7.96. log*P*: 6.44 (calcd), 2.40 (obsd). dGW: −58.7 KJ. UV–vis max: 339 nm (9592).

Synthesis of 2-{(3,5-di-*tert*-butyl-4-hydroxyphenyl)methylene}-1,3-indanedione (TX-1926). A solution of 3,5-di-*tert*-butyl-4-methoxybenzaldehyde (230 mg, 0.945 mmol), 1,3-indandione (160 mg, 1.095 mmol) and *p*-toluenesulphonic acid (110 mg, 0.58 mmol) in $CHCl_3$ (24 mL) was refluxed for 21 h. After evaporation of solvents, the reaction mixture was chromatographed on silica gel with AcOEt/hexane (1:1) to afford TX-1926 (362 mg) quantitatively: mp 154–156 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.53 (bs, 18H), 6.06 (s, 1H), 7.26 (s, 1H), 7.76–7.78 (m, 4H), 8.52 (s, 2H). IR (KBr) ν cm^{-1}: 3521, 2952, 1723, 1685, 1557, 1433, 1353, 1279, 1199, 1093, 1036, 1004, 948, 883, 812, 780, 738, 651, 604. Anal. calcd for $C_{25}H_{28}O_3$ (M_r 362.5): C, 79.53; H, 7.23. Found: C, 79.43; H, 7.40. log*P*: 6.30 (calcd), 2.39 (obsd). dGW: −59.4 KJ. pKa: 7.85. UV–vis max: 508 nm (15,157).

Synthesis of 2-{(3,5-di-*tert*-butyl-4-methoxyphenyl)methylene}-malononitrile (TX-1927).[35] Yellow solid: mp 104–105 °C. ^1H NMR ($CDCl_3$, 400 MHz): δ 1.45 (bs, 18H), 3.75 (s, 3H), 7.71 (s, 1H), 7.84 (s, 2H). IR (KBr) ν cm^{-1}: 2964, 2226, 1699, 1589, 1557, 1461, 1442, 1408, 1398, 1293, 1266, 1211, 1118, 1003, 927, 891, 632, 619. EI–MS (*m*/*z*): 296 (M^+), 281 [$M^+ - 15(CH_3)$]. Anal. calcd for $C_{19}H_{24}N_2O$(M_r 296.4): C, 76.99; H, 8.16; N, 9.45. Found: C, 76.73; H, 8.14; N, 9.22. logP: 5.52 (calcd), 2.83 (obsd). dGW: −60.6 KJ. UV–vis max: 336 nm (9979), 278 nm (24,107).

Protein kinase assay

Materials. [γ-^{32}P]ATP was purchased from NEN Research Products (Wilmington, DE, USA).

Multiple protein kinase assay[36]

Assay for PKA, PKC, Src-K, eEF2-K. v-*src* Transformed NIH3T3 cells were incubated for 10 min on ice in a hypotonic buffer (1 mM Hepes, pH7, 4.5 mM $MgCl_2$, and 25 μg/mL each of the protease inhibitors antipain, leupeptin and pepstatin A). The swollen cells were homogenized by vortexing for 2 min at room temperature. Following an increase of HEPES to 20 mM, the homogenate was centrifuged at 500 *g* for 5 min to sediment nuclei. To this supernatant, addition was made to give a final concentration of 20 mM HEPES, pH 7.4,

10 mM $MgCl_2$, 0.1 mM Na_3VO_4, 10 mM ß-glycerophosphate, 1 mM NaF and 2.5 mg/mL protein, 1 μM phorbol myristate 13-acetate (PMA) and 20 μM cAMP. Protein kinase inhibitors were dissolved in DMSO and the final concentration of DMSO in the reaction mixture was 10% (v/v). The kinase reaction was initiated by addition of [γ-^{32}P]ATP (12.5 μM, 10 μCi), and the solution was incubated for 15 min at 25 °C. The reaction was terminated by the addition of SDS-PAGE sample buffer. The phosphorylated proteins were separated by SDS-PAGE (9% w/v gels). To detect protein tyrosine kinase (PTK) activity, the dried gels were further treated with 1 N KOH at 55 °C for 2 h. The results were visualized by autoradiography or Fuji Film Bio-image Analyzer BAS 2000. From the enzyme activity (% of control) as a function of drug concentration, the IC_{50}value was estimated as the index of the enzyme inhibition of drug.

Assay for EGFR-K.[37] To measure EGF-K activity, A431 human epithelial carcinoma cells were solubilized and cell debris was removed. Cell lysate was incubated with or without EGF at 25 °C for 30 min, and the reaction was started by addition of [γ-^{32}P]ATP and incubated at 0 °C for 10 min. After the reaction was stopped, the mixture was washed. To estimate phosphorylation of the receptor, the ^{32}P radioactivity was measured with a scintillation counter.

Mitochondrial toxicity

Uncoupling activity. Mitochondria were isolated from adult male Wistar rats as reported by Myers and Slater.[38] The amount of mitochondrial protein was determined by the Biuret method with bovine serum albumin as the standard. The respiration of mitochondria was monitored polarographically with a Clark-type oxygen electrode (Yellow Spring, YSI 5331). The incubation medium consisted of 200 mM sucrose and 2 mM $MgCl_2$ in 10 mM potassium phosphate buffer (pH 7.4). Mitochondria were added at 0.7 mg protein/mL in a total volume of 2.53 mL succinate (10 mM, plus rotenone at 1 μg/mg protein) was the respiratory substrate. The activity was determined by measuring changes in the rate of state 4 respiration upon addition of the test compound.

ATP synthesis inhibition. ATP synthesis in mitochondria was determined as reported by Nishimura et al.[39] by measuring the increase in pH of the medium associated with ATP synthesis at 25 °C in medium consisting of 200 mM sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 3 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.68 mL. The reaction was started by adding 400 μM ADP to the mitochondrial suspension (0.7 mg protein/mL) energized with 5 mM succinate (plus rotenone at 1 μg/mg protein). The pH change was monitored using a pH meter (Horiba, Kyoto, Japan).

Tumor cell growth assay using MTT

The HCT116 human colon tumor cells or human hepatoma Hep G2 cells were maintained at exponential growth. MTT [3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] was purchased from Dojindo Laboratories (Kumamoto, Japan). Optical density (OD) was measured on a microplate reader (BioRad model 450, Japan Bio-Rad Laboratories, Tokyo, Japan) using a 570 nm filter with blanking at 700 nm. The MTT assay was performed by suspending the cells in Eagle's MEM containing 7% $NaHCO_3$, and 4.7% FCS (pH 7.4), then pouring 300 μL into the wells of a 48-well culture plate. Drugs in ethanol (2.5 μL) were added and the cells were incubated at 37 °C for 48 h. Thereafter, drugs were washed out with culture medium. The culture medium (270 μL) and 30 μL of the MTT reagent (MTT 5 mg/mL in phosphate buffered saline without potassium and magnesium ions) were added and the cells were incubated for 3 h at 37 °C. Formazan was extracted with 300 μL of 0.04 N HCl in isopropanol and the OD was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad, Model 550). From the surviving fraction (OD% control) as a function of drug concentration, the IC_{50} value was estimated as the index of the inhibition of drug to tumor cell growth.

Hepatotoxicity assay[40]

Preparation of primary cultures of rat hepatocytes. Hepatocytes for primary culture were prepared from male rats of the SD strain (body weight: 150 g, 6 weeks old) by a collagenase perfusion method. Hepatocytes were isolated and then inoculated, at a density of 1.31×10^5 cells/cm^2 in a 12-well tissue culture polystyrene plate.

MTT reduction assay in primary cultures of hepatocytes. After 2 h, the cells were rinsed three times with a William's E medium, the medium with 10% fetal bovine serum and drugs in EtOH (2.5 μL) were added and cultured at 37 °C under 5% CO_2 atmosphere. After 24 h, the medium was removed, then 490 μL of medium and 10 μL MTT (5 mg/mL) in phosphate buffered saline (PBS) were added to each well and the plates were incubated at 37 °C for 3 h to produce formazan. The medium was then removed from the culture wells, and the cells were gently rinsed three times with Dulbecco's PBS. Then 100 μL 0.04 N HCl in isopropanol was added to each well to extract the formazan in the cells. The fromazan extracts were transferred to a 96-well plate and absorbance was measured at 570 nm in a microplate reader.

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