Cancer Chemother Pharmacol (1992) 29: 266–272

Cancer Chemotherapy and Pharmacology

Antitumor effect of KT6124, a novel derivative of protein kinase inhibitor K-252a, and its mechanism of action

Shiro Akinaga[1], Tadashi Ashizawa[1], Katsushige Gomi[1], Hiroe Ohno[1], Makoto Morimoto[1], Chikara Murakata[2], and Masami Okabe[1]

[1] Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shizuoka, Japan
[2] Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan

Received 18 July 1991/Accepted 25 September 1991

Summary. Novel derivatives of K-252a, (8R*,9S*,11S*)-(–)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo [a,g]-cycloocta[cde]trinden-1-one, an inhibitor of protein kinases and calmodulin-dependent phosphodiesterase, were synthesized and evaluated for their antitumor activity in vitro and in vivo. Of ten derivatives tested, four were active against the P388 murine leukemia i.p.-i.p. system, although K-252a was inactive. Among these derivatives, KT6124 was selected for further biological evaluation studies because its efficacy was the highest. KT6124 was also active against sarcoma 180 and B16 melanoma. It exerted a relatively broad spectrum of antiproliferative activity against 20 human tumor cell lines in vitro. To determine the mechanism(s) of action underlying the antitumor activity of KT6124, we tested the drug for inhibition of protein kinases, including Ca^{2+}- and phospholipid-dependent protein kinase (PKC), in intact A431 human epidermoid carcinoma cells in comparison with the PKC-inhibitory activity of K-252a. KT6124 did not antagonize the action of phorbol 12-myristate 13-acetate (PMA) in A431 cells, whereas K-252a did, suggesting that KT6124 may not act on protein kinases in the cells. The interaction of KT6124 with DNA in living cells was examined by the alkaline elution method. KT6124 apparantly exhibited DNA scission both dose- and time-dependently in the target cells. The DNA breakage was dependent on proteinase K treatment, suggesting its possible interaction with DNA-related enzyme(s). These results indicate that KT6124 exerts antitumor activity by acting on DNA or on DNA-related enzyme(s) in tumor cells rather than via the inhibition of protein kinases.

Introduction

K-252a (8R*,9S*,11S*)-(–)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H, 11H-2,7b,11a-triazadibenzo[a,g]-cycloocta[cde]trinden-1-one (Fig. 1), isolated from a culture broth of *Nocardiopsis* sp., has been found to inhibit Ca^{2+}- and phospholipid-dependent protein kinase (PKC) [9, 10], cyclic AMP-dependent protein kinase (PKA) [10], cyclic GMP-dependent protein kinase [10], myosin light-chain kinase [16], and calmodulin-dependent phosphodiesterase [12]. The compound has also shown antiallergic and anti-inflammatory effects in vitro and in vivo in additional studies [18].

Recently, several compounds that, like K-252a, contain the indolocarbazole chromophore have been reported to possess antitumor activity in vitro and in vivo [1, 2, 11, 13, 21, 23]. Among these compounds, the staurosporine derivatives CGP 41 251 [13] and UCN-01 [1, 21] were found to exhibit their antitumor activity through the possible inhibition of protein kinases, including PKC [1, 13, 21]. However, rebeccamycin [2] and AT2433 [11] exert their antitumor activity via interaction with DNA but demonstrate little inhibition of PKC [22].

Although K-252a exhibited apparent antiproliferative activity against HeLa S_3 human cervical carcinoma cells in vitro, it failed to show any antitumor activity against murine P388 leukemia in vivo (see Table 1). Since K-252a displays a unique chemical structure and a strong tendency to inhibit PKC, which is believed to participate in the growth of tumor cells and the promotion of tumor development [5, 17, 19], we synthesized a series of K-252a derivatives and examined their antitumor activity using in vitro and in vivo screening models. Among the ten derivatives, KT6124 was selected because its antitumor activity against P388 leukemia was the highest. The spectrum of antitumor activity exerted by KT6124 in vitro and in vivo and its mode of action are described in this report.

Offprint requests to: S. Akinaga, Pharmaceutical Research Laboratories, Kyowa Hakko Kogyo Co., Ltd., Shimotogari 1188, Nagaizumi-cho, Sunto-gun, Shizuoka-ken 411, Japan

Materials and methods

Drugs. K-252a was produced in our laboratories by fermentation as previously described [9]. Mitomycin C (MMC) and Adriamycin (ADM) were obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo). Derivatives of K-252a, including KT6124, were prepared from K-252a as follows [4, 15]. Compound 1 was obtained by the reduction of K-252a with $LiAlH_4$ and was converted into compound 3 in four steps: (1) TsCl, Et_3N, DMAP in THF; (2) NaH in THF; (3) NaN_3 in DMF; and (4) $LiAlH_4$ in THF. Conversion of compound 3 into compound 9 was achieved in three steps: (1) $ClCO_2Bn$, $NaHCO_3$ in THF-H_2O; (2) CrO_3 in pyridine; and (3) H_2, Pd-C in DMF. Compound 2 was obtained by the hydrolysis of K-252a with 3 *N* NaOH in dimethylformamide and was converted into compound 4 in four steps: (1) Ac_2O in pyridine; (2) $SOCl_2$; (3) $NH_2OH \cdot HCl$, Et_3N in $CHCl_3$; and (4) 1 *N* NaOH in MeOH. Conversion of compound 4 into compound 7 was achieved in three steps: (1) Ac_2O in pyridine, (2) CrO_2 in pyridine, and (3) 28% NH_4OH in DMF. Compound 5 was prepared by Sarett's oxidation of K-252a followed by reduction with $NaBH_4$. Treatment of compounds 5 and 9 with $NH_2NH_2 \cdot H_2O$ in dioxane at 100° C yielded compounds 6 and 8, respectively. Compound 10 was obtained from compound 1 in six steps: (1) $MeC(OMe)_3$,CSA in $CHCl_3$; (2) CrO_3 in pyridine; (3) NaH,$ClCO_2Et$ in THF; (4) SiO_2; (5) $NH_2OH \cdot HCl$, Et_3N in DMF; and (6) 2 *N* NaOH in THF-MeOH.

Cell culture. The origins of all human tumor cell lines used in this study except for myelogenous leukemia HL-60 cells have been described elsewhere [14]. HL-60 cells were obtained from the American Type Culture Collection through Dainihon Pharmaceutical Co. (Osaka).

Animals and tumors. Murine P388 lymphocytic leukemia cells were passaged in adult male DBA/2 mice and used for the experiments in adult male BALB/c × DBA/2 F_1 (CDF_1) mice weighing 20–25 g. Sarcoma 180 cells were passaged and used for the experiments in adult male ddY mice weighing 20–25 g. B16 melanoma cells were passaged in adult male C57BL/6 mice weighing 20–25 g. These animals were obtained from SLC (Shizuoka, Japan). P388 leukemia and B16 melanoma were supplied by the National Cancer Institute (Bethesda, Md.) through the Cancer Chemotherapy Center of the Japanese Foundation for Cancer Research (Tokyo). Sarcoma 180 was kindly supplied by the National Cancer Center (Tokyo).

Evaluation of antitumor activity. Sarcoma 180 cells were inoculated s.c. in the axillary region of ddY mice. For evaluation of antitumor activity against s.c.-inoculated tumors, the tumor volume was calculated by the following formula according to the method of the National Cancer Institute [3] after the length and width of the tumors had been measured:

$$\text{Tumor volume (mm}^3\text{)} = \frac{\text{Length (mm)} \times [\text{width (mm)}]^2}{2}.$$

The criteria for effectiveness included a treated vs control (T/C) value of $\leq 0.5\%$, and statistical significance was determined using Mann-Whitney's *U*-test. The antitumor activity against i.p.-inoculated tumors was evaluated after calculation of the percentage of increase in life span [ILS(%)] derived from 60-day observation of the duration of survival (in days) of tumor-bearing mice.

In vitro antiproliferative activity. The various types of cells were precultured for 24 h in 96-well microplates (Nunc, Roskilde, Denmark) containing 0.1 ml culture medium at 37° C in a humidified atmosphere comprising 5% CO_2 in air. The drug was added to the plates in serial 3-fold dilution ($n = 3$) and the plates were incubated for another 72 h. For 1-h pulse experiments, the cells were exposed to drugs for 1 h, transferred to fresh medium, and incubated for another 71 h. The antiproliferative activity of the drug was evaluated according to the inhibition of uptake of neutral red dye into the cells. Briefly, after the above incubation, the culture medium was discarded and 0.1 ml 0.02% (w/v) neutral red-containing medium was added to each well. Following the 1-h incubation, the solution was discarded and each well was washed with 0.1 ml 0.9% (w/v) NaCl solution. The neutral red dye was extracted using 0.1 ml 30% (v/v) ethanol solution with 0.001 *N* HCl, and the absorbance at 550 nm was measured by a Microplate Reader (Corona Electric Co., Ibaragi, Japan). The antiproliferative activity of the drug was expressed as the IC_{50} value (the concentration required for 50% inhibition of dye uptake).

EGF-binding assay. A431 cells were seeded at an initial density of 8×10^4 cells/cm^2 in Nunclon 24-well multidishes (Nunc) containing 1 ml culture medium. After 48 h preincubation at 37° C, the cells were treated with various concentrations of the drug for 1 h at 37° C. Next, phorbol 12-myristate 13-acetate (PMA) was added directly to the culture medium at a final concentration of 100 nM, and the cells were incubated for 2 h at 37° C; this step was omitted for control cells. After the cells had cooled to 4° C, they were incubated with (3-[^{125}I]-iodotyrosyl) human epidermal growth factor (EGF; 1,675 Ci/mmol; Amersham Japan) at 0° C for 3 h. Unbound [^{125}I]-EGF was removed by washing the cells twice with 2 ml phosphate-buffered saline (PBS), and cell-associated radioactivity was determined using a γ-auto-well counter (Packard, Meriden, Conn.) following solubilization with an aqueous solution of 1% (w/v) sodium dodecyl sulfate (SDS).

Effect on macromolecular synthesis. After 20 h preculture of 3×10^4 HeLa S_3 cells in Nunclon 24-well multidishes (Nunc), the cells were incubated with the drug for 1 h at 37° C and the culture medium was supplemented at 0.5 μCi/ml with [6-^{3}H]-thymidine (81 Ci/mmol), [5-^{3}H]-uridine (26 Ci/mmol), or L-[4,5-^{3}H]-leucine (58 Ci/mmol), respectively, and incubated for 1 h additional at 37° C. All of the precursors were obtained from Amersham Japan. The radioactivity incorporated into the 0.5% (w/v) trichloroacetic acid (TCA)-insoluble fraction was counted in a liquid scintillation counter (Packard) following solubilization with 1 *N* NaOH.

Alkaline elution method. Alkaline elution was performed according to the method of Kaneko et al. [8]. After 20 h preculture of 4×10^4 HeLa S_3 cells on 35-mm dishes (Nunc), the cells were labeled with 0.1 μCi [^{14}C]-thymidine/ml (60 mCi/mmol; New England Nuclear) for 24 h and then cultured in fresh medium for 1 day until they leached subconfluently. The cells were exposed to the drug at 37° C for either 1 h or a period indicated in Fig. 6 and were then washed once with PBS and transferred to fresh medium.

After all cells had been collected on a polycarbonate filter (pore size, 2 μm; diameter, 25 mm; Nuclepore Corp., Pleasanton, Calif.), they were treated with proteinase K (0.5 mg/ml; E. Merk, Darmstadt, FRG) in the presence of 2% SDS (Wako Pure Chemical Industries Ltd., Osaka)/25 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na; Nakarai Chemical Industries Ltd., Osaka; pH 9.6) for 1 h at room temperature in a dark environment for the digestion of DNA-bound proteins. The filter was washed twice with 3 ml 20 mM EDTA solution (pH 10) and then eluted with a solution of tetrapropylammonium hydroxide (Eastman Kodak, Rochester, N. Y.) and 20 mM EDTA (pH 11.9) at a rate of 0.05 ml/min. Fractions were collected directly into scintillation vials at 90-min intervals. The radioactivity of each fraction was expressed as a proportion of the total radioactivity.

Assay of PKC activity. The PKC enzyme activity in P388 cells was measured by the method of Ido et al. [7] except that EGF-receptor peptide, Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu (Amersham Japan) was used instead of histone III S as an exogenous substrate. Briefly, P388 cells (1.5×10^7 cells/tube) in growth medium were treated with drug for 1 h at 37° C. The cells were washed once with ice-cold PBS and then homogenized using a Dounce homogenizer in ice-cold extraction buffer consisting of 25 mM TRIS-HCl (pH 7), 2 mM ethylenedioxy-bis(ethylamine)-tetraacetic acid (EGTA), 50 mM mercaptoethanol, and 0.01% leupeptin (Sigma, St. Louis, Mo.). The homogenates were centrifuged at 100,000 *g* for 60 min at 4° C. The PKC activity in the cytosol fraction prepared as described above was assayed in the presence or absence of Ca^{2+} and PMA using the PKC-detection system (Amersham Japan) according to the manufacture's directions.

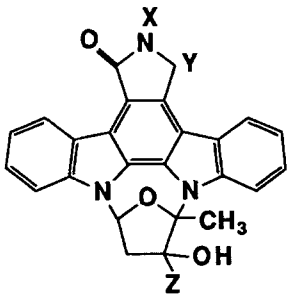

Fig. 1. Structure of K-252a derivatives:

Compounds	Substitutions		
	X	Y	Z
K-252a	H	H_2	$COOCH_3$
1	H	H_2	CH_2OH
2(K-252b)	H	H_2	COOH
3	H	H_2	CH_2NH_2
4	H	H_2	CONHOH
5	H	=O	CH_2OH
6(KT6124)	NH_2	=O	CH_2OH
7	H	=O	CONHOH
8	NH_2	=O	CH_2NH_2
9	H	=O	CH_2NH_2
10	OH	=O	CH_2OH

Results

Antiproliferative activity against HeLa S_3 cells

The antiproliferative activity of the K-252a derivatives (Fig. 1) was compared with that of K-252a. As shown in Table 1, seven of the ten derivatives tested were more active than K-252a against HeLa S_3 cells in vitro, whereas the other three compounds were less active than the parent drug.

Antitumor activity against P388 cells

All ten derivatives of K-252a were examined for their antitumor activity against P388 ascitic tumor by primary screening. As shown in Table 1, four derivatives (compounds 1, 6, 7, and 10) were found to be active (ILS, >30%) against P388 tumor, although the parent drug was inactive in this model. Compound 6 (KT6124) was the most active of these, its activity being comparable with that of mitomycin C (MMC). Thus, KT6124 was selected for the further evaluation of antitumor activity in vitro and in vivo.

Antitumor activity against murine tumors

The antitumor activity of KT6124 against murine ascitic tumors, P388 leukemia, sarcoma 180, and B16 melanoma is shown in Table 2. KT6124 was found to exhibit statistically significant antitumor activity against these tumors. It produced a marked increase in the survival of mice bearing

Table 1. Antiproliferative and antitumor activity of K-252a derivatives against HeLa S_3 human cervix carcinoma and murine P388 leukemia

Compounds	HeLa S_3 IC_{50} (μM)	P388	
		Optimal dose (mg/kg)	ILS (%)
K-252a	0.42	20	7
1	0.15	50	33
2(K-252b)	>2	50	0
3	0.034	12.5	20
4	0.3	6.25	23
5	0.14	50	9
6(KT6124)	0.32	100	61
7	0.83	12.5	40
8	>2	100	29
9	0.069	12.5	14
10	0.35	100	42

HeLa S_3 cells were cultured in 96-well plates on day 0, treated with the compounds on day 1, and then cultured for 72 h. The antiproliferative activity was determined as described in Materials and methods. P388 cells (1×10^6/mouse) were inoculated i.p. on day 0, and drugs were injected i.p. on day 1

sarcoma 180, which was comparable with that of MMC. The activity of KT6124 against B16 melanoma was lower than that of MMC. Against solid Sarcoma 180, KT6124 exhibited statistically significant antitumor activity (Table 3) that was also comparable with that of MMC.

Spectrum of the antiproliferative activity of KT6124

The antiproliferative activity of KT6124 against 20 human tumor cell lines is shown in Table 4. KT6124 exerted strong antiproliferative activity against all human tumor cell lines, giving a mean IC_{50} value of 0.49 μM. The sensitivity of HL-60, Calu-1, and COLO205 to KT6124 was slightly higher than that of the other cell lines.

Effect on the down-modulation of the EGF receptor caused by PMA

KT6124 and K-252a were tested for their effect on the PMA-induced reduction in the binding of [^{125}I]-EGF to the EGF receptor on intact A431 epidermoid carcinoma cells, since the receptor on these cells has been reported to be phosphorylated at the Thr^{654} position by PKC following PMA treatment, resulting in down-modulation of the receptor [6]. As shown in Fig. 2, K-252a but not KT6124 inhibited the PMA-induced reduction in [^{125}I]-EGF binding to A431 cells concentration-dependently. These results suggest that K-252a may inhibit protein kinases, including PKC, in intact cells, whereas KT6124 does not.

Effect on the PKC activity in P388 cells

To exclude the possibility that PKC inhibition may have contributed to the cytotoxicity of KT6124, the PKC activi-

Table 2. Antitumor activity of KT6124 against murine ascitic tumors

Tumors	Drugs	Dose (mg/kg)	Mean survival (days)	ILS (%)	>60-day survivers
P388	–	0	10.2± 0.4	0	0/5
	KT6124	100	16.4± 0.5*	61	0/5
		50	15 ± 0.9*	47	0/5
		25	14 ± 1.3*	37	0/5
	MMC	4.0	16.2± 0.4*	59	0/5
Sarcoma 180	–	0	15.3± 6.3	0	0/5
	KT6124	100	>41.2±15.6*	>269	2/5
		50	>32.8±14.4*	>214	1/5
		25	>36.6±19.1*	>239	2/5
	MMC	4.0	>37.2±20.2**	>243	2/5
B16	–	0	13.7± 1.5	0	0/5
	KT6124	100	19 ± 1.1*	39	0/5
		50	15.6± 0.8**	14	0/5
		25	14.2± 1.2	4	0/5
	MMC	4.0	30.6± 2.5*	123	0/5

P388 (1×10^6), sarcoma 180 (5×10^6), and B16 (0.5 ml 10% homogenate/mouse) cells were inoculated i.p. on day 0. Drugs were injected i.p. on day 1
* $P < 0.01$, ** $P < 0.05$ vs control values (Mann-Whitney's *U*-test)

Table 3. Antitumor activity of KT6124 against murine sarcoma 180 solid tumors

Drugs	Dose (mg/kg)	Mean tumor volume (mm^3)	T/C (%)
–	0	1,748±226	100
KT6124	200	574±347*	33
	100	1,434±310	82
	50	1,683±281	96
MMC	6	547±206*	31

Sarcoma 180 cells (5×10^6/mouse) were inoculated s.c. on day 0, and the drugs were injected i.p. on day 1. Tumor volume was measured on day 7 as described in Materials and methods, and T/C (treated vs control) values were calculated
* $P < 0.01$ vs control values (Mann-Whitney's *U*-test)

Table 4. Spectrum of the antiproliferative activity of KT6124 against human tumor cell lines

Origin	Cell line	IC_{50} (μM)
Lung	A549	0.58
	Calu-1	0.1
	PC-10	0.95
	PC-12	0.45
	PC-13	0.47
Stomach	KATO III	0.69
	MKN-28	1.48
	MKN-45	0.32
	NU-GC-3	1.02
Colon	COLO205	0.15
	COLO320DM	0.79
	DLD-1	1.2
	WiDr	0.58
Bladder	T24	0.49
Sarcoma	HT1080	1.15
Kidney	ACHN	0.58
Breast	MCF-7	0.51
Uterine	HeLa S_3	0.32
Vulva	A431	0.79
Leukemia	HL-60	0.037

Cells were cultured in 96-well plates on day 0, treated with KT6124 on day 1, and then cultured for 72 h. The antiproliferative activity was determined as described in Materials and methods

ty in intact KT6124-treated P388 cells was assayed directly using Amersham's PKC-detection system. The PKC enzyme activity in P388 cells was 10.6 pmol min^{-1} 10^{-6} cells. Following treatment of the cells with KT6124 at a concentration of as much as 10 μM, the PKC activity in the cells was not inhibited at all (data not shown).

Effect on macromolecular synthesis in HeLa S_3 cells

Macromolecular synthesis in HeLa S_3 cells was examined using the radiolabeled-precursor uptake method after 2 h treatment with KT6124 or K-252a. Although both drugs markedly inhibited the uptake of thymidine and uridine into these cells, they hardly affected that of leucine (Fig. 3).

Induction of DNA single-strand breakage in HeLa S_3 cells

The DNA single-strand breakage induced by KT6124 in HeLa S_3 cells was measured by the alkaline elution method. KT-6124 decreased the retention of [^{14}C]-DNA on the filter in a dose- (Fig. 4) and time-dependent manner (Fig. 5), suggesting the occurrence of DNA single-strand breakage in these cells. As shown in Fig. 5, DNA single-strand breaks caused by KT6124 became apparent at a drug concentration that lay below the IC_{50} value for 1-h exposure (4.7 μM). Moreover, DNA single-strand breakage was detected as early as at 1 or 3 min after the addition of

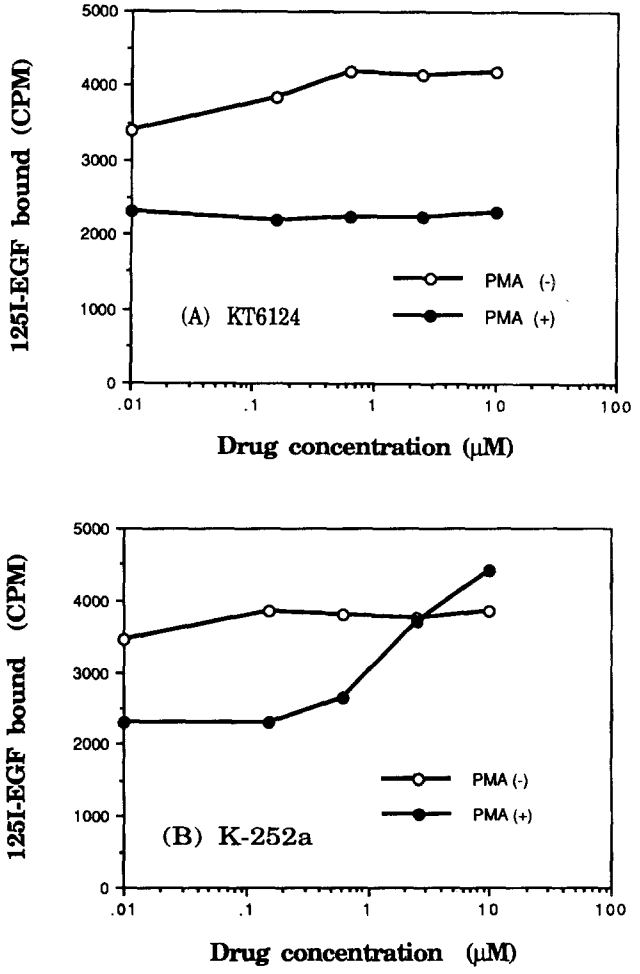

Fig. 2 A, B. Effects of KT6124 and K-252a on PMA-induced down-modulation of the EGF receptor in A431 human epidermoid carcinoma cells. A431 cells pretreated for 1 h with given concentrations of **A** KT6124 and **B** K-252a at 37° C were further incubated in the presence (○) or absence (●) of 100 nM PMA for 2 h at 37° C. After the cells had cooled to 0° C on ice, the binding of [^{125}I]-EGF to the cells was tested as described in Materials and methods

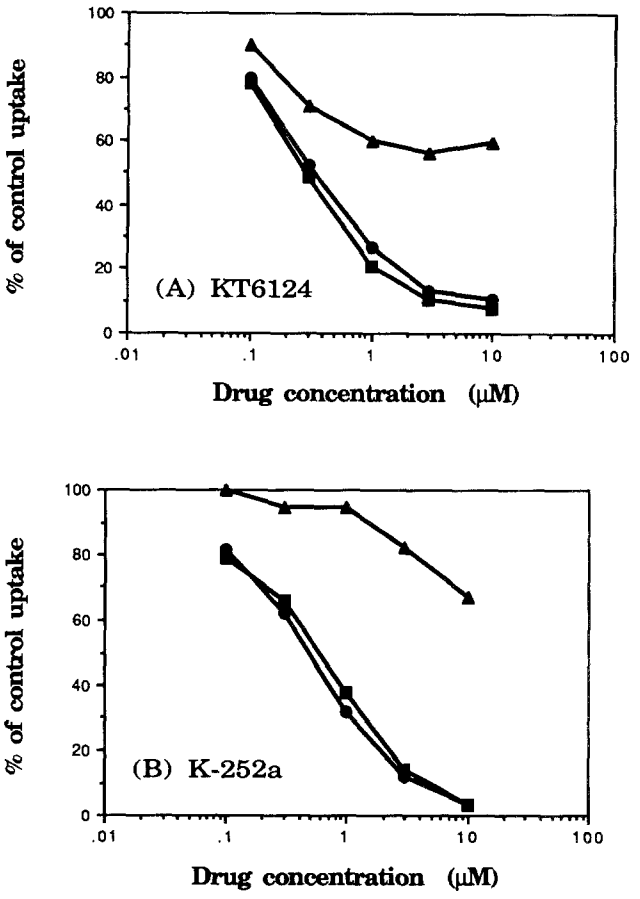

Fig. 3 A, B. Effects of KT6124 and K-252a on macromolecular synthesis in HeLa S_3 cells. The cells were exposed for 1 h to **A** KT6124 or **B** K-252a at various concentrations at 37° C. [6-^{3}H]-Thymidine (●), [5-^{3}H]-uridine (■), or L-[4,5-^{3}H]-leucine (▲) was added to the culture, which was further incubated for 1 h at 37° C. The incorporation of precursors into the cells is expressed as a percentage of control values

KT6124, and the degree of DNA breakage increased with the duration of treatment.

Proteinase K dependence of DNA single-strand breakage

To elucidate the mechanism underlying KT6124-induced DNA single-strand breakage, we investigated the proteinase K dependence of DNA single-strand breakage and compared the findings with those obtained for ADM as a positive control, which has been reported to cause proteinase K-dependent DNA scission [20]. As shown in Fig. 6, the proteinase K-dependent DNA scission induced by KT6124 was similar to that caused by ADM, although the extent of proteinase K dependence was more pronounced for KT6124 than for ADM.

Discussion

In the present study, we synthesized several derivatives of K-252a and examined their antitumor activity in vivo. Of these ten derivatives, compounds 1, 6, 7, and 10 exhibited apparent antitumor activity against P388 murine leukemia, which was insensitive to the parent drug (Table 1). Although compounds 1, 6, and 10 showed stronger antiproliferative activity against HeLa S_3 cells in vitro than did the parent drug, compound 7 was less active than K-252a (Table 1). In contrast, compounds 3, 4, 5, and 9, which were inactive against P388 leukemia, exhibited higher antiproliferative activity than did K-252a. These results suggest that antiproliferative activity in vitro does not correlate with that in vivo, at least for this series of compounds.

Among the four active compounds, KT6124 was selected for further evaluation since it showed the highest activity (Table 1), which was comparable with that of MMC (Table 2). KT6124 was found to exhibit antitumor activity against sarcoma 180 and B16 melanoma ascitic tumors, its activity being comparable with that of MMC against sarcoma 180 but lower than that of MMC against B16 melanoma (Table 2). In addition, KT6124 exerted antitumor activ-

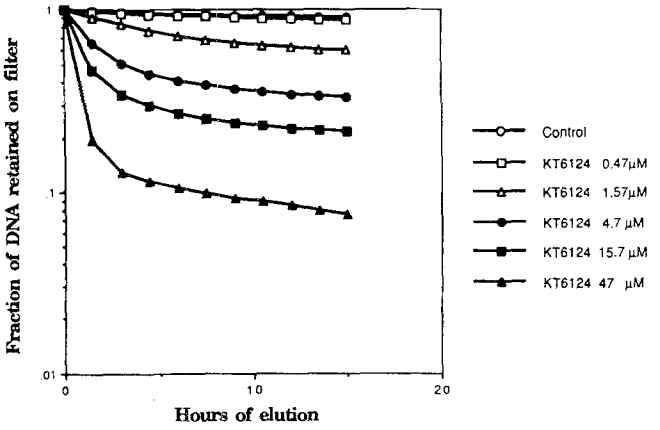

Fig. 4. DNA single-strand breakage in HeLa S_3 cells treated with various concentrations of KT6124. The cells were exposed to the drug at the indicated concentrations for 1 h at 37° C and were lysed with proteinase K-containing solution. Alkaline elution was then performed over 15 h as described in Materials and methods

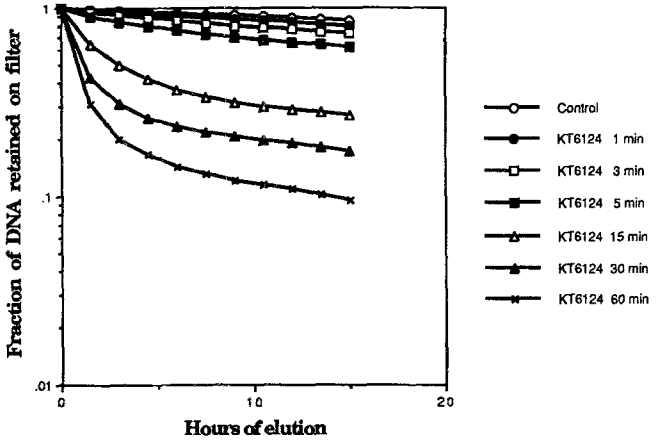

Fig. 5. DNA single-strand breakage in HeLa S_3 cells treated with 4.7 μM KT6124 for various periods. The cells were exposed to the drug for the indicated periods at 37° C and were lysed with proteinase K-containing solution. Alkaline elution was then performed over 15 h as described in Materials and methods

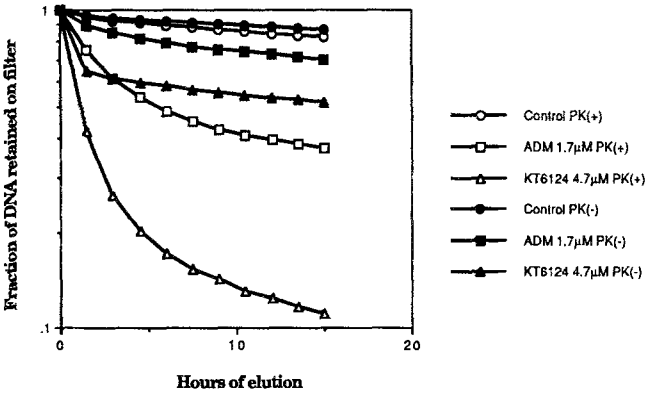

Fig. 6. Effects of proteinase K treatment on DNA single-strand breakage induced by KT6124 and ADM. The cells were exposed to the drugs at the indicated concentrations for 1 h at 37° C and were lysed in the presence (*closed symbols*) or absence (*open symbols*) of proteinase K-containing solution. Alkaline elution was then performed over 15 h as described in Materials and methods

ity against sarcoma 180 solid tumors that was also comparable with that of MMC (Table 3).

As indicated in Table 4 KT6124 showed a relatively broad spectrum of antiproliferative activity against human tumor cell lines. This finding together with the antitumor activity against experimental murine tumors described above (Tables 1–3) suggests that KT6124 could become a new candidate for use in anticancer therapy. The antitumor activity of KT6124 against human tumors in the nude mouse system is currently being investigated.

Several indolocarbazole compounds have recently been reported to show antitumor activity against experimental tumors in vivo [1, 2, 11, 13]. Among these, 4′-benzoyl staurosporine (CGP 41 251) was reported to exert its antitumor activity via the possible inhibition of protein kinases, including PKC [13]. 7-Hydroxy-staurosporine (UCN-01) was also postulated to act similarly [1]. In this regard, K-252a and KT6124 were found to inhibit the activity of PKC in a cell-free system, yielding IC_{50} values of 0.016 and 0.47 μM, respectively [4, 9, 10, 15]. Therefore, the inhibition of the PMA-induced down-modulation of the EGF receptor by these compounds was determined in intact cells in the present study. As shown in Fig. 2, K-252a (but not KT6124) antagonized the action of PMA in intact A431 cells, suggesting that K-252a inhibits the activity of protein kinases, including PKC, in these cells, whereas KT6124 does not. In this assay system, UCN-01 has also caused concentration-dependent inhibition of the PMA-induced down-modulation of the EGF receptor [1]. To exclude a possible contribution by PKC inhibition to the antiproliferative activity of KT6124, we measured the PKC enzyme activity in cells following their treatment with KT6124. However, KT6124 did not inhibit the PKC activity of the cells at a concentration of as much as 10 μM. These findings suggest that the antiproliferative activity of KT6124 may result from other mechanism(s).

Two other indolocarbazoles that exhibit antitumor activity, namely, rebeccamycin [2] and AT2433 [11], have been reported to possess a reduced potential for the inhibition of PKC in a cell-free system [22]. Rebeccamycin also selectively inhibits the uptake of [^{3}H]-thymidine into tumor cells and induces DNA single-strand breakage in intact cells [2]. The effect of KT6124 on macromolecular synthesis, including DNA synthesis, in HeLa S_3 cells was determined by the precursor uptake method (Fig. 3). KT6124 was found to inhibit both DNA and RNA synthesis in the cells in a manner similar to that shown by K-252a. DNA single-strand breakage induced by KT6124 was examined in intact cells by the alkaline elution method in a further assessment of the action of KT6124 on DNA. As shown in Figs. 4 and 5, KT6124 induced apparent DNA single-strand breaks in the target cells in a manner similar to that previously observed for rebeccamycin [2]. Moreover, this DNA single-strand breakage was apparently protein-concealed, as is that caused by ADM [20] (Fig. 6). This suggests that KT6124 may act on DNA-related enzyme(s) in the nuclei of tumor cells and induce DNA fragmentation. The mechanism underlying the possible action of KT6124 on DNA-related enzyme(s) remains to be

elucidated. However, these results suggest that KT6124 exerts cytotoxic activity through its action on DNA but not via the inhibition of protein kinases, including PKC.

On the basis of the overall results of our experiments, KT6124 will be evaluated as a new anticancer drug exhibiting a unique chemical structure and a unique mode of action. Further studies on the spectrum of its antitumor activity and the precise mechanism(s) of action involved are under way.

Acknowledgements. We are grateful to Miss Shinobu Koyama for her excellent technical assistance.

References

1. Akinaga S, Gomi K, Morimoto M, Tamaoki T, Okabe M (1991) Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. Cancer Res 51: 4888
2. Bush JA, Long BH, Catino JJ, Brander WT, Tomoita K (1987) Production and biological activities of rebeccamycin, a novel antitumor agent. J Antibiot 40: 668
3. Geran RI, Greenberg NH, MacDonald MM, Schumacher AM, Abbot BJ (1972) Protocols for screening chemical agents and natural products against animal tumors and other biological activities. Cancer Chemother Rep 3: 1
4. Hirata T, Mochida K, Murakata C, Takahashi M, Kase H, Yamada K, Iwahashi K, Sato A, Kasai M, Kobayashi E, Morimoto M, Akinaga S (1988) Japan Kokai Koho 63-295 589 (CA: 111 (21) 194456 g) "Preparation of K-252 derivatives as protein kinase C inhibitors and formulations containing them"
5. Housey GM, Johnson MD, Hsiao HLW, O'Brian CA, Murphy JP, Kriscmeier P, Weinstein IB (1988) Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. Cell 52: 343
6. Hunter T, Ling N, Cooper JA (1984) Protein kinase C phosphorylation of the EGF receptor at a threonine residue close to the cytoplasmic face of the plasma membrane. Nature 311: 161
7. Ido M, Asao T, Sakurai M, Inagaki M, Saito M, Hidaka H (1986) An inhibitor of protein kinase C, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H7), inhibits TPA-induced reduction of vincristine uptake from P388 murine leukemia cell. Leuk Res 10: 1063
8. Kaneko M, Nakayama T, Kodama M, Nagata C (1984) Detection of DNA lesions in cultured human fibroblasts induced by active oxygen species generated from a hydroxylated metabolite of 2-naphthylamine. Jpn J Cancer Res 75: 349
9. Kase H, Iwahashi K, Matsuda Y (1986) K-252a, a potent inhibitor of protein kinase C from microbial origin. J Antibiot 39: 1059
10. Kase H, Iwahashi K, Nakanishi S, Matsuda K, Yamada K, Takahashi M, Murakata C, Sato A, Kaneko M (1987) K-252 compounds, novel and potent inhibitors of protein kinase C and cyclic nucleotide-dependent protein kinases. Biochem Biophys Res Commun 142: 436
11. Matson JA, Claridge C, Bush JA, Titus J, Brander WT, Doyle TW, Horan AC, Patel M (1989) AT2433-A1, AT2433-A2, AT2433-B1 and AT2433-B2, novel antitumor antibiotic compounds produced by *Actinomadura melliayra*. J Antibiot 42: 1547
12. Matsuda Y, Nakanishi S, Nagasawa K, Iwahashi K, Kase H (1988) The effect of K-252a, a potent microbial inhibitor of protein kinase, on activated cyclic nucleotide phosphodiesterase. Biochem J 256: 75
13. Meyer T, Regenass U, Fabbro D, Alter E, Rosel J, Muller M, Caravatti G, Matter A (1989) A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. Int J Cancer 43: 851
14. Morimoto M, Ashizawa T, Ohno H, Azuma M, Kobayashi E, Okabe M, Gomi K, Kono M, Saitoh Y, Kanda Y, Arai H, Sato A, Kasai M, Tsuruo T (1991) Antitumor activity of 7-*N*-{{2-{[2-(λ-L-glutamylamino)ethyl]dithio}ethyl}}-mitomycin C. Cancer Res 51: 110
15. Murakata C, Sato A, Takahashi M, Kobayashi E, Morimoto M, Akinaga S, Hirata T, Mochida K, Kase H, Yamada K, Iwahashi K (1988) US patent 4923986 (CA: 110 (9) 75861 e) "Preparation of K-252 derivatives as anticancer agents". United States Patent Office, Washington, D. C.
16. Nakanishi S, Yamada K, Kase H, Nakamura S, Nonomura Y (1988) K-252a, a novel microbial product, inhibits smooth muscle myosin light chain kinase. J Biol Chem 263: 6215
17. Nishizuka Y (1988) The molecular heterogeneity of protein kinase C and its implication for cellular regulation. Nature 334: 661
18. Ohmori K, Ishii H, Manabe H, Satoh H, Tamura T, Kase H (1988) Antiinflammatory and antiallergic effects of a novel metabolite of *Nocardiopsis* sp. as a potent protein kinase C inhibitor from microbial origin. Arzneimittelforschung 38: 809
19. Persons DA, Wilkinson WO, Bell RM, Finn OJ (1988) Altered growth control and enhanced tumorigenicity of NIH 3T3 fibroblasts transformed with protein kinase C-1 cDNA. Cell 52: 447
20. Ross WE, Glaubiger DL, Kohn KW (1978) Protein-associated DNA breaks in cells treated with Adriamycin or ellipticine. Biochim Biophys Acta 519: 23
21. Takahashi I, Saitoh Y, Yoshida M, Sano H, Nakano H, Morimoto M, Tamaoki T (1989) UCN-01 and UCN-02, new selective inhibitors of protein kinase C: II. Purification, physico-chemical properties, structural elucidations and biological activities. J Antibiot 42: 571
22. Tamaoki T, Nakano H (1990) Potent and specific inhibitors of protein kinase C of microbial origin. Biotechnology 8: 732
23. Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimoto M, Tomita F (1986) Staurosporine, a potent inhibitor of phopholipid/Ca^{2+}-dependent protein kinase. Biochem Biophys Res Commun 135: 397