Modification of Pyrimidine Derivatives from Antiviral Agents to Antitumor Agents

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Abstract. *2,4-Diaminopyrimidine derivatives, that were originally developed as antiviral agents, were modified to antitumor agents by: i) introducing an amino group at C-5 on the pyrimidine ring, ii) changing the alkyl group and the ring size of the cycloalkyl group on the β-position of the ω-hydroxyalkylamino group, iii) replacing the phenylalkyl group on the cycloalkyl group with the 3,4,5-trimethoxyphenylalkyl group, iv) the esterification of the primary alcohol with diethyl phosphate and v) introducing the thiomethyl group at C-2 on the pyrimidine ring. Among the 21 compounds prepared,* ***6****, which has cyclobutyl at the β-position, exhibited potent activity towards P-388 leukemia. In addition,* ***14****, with methoxyl groups on the phenyl ring and* ***17****, with the thiomethyl group on the pyrimidine ring, showed specific inhibition for the EGFR protein kinase. Moreover,* ***15*** *and* ***16****, which carry the diethyl phosphoryl group on the primary alcohol, exhibited inhibitory activity towards P-glycoprotein.*

We have designed and synthesized more than eighty 2,4-diaminopyrimidine derivatives, including **1-11**, as antiviral agents based on the background that several antiviral agents used in clinical studies, *e.g.*, acyclovir and oxetanocin G, bear the guanine unit as a nucleic base, and 2,4-diaminopyrimidine resembles a part of the guanine unit (Figure 1) (1).

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Key Words: Pyrimidine derivatives, antiviral agents, antitumor agents.

During the study on the structure-activity relationships of the antiviral agents (**1-4**), we found that compounds **2** and **3**, which showed potent inhibitory activity toward influenza viruses, inhibited the growth of Madin-Darby canine kidney (MDCK) cells in the growing state while having no effects in the stationary state. Moreover, the derivatives with the phenylalkyl or phenyl groups on the cyclobutane ring (**5-8**) enhanced both the antiviral activities and the inhibitory activity toward MDCK cell growth. Based on this background, the structure and activity relationships of 2,4-diaminopyrimidine derivatives were studied in the search for novel compound as antitumor agents.

Materials and Methods

Chemical synthesis of compounds. Compounds **1-11** were prepared by the method in the literature (1). The 2-amino-3-chloro-4(-ω-hydroxy) alkylamino derivatives (**21a, 21b**) were all prepared by the reaction with the corresponding δ-hydroxy-alkylamine derivatives (**20a, 20b**) and 2-amino-4,6-dichloropyrimidine. The ω-hydroxyalkylamine derivatives (**20a, 20b**) were prepared from δ-butyrolactone (**19a, 19b**) in 5 steps, as shown in Figure 2. The 2,5-diamino-4(-ω- hydroxyalkylamino derivative (**22**) was prepared from the corresponding compound **21b** by the diazo-coupling reaction and reduction. The same conditions were used to prepare compounds **12 - 14** from 2-amino-4,6-dichloropyrimidine and the corresponding ω-hydroxyalkylamine derivatives, which were prepared in a similar way as **20b** by exchanging 4-methoxyphenethyl chloride, 3,4-dimethoxyphenethyl chloride or 3,4,5-trimethoxyphenethyl chloride with phenethyl chloride. Compounds **15** and **16** were prepared by the respective reactions of **6** and **12** with diethyphosphoryl chloridate in triethylamine. Compounds **17** and **18** were prepared from 2-thiomethyl-4,6-dichloropyrimidine and the corresponding ω-hydroxyalkylamine derivative by refluxing in ethanol. The nuclear magnetic resonance spectra and mass

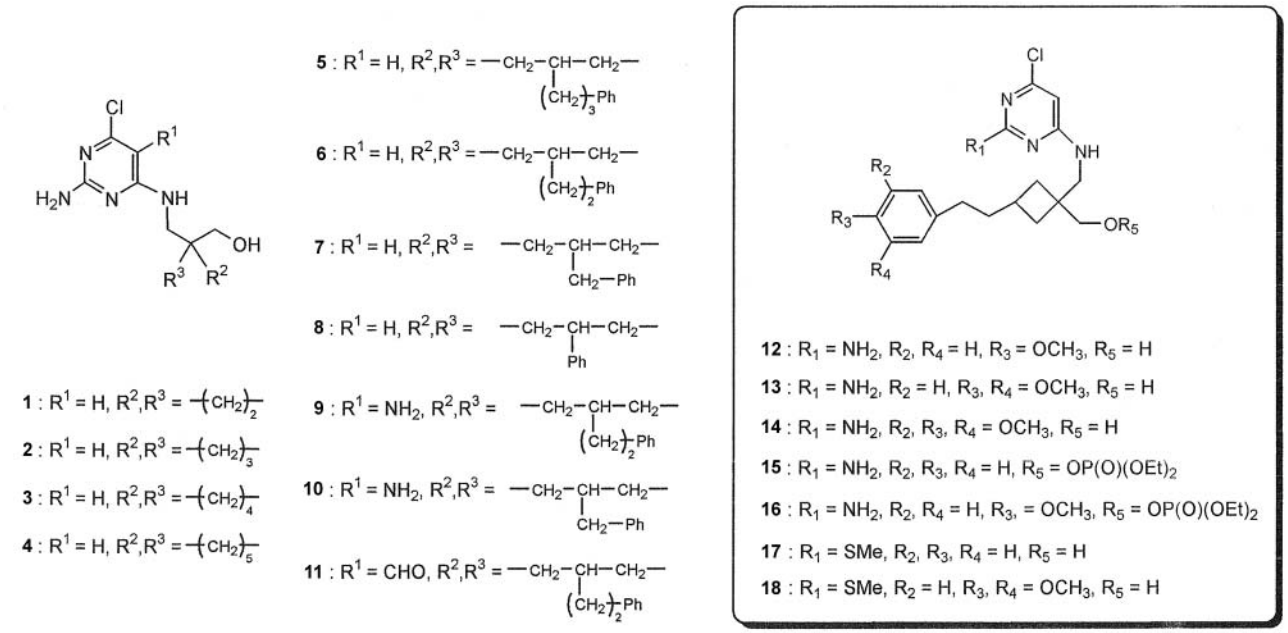


Figure 1. *Strucures of 2,4-diaminopyrimidine derivatives.*

spectra of all the compounds synthesized were consistent with their assigned structures (Figure 2).

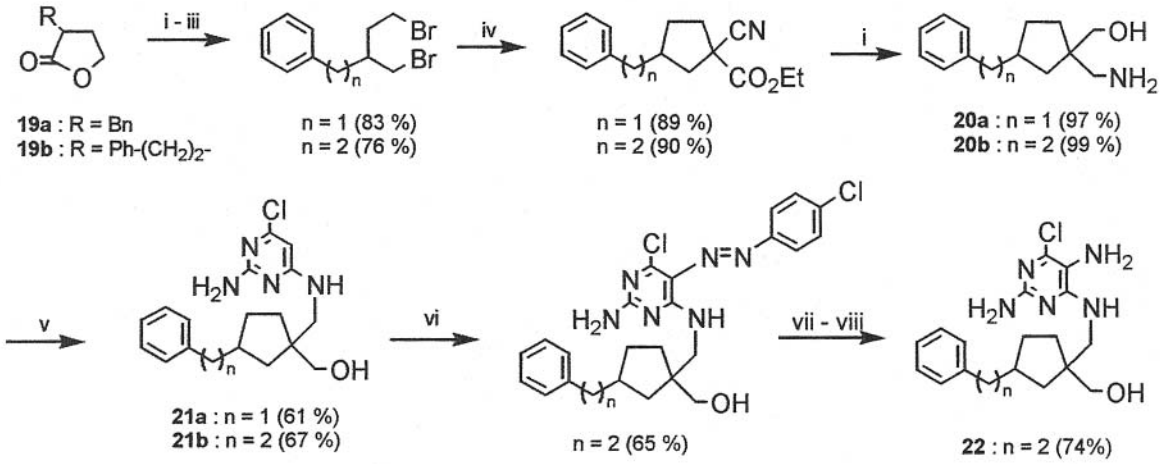
Human cancer cell (HCC) panel test. A panel of human cancer cell lines was developed according to the NCI method (2) with modifications (3-7). The panel consisted of 39 cell lines including various type of cancer cells (Figure 3). Six stomach cancer cell lines were employed, bearing in mind the high incidence of this cancer in Japan. Three breast cancer cell lines (HBC-4, HBC-5 and BSY-1), established at the Japan Foundation of Cancer Research, were used. Other cell lines were also included in the NCI 60 cell line panel. All the cell lines were cultured in RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) with fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 mg/mL), at 37˚C under 5% CO_2.

Growth inhibition experiments were done to assess the chemosensitivity to anticancer drugs. Growth inhibition was measured by determing the changes in the amounts of total cellular protein after 48 h of treatment using the sulforhodamine B assay. The GI_{50} values, which represent the 50% growth inhibition concentration, were evaluated as described before (2, 3). Several experiments were performed to determine the median GI_{50} value for each drug. Absolute values were then log transformed for further analysis.

To analyze the correlation with the fingerprints – differential growth inhibitory activities against the cell lines – the COMPARE algorithm was developed according to the method described by Paull and coworkers (8). Pearson correlation coefficients were calculated using the formula $r=[\Sigma(x_i-x_m)(y_i-y_m)]/[\Sigma(x_i-x_m)^2\Sigma(y_i-y_m)^2]^{1/2}$, where x_i and y_i are log GI_{50} of drug A and drug B, respectively, against each cell line, and x_m and y_m are the mean values of x_i and y_i, respectively.

Multiple protein kinase assay. The kinase activities of PKA, PKC, eEF-2K and PTK were assessed as described (9, 10). In brief, mouse NIH 3T3 cells transformed with v-src were suspended in a hypotonic buffer containing 1 mM Hepes-NaOH (pH 7.4), 5 mM $MgCl_2$ and 25 μg/ml each of antipain, leupeptin and pepstatin A and allowed to stand on ice for 10 min. After vigorous mixing for 1 min with a vortex mixer, the concentration of Hepes-NaOH (pH 7.4) was made equal to 20 mM. Then the lysate was centrifuged at 500 x g for 5 min at 4˚C to obtain the supernatant postnuclear fraction, which included protein kinases and their substrate proteins. Additions were made to the supernatant to give a final concentration of 1 mg/ml of protein/20 mM Hepes-NaOH (pH 7.4)/10 mM $MgCl_2$/0.1 mM Na_3VO_4/1 mM NaF/20 μM cAMP/100 μM $CaCl_2$. DMSO solution of protein kinase inhibitors (3 μl) was added to 22 μl of postnuclear fraction and the mixture was incubated for 10 min on ice. The kinase reaction was initiated by adding 5 μl of [γ-^{32}P]ATP (75 μM, 10 μCi) and the mixture was incubated for 20 min at 25˚C. At the end of the reaction, 10 μl of 4-fold concentrated SDS-PAGE sample buffer was added to the mixture and phosphorylated protein was separated by SDS-PAGE (9 % w/v gel). To detect PTK activity, the gel was further treated with 1 N NaOH for 2 h at 55˚C. The results were visualized by autoradiography. The position of the substrate protein band of each kinase was identified according to the sensitivity to various protein kinase inhibitors or activators and phosphoamino acid analysis, as previously described (9).

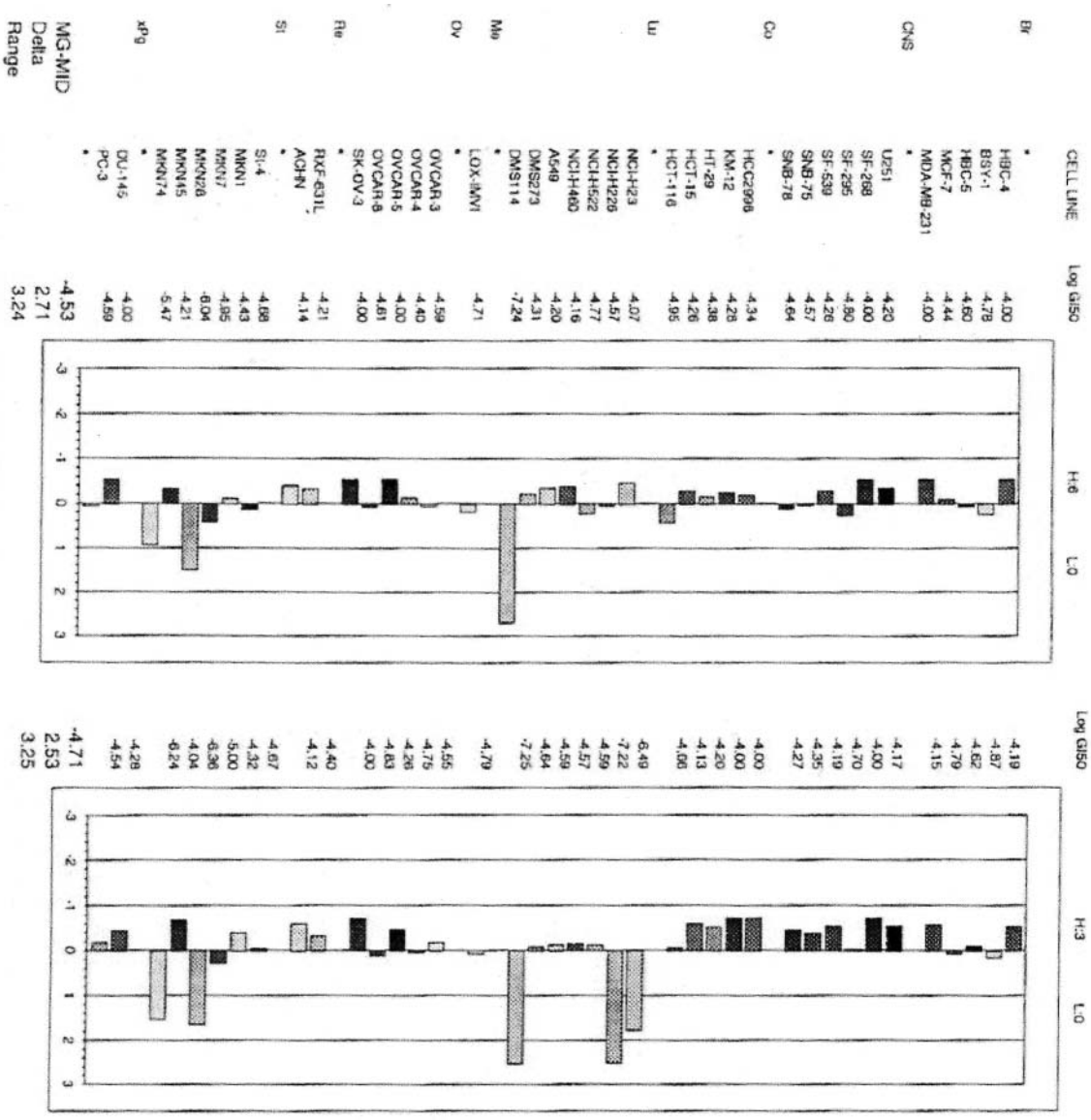
EGFR tyrosine kinase assay. For the EGF receptor tyrosine kinase assay, membrane fractions were prepared from A431 cells (20 mM HEPES, pH7.4, 0.2% Triton X-100) by the method of Hanai *et al.* (11) and the kinase assay by the method of Bertics *et al.* (12). Briefly, 15 μl of an assay mixture (20 mM HEPES, pH7.4, 5 mM $MnCl_2$)



i: $LiAlH_4$, THF, ii: CH_3SO_2Cl, Et_3N, $CHCl_3$, iii, LiBr, DMF, iv, ethyl malononitrile, v: 2-amino-4,6-dichloropyrimidine Et_3N, EtOH, vi: *p*-chloroaniline, $NaNO_2$, HCl, AcOH, vii: Zn, HCl, MeOH-EtOH, viii: HCl, MeOH

Comp	^{1}H-NMR ($CDCl_3$) δ:	HRMS *m/z*
21a	0.9 - 1.1 (1H, m), 1.2 - 1.4 (3H, m), 1.5 - 1.8 (3H, m), 2.1 - 2.3 (1H, m), 2.59 (1H, A part of AB, dd, J = 7.5, 13.2 Hz), 2.65 (1H, B part of AB, J = 7.0, 13.2 Hz), 3.24 (2H, br s), 7.2 - 7.4 (6H, m)	346.1562 (Calcd: 346.1560)
21b	0.84 - 0.91 (1H, dd, J 12.0, 9.3 Hz), 1.20 - 1.50 (3H, m), 1.61 (1H, A part of AB, d, J 6.5 Hz), 1.67 (1H, B part of AB, d, J 6.5 Hz), 1.83 - 1.94 (3H, m), 2.60 (2H, t, J 7.7 Hz), 3.04 - 3.36 (4H, m), 5.16 (4H, br s), 5.78 (1H, s), 7.14 - 7.29 (5H, m)	360.1719 (Calcd for $C_{19}H_{25}ClN_4O$: 360.1717)
22	0.97 (1H, dd, J 9.9, 13.2 Hz), 1.22 - 1.35 (2H, m), 1.62 - 1.73 (5H, m), 1.84 - 1.93 (3H, m), 2.61 (1H, br t, J 7.8), 2.70 (2H, br), 3.24 (2H, s), 3.25 (1H, A part of AB, dd, J 6.6, 14.0 Hz), 3.42 (1H, B part of AB, dd, J 6.9, 14.0 Hz), 4.64 (2H, br s), 5.89 (1H, br t, J 6.0 Hz), 7.16 - 7.30 (5H, m)	375.1835 (Calcd for $C_{19}H_{26}ClN_5O$: 375.1826)
12	1.35 (2H, br t, J 10.8 Hz), 1.70 (3H, m), 1.90 (2H, m), 2.24 (1H, m), 2.46 (2H, t, J 7.5 Hz), 3.39 (2H, br d, J 5.7 Hz), 3.40 (2H, br s), 3.79 (3H, s), 5.78 (1H, s), 6.81, 7.05 (each 2H, d, J 8.4 Hz)	376.1665 (Calcd for $C_{19}H_{25}ClN_4O_2$:376.1666)
13	1.37 (2H, br t, J 10.8 Hz), 1.73 (2H, br q, J 7.8 Hz), 1.92 (2H, m), 2.24 (1H, m), 2.47 (2H, t, J 7.2 Hz), 3.39 (2H, br d, J 8.0 Hz), 3.50 (2H, br s), 3.85, 3.87 (each 3H, t), 5.13 (3H, br), 5.78 (1H, s), 6.67 (2H, dd, J 1.8, 5.7 Hz), 6.78 (1H, d, J 8.7 Hz)	406.1766 (Calcd for $C_{20}H_{27}ClN_4O_3$: 406.1771)
14	1.39 (2H, br t, J 12.0 Hz), 1.72 (2H, br q, J 7.5 Hz), 1.94 (2H, m), 2.23 (1H, m), 2.46 (2H, t, J 7.2 Hz), 3.40 (2H, br s), 3.51 (2H, br s), 3.78 (3H, s), 3.85 (6H, s), 5.13 (2H, br), 5.27 (1H, br), 5.79 (1H, s), 6.37 (2H, s)	436.1881 (Calcd for $C_{21}H_{29}N_4O_4Cl$: 436.1877)
15	1.32 (6H, dt, J 0.9, 7.2 Hz), 1.56 (2H, m), 1.77 (2H, q, J 7.2 Hz), 1.95 (2H, m), 2.22 (2H, m), 2.38 (1H, br), 2.52 (2H, t, J 7.2 Hz), 3.42 (2H, br), 4.10 (4H, m), 5.00 (2H, br), 5.65 (1H, m), 5.81 (1H, s), 7.15 (3H, m), 7.26 (2H, m)	482.1863 (Calcd for $C_{22}H_{32}N_4O_4PCl$: 482.1849)
16	1.32 (6H, dt, J 1.2, 7.2 Hz), 1.56 (2H, m), 1.72 (2H, q, J 7.5 Hz), 1.95 (2H, tt, J 2.4, 10.8 Hz), 2.26 (2H, m), 2.46 (2H, t, J 7.5 Hz), 3.42 (2H, br), 3.78 (3H, s), 4.11 (4H, m), 5.00 (2H, br), 5.60 (1H, br), 5.80 (1H, s), 6.81, 7.04 (each 2H, d, J 8.4 Hz)	512.1953 (Calcd for $C_{23}H_{34}N_4O_5PCl$: 512.1955)
17	1.40 (2H, m), 1.73 (2H, q. J 7.2 Hz), 1.95 (2H, m), 2.24 (1H, m), 2.48 (3H, s), 2.52 (2H, m), 3.45, 3.55 (each 2H, br), 5.50 (1H, br), 6.04 (1H, s), 7.15 (3H, m), 7.25 (2H, d, J 7.5 Hz)	377.1320 (Cacld for $C_{19}H_{24}ClN_3OS$: 377.1328).
18	1.42 (2H, m), 1.71 (2H, q, J 7.5 Hz), 1.95 (2H, m), 2.24 (1H, quint, 8.0 Hz), 2.46 (2H, m), 2.49 (3H, s), 3.49, 3.56 (each 2H, br), 3.84, 3.86 (each 3H, s), 5.81 (1H, br), 6.06 (1H, s), 6.68 (2H, m), 6.77 (1H, d, J 8.4 Hz)	437.1545 (Calcd for $C_{21}H_{28}ClN_3O_3S$: 437.1540)

Figure 2. *Synthetic route of* ***21a, 21b, 22*** *and the spectral data of newly prepared compounds.*



Parameters of effective concentration and differential activity.

Cis-9	GI_{50}	TGI	LC_{50}
MG-MID	-4.53	-4.04	-4
Delta	2.71	0.53	0
Range	3.24	0.57	0

Trans-9	GI_{50}	TGI	LC_{50}
MG-MID	-4.71	-4.06	-4
Delta	2.53	2.03	0
Range	3.25	2.09	0

Results of comparison.

Rank	Compounds	*Cis*-9	*Trans*-9
1	nitrogen mustard	0.347	0.458
2	vinblastine	0.323	0.416

Figure 3. *HCC panel test of cis- and trans-isomers of compound* ***9****.*

Table I. *Antitumor activity toward P-388 mouse leukemia in vivo.*

	Dose (mg/kg)	T/C (%)		Dose (mg/kg)	T/C (%)
	400	0		400	23
6	80	90	**21a**	200	102
	40	145		100	94
	400	95		400	11
11	200	103	**21b**	200	11
	100	113		100	14
	400	102		400	11
9	200	97	**22**	200	11
	100	107		100	11
	2.5	96			

Table II. *Inhibition specificity of compounds **14** and **17** toward protein kinases.*

Compound	*c*(μg/ml)	PKA	PKC	PTK	eEF2K	Flt-1	EGFR
	100	–	–	–	–	–	+
14	10	–	–	–	–	–	–
	1	–	–	–	–	–	–
	100	–	–	–	–	–	++
17	10	–	–	–	–	–	+
	1	–	–	–	–	–	+
	0.1	–	–	–	–	–	–

containing a membrane fraction of 10 μg protein and 2 μg/ml EGF and 1.0 mg/ml angiotensin II as substrate were preincubated at 25˚C for 30 min with a sample, and the kinase reaction was started by adding 5 μl of [γ-^{32}P]ATP (1 μM, 5 μCi/assay). After 15-min incubation at 0˚C, the reaction was stopped with ice-cold 20% trichloroacetic acid (TCA), the supernatant was spotted on P81 cellulose paper (Whatmann), washed and the radioactivities remaining were measured by scintillation counter.

Flt-1 tyrosine kinase assay. VEGFR tyrosine kinase activity was measured using membrane fractions derived from Flt-1-overexpressing Tn 5 cells, as described (13).

Inhibitory activity toward P-glycoprotein (14). The source and maintenance conditions of 2780^{AD} human ovarian carcinoma cells were attained as in the literature (15, 16). Cells from an exponentially-growing culture were suspended with trypsin/EDTA (0.0025% and 0.25% w/v, respectively) or EDTA alone in Ca^{2+} and Mg^{2+} -free Hank's balanced salt solution (pH = 7.4). The cells were resuspended at densities of about 1x10^6 /ml in Dulbecco's modified essential medium without bicarbonate, and with 20 mM HEPES (Serva, Heiderberg, Germany) (pH = 7.4) and 10% heat-inactivated fetal calf serum (Flow Laboratories, Irvine, UK). Cellular ATP levels after incubation in the medium were determined by the HPLC method as described (17). Next, cells (3-4x10^5) were incubated with radiolabelled [G-^{3}H] vincristine sulfate (sp. act. 4.8 Ci/mmol) (Amersham, UK) and the test sample, or verapamil as a positive control, in polypropylene vials in 440 μl medium, washed and the radioactivity was determined as described in the literature (16).

Antitumor activity against P 388 lymphocytic leukemia. Groups of seven 8-week-old male CDF1 mice were inoculated intraperitoneally (*i.p.*) with 1x10^6 P 388 lymphocytic leukemia cells on day 0. A given amount of the test compound dissolved or suspended in saline was administered *i.p.* on days 1 and 5. Antitumor effects were evaluated in terms of T/C (%) calculated as the following equation: T/C (%) = T/C x 100, where T: the medium survival days of the treated group with the test compound; C: the medium survival days of the non-treated group.

Results and Discussion

Since the results shown above suggested a promising possibility that 2,4-pyrimidine derivatives having the cyclopentane ring together with the phenylalkyl group are also excellent candidates as anticell growth agents, we next tried to synthesize **21a** and **21b** as novel antitumor agents (Figure 2).

Meanwhile, each *cis*- and *trans*-isomer of **9** exhibited a unique pattern of anticancer activities in the HCC panel tests (Figure 3) (2). Since the HCC panel test was the developed assay system to profile the drugs in terms of their patterns of differential activity against the cell lines, and there are significant correlations between the patterns and the mode of drug actions (1, 2), the result of the HCC panel test of **9** suggested that a novel mechanism to inhibit the growth of tumor cells might be triggered by the amino group at C-5 of the pyrimidine ring. Therefore, an amino group was introduced onto the C-5 position of **21b** to give **22** (Figure 2).

Thus, all the derivatives prepared so far including **21a**, **21b** and **22** that have the phenylalkyl group on the cyclopentane ring, were subjected to the HCC panel test in advance of *in vivo* antitumor assays. Since the panel tests of **6, 9, 11, 21a, 21b** and **22** showed significant growth inhibition, the compounds were evaluated by the *in vivo* assay using P-388 mouse leukemia (Table I). However, none of the newly-synthesized derivatives (**21a-b**, **22**) exhibited promising results, and only compound **6** showed a T/C value higher than 120%, which is the minimum requisite value in order to advance towards further studies.

Based on the fact that **6** showed the most potent activity in the *in vivo* assay, its structure was modified to have methoxyl groups on the phenyl ring or to attach the diethyl phosphate group on the primary alcohol. Changing the alkylating reagent of diethyl malonate in the synthetic route of **6** (1)

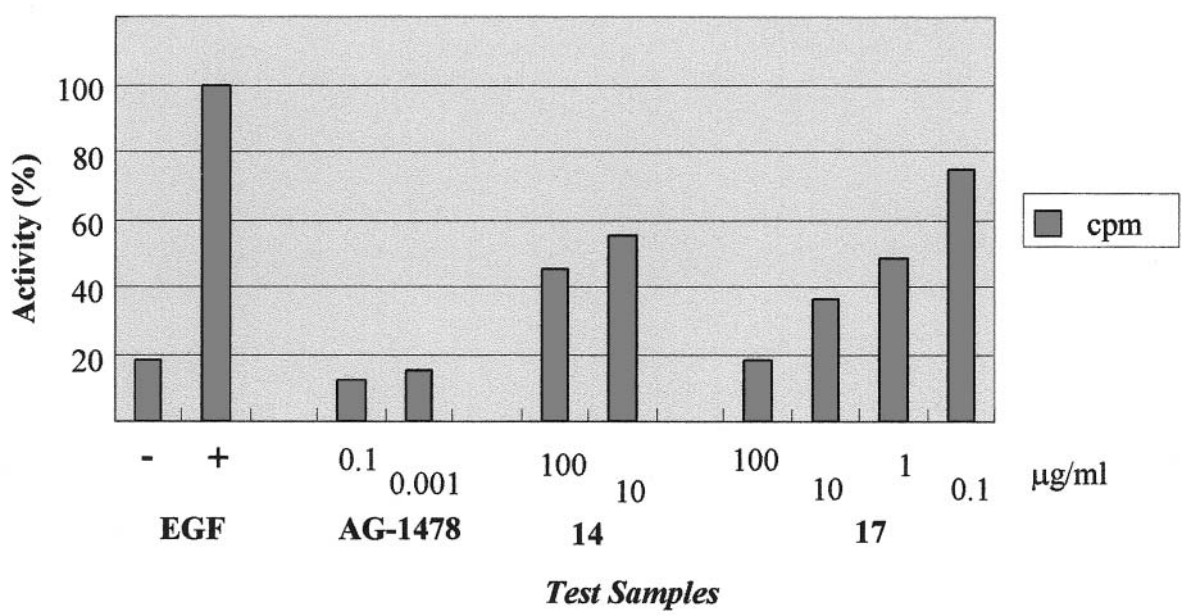


Figure 4. *EGFR kinase assay.*

from phenethyl bromide to 4-methoxyphenyl chloride, 3,4-dimethoxyphenyl chloride or 3,4,5-trimethoxyphenyl chloride, respectively, afforded **12, 13** and **14** in satisfactory yield. Phosphorylation of **6** and **12** afforded diethyl phosphates (**15, 16**), and thiomethyl-carrying derivatives (**17, 18**) on the pyrimidine ring at C-2 were also prepared by using 2-thiomethyl-4,6-dichloropyrimidine instead of 2-amino-4,6-dichloropyrimidine in the above synthetic routes (Figure 1).

After subjecting the newly prepared derivatives **(12-18)** to the HCC panel test, the inhibitory activities of **14** and **17** for protein kinases were investigated by using PKA, PKC, PTK, EGFR and Flt-1 (9, 10, 13), and **14** showed specific inhibition for the EGFR protein kinase at the range of 100 µg/mL (Table II) (11, 12). In addition, **17** also had much more potent activity. The phosphorylation activities were on a level similar to that of EGF(-) at 100 µg/ml and 50% inhibition was observed at 1 µg/ml (Figure 4).

On the other hand, both **15** and **16** showed inhibitory activities toward P-glycoprotein that excrete antitumor agents out of tumor cells (14-17). The potency of the inhibitory activity of **15** was 121% based on that of verapamil at the concentration of 0.1 µg/ml (Table III). Therefore, it can be suggested that introducing three methoxy groups to the phenyl ring or diethyl phosphate group on the primary alcohol of the 2,4-diaminopyrimidine derivatives (**1-11**) could enhance their antitumor activities.

As shown above, 2,4-diaminopyrimidine derivatives, that were designed and synthesized as antiviral agents, have potential to act as antitumor agents on modification of their structures. Further studies on the relationship between structure and bioactivities are in progress.

Table III. *Inhibitory activities of compounds **15** and **16** toward P-glycoprotein.*

Compound	c(µg/ml)	Average dpm/well	% of control	Effect	Relative %
	10	3692	398	++	121
15	1	1758	190	+	119
	0.1	1134	122	+	118
	10	3393	366	++	111
16	1	1473	159	+	99
	0.1	1165	126	+	121
	10	3050	329	++	100
Verapamil	1	1486	160	+	100
	0.1	959	103	±	100
	0	927			

Acknowledgements

This research was, in part, financially supported by the Frontier Research Program and the 21st Century Center of Excellence Program "Development of Drug Discovery Frontier Integrated from Tradition to Proteome" of the Ministry of Education, Culture, Sport and Technology, Japan. We also thank the Screening

Committee of New Anticancer Agents supported by a Grant-in-Aid for Scientific Research on Priority Area "Cancer" from the Ministry of Education, Science, Sports and Culture, Japan.

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Received October 18, 2005
Accepted November 3, 2005