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6,12-Dihydro-1-benzopyrano[3,4-*b*] [1,4]benzothiazin-6-ones: Synthesis and *mdr* Reversal in Tumor Cells

ANAMIK SHAH[1], YOGESH NALIAPARA[1], DINESH SUREJA[1], NOBORU MOTOHASHI[2], MASAMI KAWASE[3], CSILLA MISKOLCI[4], DIANA SZABO[4] and JOSEPH MOLNÁR[4]

[1]*Department of Chemistry, Saurashtra University, University Road, Rajkot-360 005, India;*
[2]*Meiji College of Pharmacy, Tanashi-shi, Tokyo 188, Japan;*
[3]*Faculty of Pharmaceutical Sciences, Josai University, Saitama, 350-0290;*
[4]*Faculty of Medicine, Institute of Microbiology, Albert Szent-Györgyi Medical University, Szeged, H-6720 Hungary*

Abstract. *Six 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones and three coumarins were systematically investigated for reversal of multidrug resistance of bacteria and cancer cells in model experiments. 7-Methylcoumarin was able to eliminate the E. coli plasmid significantly; however, the other derivatives were ineffective. Four of 6,12-dihydro-1-benzopyrano[3,4-b][1,4] benzothiazin-6-ones had a moderate effect on the multidrug resistance efflux pump of mouse lymphoma cells in vitro. Despite of the similarity of resistance mechanisms of bacteria and tumor cells, the reversal of drug resistance in bacteria and in cancer cells is not uniform because the structure- activity requirements are apparently different.*

In cancer therapy, the development of resistance, including multidrug resistance for traditionally used medicines, is now a major problem. Patients need new type of medicines or special drugs to reduce the drug resistance. Therefore, some benzo[*a*]phenothiazines were planned for synthesis in our laboratories. As shown earlier, benzo[*a*]phenothiazines have antitumor activity against some tumor cells (1), induce significant apoptosis on human myelogenous leukemic cell lines such as ML-1, U-937 and THP-1 (2), and affect the element content of tobacco tissue culture and hormone requirement (3). 6,12-Dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones are structurally very similar to the structures of benzo[*a*]phenothiazines (Figure 1). Some 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones showed an effect on the multidrug resistance efflux pump of mouse lymphoma cells *in vitro* (4). The coumarins which are included in these compounds have also shown antitumor activity by the inhibition of HIV integrase (5). Based on our estimated structure-activity relationship, it was expected that 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones may have some antitumor activity and may cause reversal on resistant tumor cells. The purpose of this paper is to show the *mdr* reversal activity of seven 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones on tumor cells with multi-drug resistance.

Correspondence to: Dr. Anamik Shah, Department of Chemistry, Saurashtra University, University Road, Rajkot-360 005, India. Tel: (+91)-281-78512; Fax: (+91)-281-77633 (local time 11.00am to 5:30pm only available)

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Materials and Methods

Melting points were determined in open glass capillaries in a paraffin bath and are uncorrected. ^{1}H-NMR spectra were performed on a JEOL JNM-GSX 500 (500 MHz) spectrometer using TMS as internal standard (δ=0). Aromatic protons are represented as ArH. Infrared (IR) spectra were recorded on a JASCO IR810 spectrometer. Mass spectra (MS) were recorded on a JEOL-JMS-DX300 spectrometer with direct inlet system at 70eV. TLC (Thin layer chromatography) was performed on a Merck Kieselgel 60 F_{254} (Merck 5549, USA).

Chemicals. Phenols were purchased from SISCO Chem Industries, Bombay, India. Phosphorus oxychloride was purchased from Spectrochem private limited Bombay, India. Zinc chloride was made anhydrous by fusion and then used. 6-Methylcoumarin [13](Aldrich, M3,620-3); 7-methylcoumarin [14](Aldrich, 22,032-9); ethyl 3-coumarincarboxylate [15](Aldrich, 39,080-1) and rhodamine 123 hydrate (R123)(Aldrich, 28394-0) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). (±)-Verapamil (Sigma, V 4629) as calcium channel blocker (3,5) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

General method of preparation of 4-hydroxycoumarins [1-6]. 4-Hydroxycoumarins [1-6] were prepared according to the methods of Shah and Bose (6,7). Phenol or substituted phenol (0.1 M) and malonic acid (0.1 M) were added to a mixture of 30 mL phosphorus oxychloride and 36 g anhydrous zinc chloride, which was preheated to 60°C and the reaction mixture was heated on a water bath at 70°C for 16-36 hours. After the reaction had finished, it was cooled and decomposed with crushed ice,

the product was crystallized and was filtered, followed by washings with water. The product was dissolved in 10% sodium carbonate, warmed if neccessary and filtered. The filtrate was slowly acidified with 3 M HCl till complete precipitation, then filtered, washed with water, dried and recrystallized from EtOH or AcOH (Scheme 1).

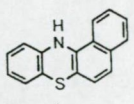
The following compounds were obtained:

a) *4-Hydroxycoumarin [1].* Yellow powders (EtOH). mp:209-210°C (Lit. 6: mp 201-203°C).
b) *6-Methyl-4-hydroxycoumarin [2].* Yellow powders (EtOH). mp:251-253°C (Lit. 8: mp 240°C).
c) *5,7-Dimethyl-4-hydroxycoumarin [3].* White powders (AcOH). mp:210-211°C (Lit. 7: mp 210-211°C).
d) *5,8-Dimethyl-4-hydroxycoumarin [4].* Yellow powders (EtOH). mp:261-262°C (Lit. 9. mp 261-262°C). ^{1}H-NMR (60 MHz, TFA) δ: 2.47 (s, 3H, CH_3), 2.49 (s, 3H, CH_3), 6.22 (s, 1H, H-3), 7.41 (br s, 1H, ArH), 7.60 (br s, 1H, ArH).
e) *7,8-Dimethyl-4-hydroxycoumarin [5].* Yellow powders (EtOH). mp:236-237°C (Lit. 7: mp 236-237°C).
f) *6-Chloro-7-methyl-4-hydroxycoumarin [6].* Yellow powders (EtOH). mp:229-230°C (Lit. 7: mp 229-230°C).

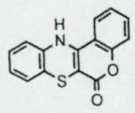
General method of preparation of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [7-12]. 6,12-Dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones [**7-12**] were prepared according to the method of K.Tabakovic *et al* (10). A mixture of appropriate 4-hydroxycoumarin [**1-6**] (0.01M) and 2-aminothiophenol (0.01M) in 25-30 mL DMSO was stirred and heated at 140-150°C for 10-13 hours. At the stage when the reaction mixture became dark, heating was stoped and the mixture slowly distilled in an excess of approximately 15-17 mL DMSO at atmospheric pressure. The reaction mass was then cooled to obtain dark colored crystallized product. This product was washed with MeOH and purified by three time washings and its purity was checked by TLC using CH_2Cl_2-MeOH (10:1) system (Scheme 1).

The following compounds were obtained:

a) *6,12-Dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [7].* Reddish brown crystals. mp:337-340°C (MeOH) (Lit. 10: mp 337-340°C).
b) *2-Methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [8].* Dark orange crystals. mp:340°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.40 (s, 3H, CH_3), 6.82-6.88 (m, 2H, ArH), 6.95-7.20 (m, 2H, ArH), 7.26 (d, 1H, *J*=8.2 Hz, ArH), 7.44 (d, 1H, *J*=8.5 Hz, ArH), 7.92 (s, 1H, ArH), 8.94 (s, 1H, NH). IR (Nujol) cm^{-1}: 3350 (NH), 1655 (C=O). MS m/e: 281 (M+, 100%). High-resolution MS: Calcd for $C_{16}H_{11}NO_2S$: 281.0511. Found: 281.0506.
c) *1,3-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [9].* Green crystals. mp:290°C (MeOH). yield: 52%. ^{1}H-NMR (DMSO-d6) δ: 2.33 (s, 3H, CH_3), 2.65 (s, 3H, CH_3), 6.68-6.73 (m, 1H, ArH), 6.79-6.85 (m, 1H, ArH), 6.87-6.98 (m, 3H, ArH), 6.98-7.40 (m, 1H, ArH), 10.10 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3300 (NH), 1630 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0653.
d) *1,4-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [10].* Light green crystals. mp: 170°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.26 (s, 3H, CH_3), 2.53 (s, 3H, CH_3), 6.35-6.45 (m, 1H, ArH), 6.65-6.76 (m, 1H, ArH), 6.80-7.10 (m, 3H, ArH), 7.28-7.37 (m, 1H, ArH), 7.96 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3300 (br, NH), 1670 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0654.
e) *3,4-Dimethyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-one [11].* Orange crystals. mp:>300°C (MeOH). yield: 50%. ^{1}H-NMR (DMSO-d_6) δ: 2.24 (s, 3H, CH_3), 2.35 (s, 3H, CH_3), 6.82-6.87 (m, 2H, ArH), 6.96-7.02 (m, 2H, ArH), 7.24 (d, 1H, *J*=8.2 Hz, ArH), 7.86 (d, 1H, *J*=8.2 Hz, ArH), 8.94 (s, 1H, NH). IR (Nujol) cm^{-1}: 3340 (NH), 1655 (C=O). MS m/e: 295 (M+, 100%). High-resolution MS: Calcd for $C_{17}H_{13}NO_2S$: 295.0667. Found: 295.0667.
f) *2-Chloro-3-methyl-6,12-dihydro-1-benzopyrano[3,4-b][1,4] benzothiazin-6-one [12].* Reddish brown crystals. mp:328°C (MeOH). yield: 45%. ^{1}H-NMR (DMSO-d_6) δ: 2.40 (s, 3H, CH_3), 6.83-6.87 (m, 2H, ArH), 6.94 (d, 1H, *J*=7.6 Hz, ArH), 6.97-7.01 (m, 1H, ArH), 7.42 (s, 1H, ArH), 8.25 (s, 1H, ArH), 8.98 (br s, 1H, NH). IR (Nujol) cm^{-1}: 3350 (NH), 1660 (C=O). MS m/e: 315 + 317 (3:1) (M^+, 100%: 38.6%). High-resolution MS: Calcd for $C_{16}H_{10}{}^{37}ClNO_2S$: 315.0121. Found: 315.0129. Calcd for $C_{16}H_{10}{}^{37}ClNO_2S$: 317.0091. Found: 317.0085.



Benzo[*a*]phenothiazine



6,12-dihydro-1-benzopyra[3,4-*b*][1,4]benzothiazin-6-ones[**7-12**]

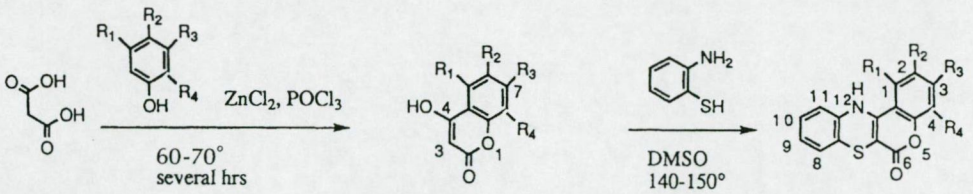
Figure 1. *Benzo[a]phenothiazine and 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [7-12].*

Biological evaluation

Measurement of antiplasmid activity. The F'lac plasmid of *E. coli* served as a convenient model in this study because the plasmid carrying colonies were easily differentiated from the plasmidless colonies on a simple eosinum methylene blue (EMB)-differential media. The antiplasmid activity of test compounds was measured on *E. coli* K12 LE140 F'lac strain in MTY broth which contained various concentrations of test compounds (11,12). After 24 hours of incubation at 37°C, various dilutions of samples were plated on EMB agar. The lactose-positive (plasmid carring) colonies and lactone-negative (plasmidless) colonies were counted (Table I).

Tissue cultures. L5178 mouse T cell lymphoma and its MDR1/A retrovirus transfected derivative were provided from Prof. Aszalos (Table II).

Cell and fluororescence uptake. MDR1/A expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain the expression of the *mdr* phenotype. The L5178 MDR cell line, and the L5178Y parent cell line were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2 x 10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquots to Eppendorf centrifuge tubes. Then, the tested compounds were added in 2.0 μL of the 2.0 mg/mL stock solution and the samples were incubated for 10 minutes at room temperature. Then, 50 μL rhodamine 123 (R123) as indicator was added to the samples (5.2 μM final concentration) and the cells were incubated for a further 20 minutes at 37°C, washed twice and resuspended in 0.5 mL phosphate-buffered saline (PBS) for analysis. The fluorescence of the cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument (cell sorter). (±)Verapamil was used as the positive control in the R123 accumulation experiments (13,14). The R123 accumulation was calculated from fluorescence of one height value using the 2nd equation $y=10^{\frac{x}{256}}$ (Table II). In the case of logarithmic transformation, the 1024 digital channels were switched to one decade at each 256 (=2^8) channels. Then, the percentage of control mean of the



1: R_1=H, R_2=H, R_3=H, R_4=H

2: R_1=H, R_2=CH_3, R_3=H, R_4=H

3: R_1=CH_3, R_2=H, R_3=CH_3, R_4=H

4: R_1=CH_3, R_2=H, R_3=H, R_4=CH_3

5: R_1=H, R_2=H, R_3=CH_3, R_4=CH_3

6: R_1=H, R_2=Cl, R_3=CH_3, R_3=H

7: R_1=H, R_2=H, R_3=H, R_4=H

8: R_1=H, R_2=CH_3, R_3=H, R_4=H

9: R_1=CH_3, R_2=H, R_3=CH_3, R_4=H

10: R_1=CH_3, R_2=H, R_3=H, R_4=CH_3

11: R_1=H, R_2=H, R_3=CH_3, R_4=CH_3

12: R_1=H, R_2=Cl, R_3=CH_3, R_3=H

Scheme 1. *Synthesis of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin -6-ones[**7-12**].*

fluorescence intensity was calculated for parental and *mdr* cell lines, compared to untreated cells. An activity ratio was calculated by the following equation (13,15)(Table II):

$$\text{Ratio} = \frac{(\textit{mdr}\text{ treated}/\textit{mdr}\text{ control})}{(\text{parental treated}/\text{parental control})}$$

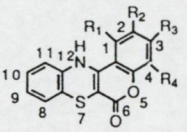
Results and Discussion

Antiplasmid activity by elimination of F'lac. Antiplasmid activity by elimination of F'lac was tested and only one compound the 7-methylcoumarin [**14**] had a remarkable effect, while three other derivatives [**8**], [**11**] and [**12**] had slight effect. The other coumarins [**7, 9, 10, 13, 15**] were ineffective (Table I).

The activity of 7-methylcoumarin [**14**] is probably related to the electrophilic-superdelocalization induced by methyl substitution at 7 position. If this correlation were true then we would expect similar effects in case of compounds [**9, 11, 12**]; however, these compounds were weak. The effect of [**9**] was reduced by methylation at position 1 due to a compensating electronic distribution.

mdr Reversal on tumor cells. The effect of 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones [**7-12**] and coumarins [**13-15**] was tested on the *mdr* reversal on tumor cells. The coumarins used in this study were slightly effective (Table II). Three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones of [**8**] (Fluorescence activity ratio, 1.56), [**9**] (Fluorescence activity ratio, 1.43) and [**10**] (Fluorescence activity ratio, 1.11) at 20 μg concentration had a moderate activity on *mdr* reversal (Fluorescence activity ratio>1). However, three 6,12-dihydro-1-benzopyrano[3,4-*b*][1,4]benzothiazin-6-ones [**7,11,12**] (Fluorescence activity ratios, 0.57,

Table I. *Antiplasmid activity of 6,12-dihydro-1-benzopyrano[3,4-b][1,4]benzothiazin-6-ones [**7-12**] and related coumarins [**13-15**].*



Compd's No.	R_1	R_2	R_3	R_4	Elimination of F'lac (μg/mL)	Antiplasmid effect (%)
7	H	H	H	H	200	0
8	H	CH_3	H	H	>200	0.05/at 160 μg
9	CH_3	H	CH_3	H	200	0
10	CH_3	H	H	CH_3	200	0
11	H	H	CH_3	CH_3	200	0.1/at 160 μg
12	H	Cl	CH_3	H	>200	0.9/at 180 μg
13 (6-methylcoumarin)					200	0
14 (7-methylcoumarin)					95	32.0/at 70 μg
15 (ethyl 3-coumarin-hyphen carboxylate)					200	0

Table II. *The effect of 6,12-dihydro-1-benzopyrano[3,4-b][1-4]benzothiazin-6-ones [7-12] and related coumarins [13-15] on the mdr reversal on L-5178 tumor cells with multidrug resistance.*

Compound's number	Concentration (μg)	Forward scatter count (cell size)	Side scatter count (granulation of cell)	Fluorescence one height[a]			Fluorescence activity ratio[c]
				x^b	$\frac{x}{256}$	$y=10^{\frac{x}{256}}$	
par[d)]	control	501.31	335.65	781.9520	3.0545	1133.80	–
mdr + R123[e)]	control	552.47	385.44	326.0672	1.2737	18.78	1.00
(±)-veraparnil[f)]	8 μg	540.50	381.20	563.8161	2.2024	159.37	8.49
7	2 μg	551.31	375.07	246.9632	0.9647	9.22	0.49
	20 μg	533.43	400.03	263.5264	1.0294	10.70	0.57
8	2 μg	557.39	375.09	263.6261	1.0298	10.71	0.57
	20 μg	541.54	388.52	375.3984	1.4664	29.27	1.56
9	2 μg	553.48	379.05	298.3680	1.1655	14.64	0.78
	20 μg	544.72	375.61	366.0288	1.4298	26.90	1.43
10	2 μg	551.91	385.77	291.9680	1.1405	13.82	0.74
	20 μg	543.69	380.65	337.7408	1.3193	20.86	1.11
11	2 μg	540.58	368.60	235.6851	0.9206	8.33	0-44
	20 μg	553 07	374.34	269.1072	1.0512	11.25	0.60
12	2 μg	550.50	386.64	251.5712	0.9827	9.61	0.51
	20 μg	544.76	382.16	275.4304	1.0759	11.91	0.63
13	2 μg	558.61	396.64	254.8736	0.9956	9.90	0.53
	20 μg	559.92	389.56	252.8512	0.9877	9.72	0.52
14	2 μg	559.13	393.86	265.9840	1.0390	10.94	0.58
15	2 μg	522.56	397.48	264.1408	1.0318	10.76	0.57
	20 μg	513.96	371.64	275.8144	1.0774	11.95	0.64

[a]Ref.15. [b]x: Measured fluorescence value at linear scale [μg, uptake of R123].
[c]The R-123 accumulation was calculated from fluorescence of one height value using 1st equation $=10^{\frac{x}{256}}$; the fluorescence activity ratios were calculated according to the formula given below;

$$\text{Ratio} = \frac{(\textit{mdr} \text{ treated}/\textit{mdr} \text{ control})}{(\text{parental treated}/\text{parental control})}$$

[d]par: parental without multidrug resistant gene; [e]*mdr*: parental with multidrug resistant gene.
[f](±)-verapamil: a control for *mdr* reversal.

0.60 and 0.63, respectively) without methyl or benzo group at positions 1 or 2 together reduced the rhodamine accumulation in tumor cells, probably by inducing the efflux pump mechanism or by causing a direct membrane injury (Table II). Position 4 must be free or low in electron density for the *mdr* reversal effect. The one exception is [**10**], in which the substitutions at positions 1 and 4 neutralized each other.

The ineffectivity of the compounds was probably correlated with the lower or reduced cell size, however, granulation did not change remarkably in the cells. There was no toxic effect in the applied concentration, and the cell size did not change in flow cytometry.

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Plasmid Elimination and Immunomodulation by 3-Benzazepines *in Vitro*

NOBORU MOTOHASHI[1], MASAMI KAWASE[2], SETSUO SAITO[2], CSILLA MISKOLCI[3], LIVIA BEREK[3] and JOSEPH MOLNÁR[3]

[1]*Meiji Pharmaceutical University, Tokyo, Japan;* [2]*Faculty of Pharmaceutical Sciences, Josai University, Saitama, Japan;* [3]*Department of Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary*

Abstract. *For studying the mechanisms of biological activity on 3-benzazepines, antimicrobial effect, F'lac plasmid elimination activity (a plasmid curing effect on F'lac plasmid) and antibody-dependent cellular cytotoxicity (ADCC) test were performed. A weak antiplasmid effect was found at sub-inhibitory concentrations. A combination of [KF4] with verapamil [2] did not alter the ineffectivity, however, [KF4] could inhibit the antiplasmid effect of promethazine, as compared to the control (promethazine alone) plasmid curing effect. A competition between promethazine and [KF4] might exist in plasmid elimination effect. ADCC activity of human leukocytes was enhanced by KF1, KF2, KF3, DA and NE at 1.0 μg/mL concentrations. The majority of 3-benzazepines [KS02, KM57, KN50, KE04, KI10, KP80] was ineffective for plasmid curing, however, inhibited the ADCC reaction, but they did not show a real dose-dependent effect.*

3-Benzazepines have been tested for anti-plasmid. DNA-complexing, reversal of multidrug resistance (MDR) of tumor cells, inhibitory effects of reverse transcriptase of Moloney leukemia virus, and cytotoxic activity for HL-60 cells (1, 2). A phenothiazine and its derivatives have a plasmid curing effect on F'lac plasmid and other plasmids of a multiple antibiotic resistant *E. coli* (3, 4, 5). The purpose of this paper was to investigate the synergism, additive effect and antagonism on plasmid-eliminating activity of 3-benzazepines with known resistance modifiers such as promethazine or verapamil. Compound [KP80] (ST450570) seems to have reasonable activities against all 60 cancer cell lines (7).

Materials and Methods

Chemicals. KS02, KM57, KN50, KE04, KI10, KP80, KF1-KF4, dopamine (DA) and norepinephrine (NE) were obtained previously (9). The following chemicals and reagents were obtained from the indicated companies: promethazine (pipolphen) (EGYT, Budapest, Hungary) and verapamil [VP] (Chinoin, Budapest, Hungary) (Table I).

Correspondence to: Prof. Noboru Motohashi, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose-shi, Tokyo, 204-8588, Japan. Tel and Fax: (+)-81-424-95-8953.

Key Words: 3-Benzazepines, plasmid elimination.

Bacterial strain. Escherichia coli K12 LE140 (T_6^r, T_1^s, Sm^R, lac$^-$ delta, Su^-, I^R, Mal^-).

Culture media. Tryptone-yeast extract (MTY) liquid media was used for the cultivation and the determination of live cell counts in the cultures. Eosin-methylene blue (EMB) agar was used for the differentiation of *lac* negative (*lac*$^-$) and lac positive (*lac*$^+$) colonies (8).

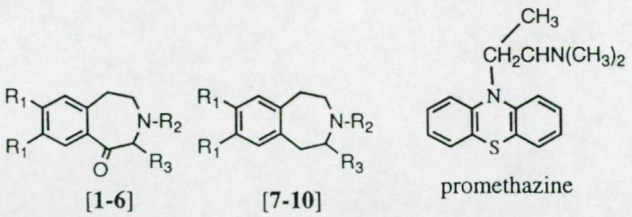
Assay for F'lac plasmid elimination activity. An overnight preculture of *Escherichia coli* K12 LE140 was diluted 10^{-4} and inoculated in 0.05 mL aliquots (approximately 5 x 10^3 cells) into 5.0 mL MTY nutrient broth. The various concentrations of promethazine (20-100 μg/mL), KSO2, KM57, KN50, KE04, KI10, KP80, KF1-KF4, DA and NE (20-200 μg/mL) were added to the bacterial cultures. Combinations of promethazine (20-100 μg/mL) plus [KF4] (100 μg/mL), or combinations of verapamil (200 μg/mL) plus [KF4] (10-100 μg/mL) were added respectively, and the tubes were incubated at 37°C for 24 hours without shaking. Two dilutions with 10^{-4} and 10^{-5} concentrations were prepared from tubes showing growth and plated in 0.1 mL amounts on EMB agar. The plates were incubated at 37°C for 24 hours, then counted for *lac*$^-$ plasmidless (pink) and *lac*$^+$ plasmid containing (deep violet) colonies. The percentage (%) of plasmid elimination was counted.

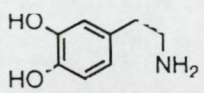
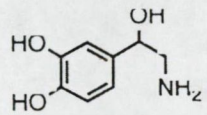
Isolation of mononuclear cells. The compounds were dissolved in DMSO, final concentrations were 1.0, 5.0, 10.0 μg/mL, in all triplicate test cultures. Both control cultures and test cultures contained DMSO in the same concentration. Human peripheral blood mononuclear cells (PBM) were isolated from five healthy blood donors by Ficoll-Uromiro (Pharmacia LKB, Bracco Ind. Chim, France) gradient centrifugation. The cells were washed and resuspended in RPMI-1640 medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (Gibco, Grand Island. NY, U.S.A.), 100 IU/mL penicillin, 100 μg/mL streptomycin sulfate and 2 mM L-glutamine. Monocyte-free peripheral blood mononuclear cells (PBM-Mo$^-$) was prepared by treating the freshly drawn heparinized blood at 37°C for 5 min with carbonyl iron powder, before separation on Ficoll-Uromiro gradient. Viability of the cells incubated with the drugs were determined by trypan blue stain after 4 hr incubation (6).

Antibody-dependent cellular cytotoxicity (ADCC) test. Human O Rh-positive red blood cells (used as target) and PBM-Mo$^-$ (used as effector) were mixed at 1:10 ratio. The reaction was mediated by red blood cell specific anti-Rh(D) antibody (6). The cultures were incubated at 37°C for 16 hr and the amount of released ^{51}Cr into the culture supernatants was determined with gamma-counter. From the average of triplicate

0250-7005/99 $2.00+.40

Table I. *Structures of six 2,3,4,5-tetrahydro-1H-3-benzazepinones [1-6], four 2,3,4,5-tetrahydro-1H-3-benzazepines [7-10], two catechols [11, 12] and promethazine used in this study*[a].



Compound	R_1	R_2	R_3
Benzazepinones			
KS02[**1**]	MeO	Ms	Me
KM57[**2**]	MeO	Tf	Me
KN50[**3**]	H	Tf	H
KE04[**4**]	MeO	Tf	H
KI10[**5**]	MeO	Tf	Me_2CH
KP80[**6**]	MeO	Tf	Ph
Benzazepines			
KF1[**7**]	HO	H	CF_3
KF2[**8**]	HO	Me	CF_3
KF3[**9**]	HO	H	H
KF4[**10**]	MeO	H	CF_3
Dopamines			
DA [**11**]			
NE [**12**]			

a) abbreviations. Me: CH_3; Ms: SO_2CH_3; Tf: SO_2CF_3.

released values, the percentage of cytotoxicity was calculated by the following formula.

$$\% \text{ Cytotoxicity} = 100 \times \frac{\text{test } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}{\text{maximum } ^{51}\text{Cr release} - \text{spontaneous } ^{51}\text{Cr release}}$$

Spontaneous release indicated those cultures without anti-D antibody. The results are expressed in percent of control cultures.

Results and Discussion

Antimicrobial effect. The antimicrobial action of 3-benzazepines is very small. The majority of compounds had no MIC values below 200 μg/mL. Antiplasmid effects and ion mutant selection are insignificant even at 320 μg/mL (data not shown).

The antiproliferative effects on parental control (Par) and MDR tumor cell lines were the same, resulting in a high

Table II. *F'lac plasmid elimination of 3-benzazepines and related compounds.*

Compound	Concentration (μg/mL)	Plasmid elimination (mean %)
control	0	0.00
promethazine	20	0.00
	40	0.00
	60	20.95
	80	84.00
	100	-(MIC)
Benzazepinones		
KS02	20	0.45
	40	0.00
	100	0.00
	200	18.75
KM57	20	0.59
	40	0.15
	100	0.00
	200	13.76
KN50	20	0.00
	40	0.00
	100	0.37
	200	21.88
KE04	20	0.01
	40	0.00
	100	0.00
	200	0.00
KI10	20	13.33
	40	0.20
	100	0.00
	200	0.00
KP80	20	0.00
	40	0.00
	100	0.05
	200	-(MIC)
Benzazepines		
KF1	20	0 77
	40	0.13
	100	2.44
	200	-(MIC)
KF2	20	0.00
	40	0.14
	100	1.60
	200	-(MIC)
KF3	20	0.00
	40	0.045
	100	1.20
	200	-(MIC)
KF4	20	0.00
	40	0.00
	100	0.00
	200	0.00
Dopamines		
DA	20	0.00
	40	0.00
	100	0.38
	200	0.00
NE	20	0.00
	40	0.00
	100	0.00
	200	0.14

Table III. *E. coli F'lac plasmid elimination by a combination of promethazine with KF4 [10].*

Compound	Concentration (μg/mL)	Plasmid elimination (%)
control	0	0.00
promethazine	20	0.00
	40	0.10
	60	3.52
	80	70.6
	100	-(MIC)
promethazine + KF4[**10**]	20 + 100	0.00
	40 + 100	0.00
	60 + 100	0.00
	80 + 100	0.00
	100 + 100	0.00
Verapamil + KF4[**10**]	200 + 10	0.00
	200 + 20	0.00
	200 + 50	0.00
	200 + 100	0.00

Table IV. *ADCC activity in the presence of 3-benzazepines.* [1,2]

Compound	ADCC activity (% of control)		
	1 (μg/mL)	5 (μg/mL)	10 (μg/mL)
control	62	51	44
Benzazepinones			
KS02[**1**]	74	85	93
KM57[**2**]	79	85	83
KN50[**3**]	51	62	70
KE04[**4**]	60	57	66
KI10[**5**]	30	34	23
KP80[**6**]	83	78	74
Benzazepines			
KF1 [**7**]	104[3]	127[3]	125[3]
KF2[**8**]	91	111[3]	97
KF3[**9**]	101[3]	123[3]	106[3]
KF4[**10**]	96	119[3]	84

[1]The target-effector ratio was 1: 10. [2]Basic ADCC activity: without DMSO = 50%. [3]Enhancing effect of 3-benzazepines: over 100%.

toxicity. The growth inhibitory effect was found in the experiment as follows: 50% growth inhibitory concentration (GI_{50}) as follows. lower than GI_{50} = 0.2 μg/mL: KF1, KF2, KF3, DA, NE; GI_{50} = 3.5 μg/mL: KS02, KM57, KN50, KE04, KI10, KP80, KF4. At 5.0 μg/mL, the inoculated cells could not grow after three days (Table II). 100% inhibitory concentrations for KF1, KF2, KF3, DA and NE was 1 μg/mL.

Combination of KF4 with verapamil. The combination of [KF4] with verapamil did not alter the ineffectivity on plasmid elimination (Table III). The effect on reversal of MDR was dependent on tertiary nitrogen (N)-atom after methyl substitution at 3nd position (R_2) of 3-benzazepine ring. For reverse transcriptase (RT) inhibition, the R_1 can be hydroxy, however, methoxy substituent at R_1 cancelled this effects in case KF4, despite the fact that methoxy substituent favours for DNA complexing effect (2).

Antiplasmid effect. Compound [KF4] could inhibit the antiplasmid effect of promethazine, compared to control (promethazine alone) plasmid curing effect. Compound [KF4] alone could not eliminate the metabolic plasmid of *E. coli* in the presence of 200 μg/mL of verapamil (Tables II and III). This suggests that competition between promethazine and [KF4] exists in plasmid elimination effect. Nevertheless, a combination of promethazine with verapamil of a known multidrug resistance (MDR) modifier was not synergistic (data not shown).

ADCC activity (Interpretation of immunomodulating effect of the drug require information about direct cytotoxic potential of compounds). A trypan blue indicator assay was used to determine the relative number of viable cells in the tested cell population. The cell viability was between 85% and 90% with all compounds. The reaction was calculated on viable cell number (Table IV).

The majority of 3-benzazepines [KS02, KM57, KN50, KE04, KI10, KP80] inhibited the ADCC reaction, but they did not show a real dose-dependent effect. Compound [KI10] had a significant inhibitory effect which could be detected even at low concentrations. Four other 3-benzazepines [KF1-KF4] caused a low stimulatory effect on ADCC reaction, especially [KF1] (Table IV).

Interestingly, four compounds [KF1-KF4] have some enhancing effects on ADCC activity that can be considered as immunomodulatory effects, although five other compounds

[KS02, KM57, KN50, KE04, KP80] except [KI10] had inhibitory effects (Table IV). Based on the modulation of ADCC, the ten 3-benzazepines tested can be classified into two groups, such as four compounds with enhancing the ADCC activation of 3-benzazepines [KF1-KF4] and the other six 3-benzazepines [KS02, KM57, KN50, KE04, KI10, KP80], which reduced the ADCC.

As new biological effects of 3-benzazepines are confirmed, additional targets for possible plasmid elimination are generated. The insights gained in this paper provide optimism for effective anticancer chemotherapy in the future.

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Guanine–cytosine rich regions of plasmid DNA can be the target in anti-plasmid effect of phenothiazines

Csilla Miskolci [a,*], Imre Labádi [b], Teruo Kurihara [c], Noboru Motohashi [d], Joseph Molnár [a]

[a] *Institute of Microbiology, Albert Szent-Györgyi Medical University, Dóm Tér 10, H-6720 Szeged, Hungary*
[b] *Department of Inorganic and Analytical Chemistry, Attila József University, Dóm Tér 10, H-6720 Szeged, Hungary*
[c] *Department of Organic Chemistry, Faculty of Science, Josai University, Sakado-shi, Saitama 350-0290, Japan*
[d] *Meiji College of Pharmacy, Tanashi-shi, Tokyo 188-0001, Japan*

Abstract

The antiplasmid effects of promethazine on *E. coli* is the consequence of special complex formed with a covalently closed circular (ccc) form of plasmid DNA. The exact target in this macromolecule, however, was not clarified until recently. Caffeine and the chemically similar guanosine-5′- monophosphate (GMP) could compete with the antiplasmid effect of promethazine, showing that promethazine or other phenothiazines preferentially bind to xanthine type molecules. Among the xanthines, GMP was more effective at complex-forming than adenosine-5′-monophosphate (AMP). In addition, the Z-DNA was more susceptible than B-DNA. Therefore, one could suppose that guanine–cytosine (G–C) rich regions have higher affinity than adenine–thymine (A–T) rich region on phenothiazines. Because the G–C rich regions have a special role in the DNA stability via three hydrogen bonds, we suppose that these regions could have a key role in some biological effects such as antiplasmid and anticancer activity.

Keywords: Plasmid; Promethazine; Caffeine; Guanosine monophosphate (GMP)

1. Introduction

The phenothiazines have antibacterial, antiplasmid and antimutagenic effects on various bacteria [1–4]. It was suggested that these effects can be correlated with the binding of phenothiazines to nucleic acids by intercalation [5,6], or other type of charge transfer (CT) complex formation [7]. This suggestion is based on experimental evidence for complex formation between phenothiazines and macromolecules, such as nucleic acids, bacterial endotoxin [8], and protein A [9]. However, in most cases, the types of the binding are not exactly known. To clarify the effect of phenothiazines on bacteria, a study of possible type of interaction between phenothiazines and nucleic acids was started.

The determination of the exact binding points is difficult because the macromolecules, such as nucleic acid or proteins, have different groups which can take part in their binding. Consequently, interactions of different types have similar energy and cause the similar changes between or among the corresponding molecules.

Phenothiazine molecules are heterocyclic and contain hydrogen (H), carbon (C), nitrogen (N) and sulfur (S) atoms for the formation of the coordination bonds. Phenothiazines used in medicine consist of an aliphatic linker with cationic tertiary N atom at the end of the linker and side groups such as OH, Cl or CF3. A coordination bond may be formed by a $\pi-\pi$ stacking interaction, ionic interaction and hydrogen bonds among the linker and side groups of phenothiazines, and macromolecules. An interaction between nucleic acid and phenothiazine may be formed by the binding between base moiety, external sugar and phosphate moiety, or by the multiple bindings among linker and groups simultaneously. The different phenothiazines may prefer different parts of nucleic acids, consequently forming the different types of association. Each phenothiazine could produce different biological effects by

* Corresponding author. Tel.: +36-62-455115; fax: +36-62-455113.

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the association between the macromolecules with each specific linker and side groups. Phenothiazines have no mutagenicity, but have antimutagenic effects on bacteria. They have some specificity to covalently closed circular (ccc) form of plasmid DNA, while open circular (oc) or linear (lin) forms are less sensitive [10,11]. Xanthine analogs of some electron rich acceptors can compete with the antiplasmid effects of phenothiazines [7,12], but the molecular structure of target in bacteria has not been shown clearly. The interaction of phenothiazines with guanosine mononucleotide has been described [13].

It is supposed that the antiplasmid effect of phenothiazines is due to an intercalation or formation of a charge transfer (CT) complex. The exact binding targets could not be still determined. The cationic N part on a phenothiazine linker and negative phosphate group could make an association by a hydrogen bond. This hypothesis is supported by the calculation of the geometry and energies on chlorpromazine, 2′-deoxyguanosine-5′-monophosphate and the adduct [14].

Additionally, a presence of stacking (interaction) between nucleic acid bases and phenothiazine molecules was also suggested [14]. Nucleic acid bases and their related aromatic heterocycles may take part in stacking interaction when the aromatic sheets of two or more molecules are placed parallel to each other at 3.4–3.5 A [15]. The different molecules have different stacking affinity. Compounds having many heteroatoms with the polar bonds by dipole moments can have the highest ability to form a stacking interaction. The order of the polarizing power for stacking interaction is caffeine > guanine > adenine > purine > indole. Simple aromatic compounds with mostly C–C or C=C bonds, such as indole, also have high polarizing ability — with the following order: indole > adenine > purine > guanine > caffeine.

We rechecked the determination of the active sites of the stacking interaction in plasmid of bacteria in the presence of phenothiazine. The polarizing ability of phenothiazines could be responsible for the biological activities. The antiplasmid effect of promethazine was studied in the presence of caffeine and indole respectively. The competitive effect of three xanthine analogs (caffeine, indole and GMP) was tested on the plasmid elimination effect of promethazine in vitro to determine the specificity.

2. Materials and methods

2.1. Compounds

Promethazine and chlorpromazine were purchased from EGYT, Budapest, Hungary. Caffeine and indole were purchased from Fluka and Reanal. Guanosine-5′-monophosphate (GMP) and adenosine-5′-monophosphate (AMP) were kindly given by Professor Harri Lönnberg from Turku University (Fig. 1).

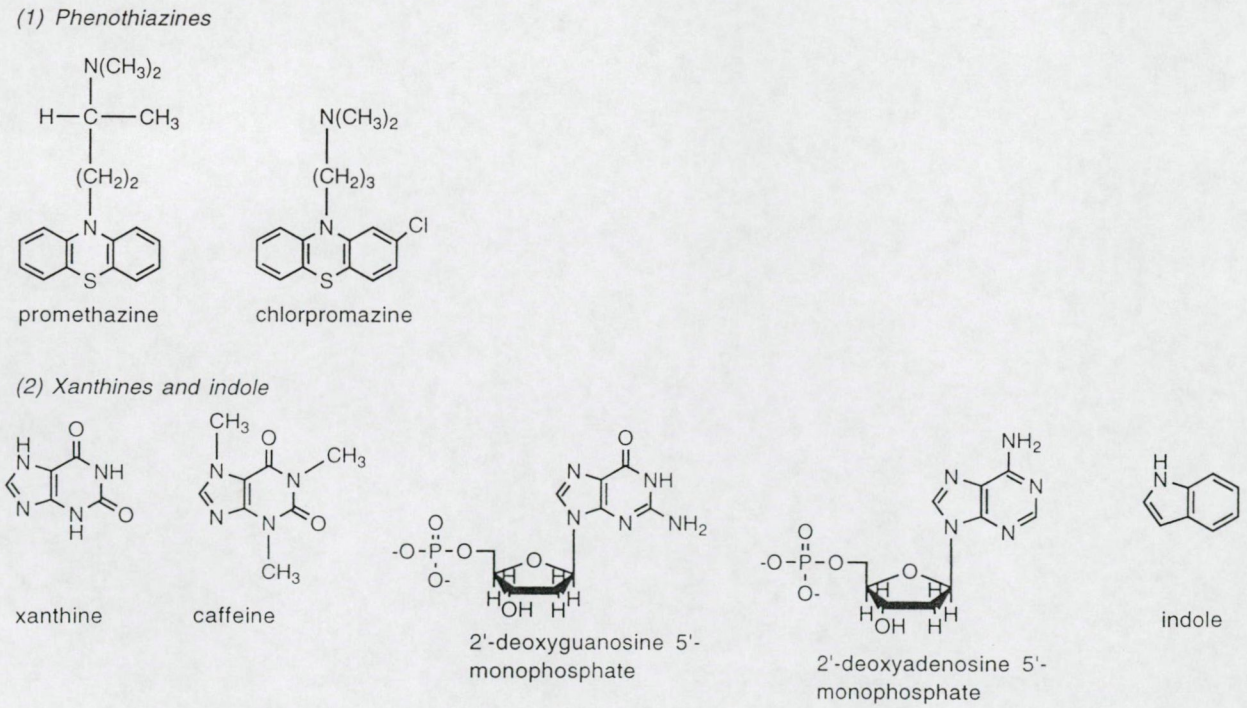


Fig. 1. Structures of phenothiazines, xanthines and indole.

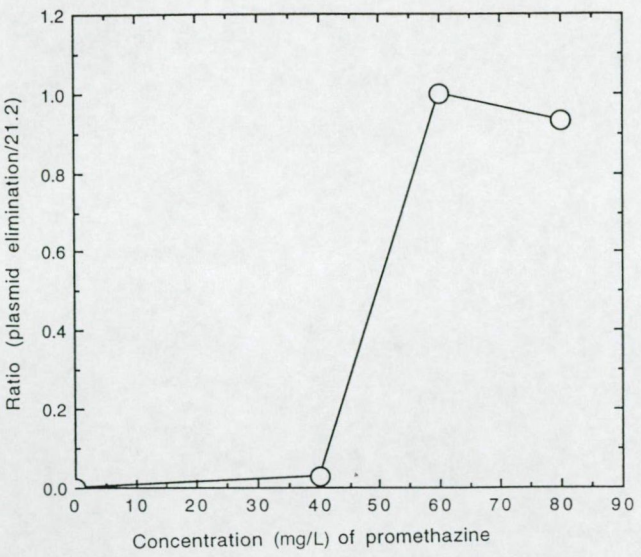


Fig. 2. Ratio of reduction of plasmid elimination by concentrations of promethazine at 0, 40, 60, 80 mg/l.

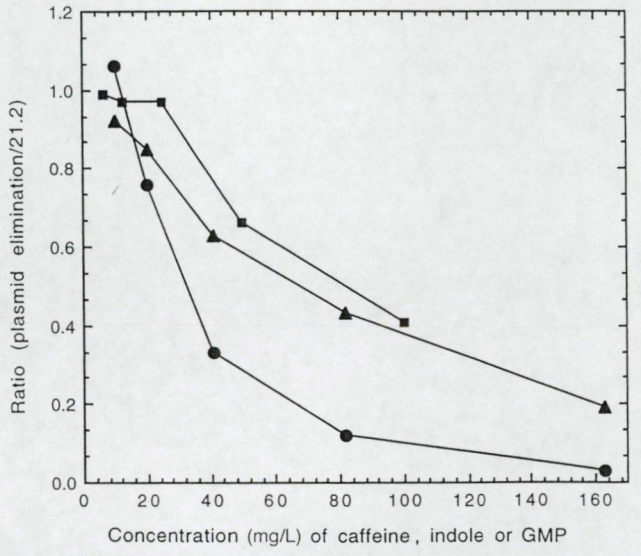


Fig. 3. Ratio of reduction for plasmid elimination by promethazine (60 mg/l) for caffeine, indole and GMP; promethazine and caffeine; promethazine and indole; promethazine and GMP.

2.2. Bacterial strain

Escherichia coli K12 LE140 (T_6r, T_1s, Sm^R, $lac^-\Delta$, SU^-, I^R, Mal^-).

2.3. Culture media

Tryptone–yeast extract (MTY) liquid media was used for the cultivation of live cell and in the determination of cell counts. Eosin-methylene blue (EMB) agar was used for the differentiation of lac negative (lac^-) and lac positive (lac^+) colonies.

2.4. Methods

An overnight culture of *E. coli* K12 LE140 was diluted to 10^{-4} and 0.05 ml aliquots (approximately 5×10^3 cells) was inoculated into 5.0 ml MTY broth. The various concentrations of caffeine (10–163 mg/l) and indole (6.25–100 mg/l) plus promethazine in 60 mg/l concentration were added, and the tubes were incubated at 37°C for 24 h without shaking. Two dilutions (10^{-4} and 10^{-5} concentrations) were prepared from tubes showing growth and plated in 0.1 ml amounts on EMB agar. The plates were incubated at 37°C for 24 h, then counted for pink (lac^-) and deep violet (lac^+) colonies. The percentage (%) of plasmid elimination was determined.

3. Results and discussion

Promethazine was effective in the elimination of plasmid in the study (Table 1 and Fig. 2).

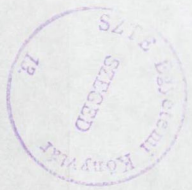
Caffeine significantly reduced the antiplasmid effect of promethazine and the extent of reduction was concentration-dependant of caffeine (Table 1 and Fig. 3). Using caffeine alone, the F′ lac plasmid was not eliminated from the bacterial cells. The observed reduction by caffeine of the plasmid elimination activity of promethazine suggested a combination effect of two compounds. A competition between caffeine and some physiological substrates which have a structural similarity could be suggested. These substrates may be a purine base of DNA, e.g. guanosine monophosphate (GMP). The combination of promethazine and caffeine (60:10) resulted in plasmid elimination (Table 1 and Fig. 3).

Indole reduced the effect of promethazine slightly, depending on the concentration. The reducing effect by GMP was more than that of indole (Table 1 and Fig. 3). A combination of promethazine and GMP did not show a significant reduction of plasmid elimination by promethazine, compared to the combination effect with caffeine. The extent of reduction of plasmid elimination was more similar in the presence of GMP than in the presence of indole (Table 1 and Fig. 3).

A third molecule, one of caffeine, GMP and indole, could influence the complex formation between promethazine and plasmid DNA in the following ways: (i) the third molecule could form a stronger complex with plasmid DNA or with a promethazine. The three compounds of this third molecule would be the competing agents against promethazine in the interaction with plasmid DNA. (ii) The third molecule could also form a stronger complex with promethazine than the plasmid DNA. Consequently, the concentration of free promethazine would decrease.

Table 1
Reduction of plasmid elimination by promethazine alone and combined with caffeine, indole or GMP

Compound	Concentration (mg/l)	Plasmid elimination (%)			Average of plasmid elimination (%)	Number of colony			Average of number of colony	Ratio (plasmid elimination average/21.2)
Promethazine	0	0.00	0.00	0.00	0.00	5150	7051	6861	6687	0.00
	40	0.00	1.30	0.50	0.60	2501	3164	1815	2493	0.00
	60	20.30	25.60	18.20	21.20	596	472	509	526	1.00
	80	21.95	19.80	17.10	19.61	103	115	201	140	0.93
Promethazine + caffeine	60+10	22.30	26.06	19.02	22.46	615	423	550	529	1.06
	60+20	21.80	12.80	14.48	16.12	639	501	504	548	0.76
	60+41	6.80	8.30	6.05	7.05	581	403	502	495	0.33
	60+82	1.88	3.45	2.33	2.55	565	440	475	493	0.12
	60+163	0.05	1.60	0.25	0.63	563	425	490	493	0.03
Promethazine + indole	60+6.25	19.80	24.30	19.05	21.05	513	525	483	507	0.99
	60+12.50	18.10	25.10	18.52	20.57	581	498	427	430	0.97
	60.25	21.60	22.05	18.03	20.56	576	471	435	494	0.97
	60+50	10.50	18.20	13.45	14.05	503	440	455	466	0.66
	60+100	9.98	6.80	9.30	8.69	598	435	471	501	0.41
Promethazine + GMP	60+10	19.50	20.05	19.10	19.55	610	502	510	541	0.92
	60+20	16.30	18.20	19.76	18.08	575	481	451	502	0.85
	60+41	12.10	16.05	10.40	13.40	517	490	466	492	0.63
	60.82	12.50	5.10	9.60	9.07	581	416	413	470	0.43
	60+163	6.30	3.20	2.30	3.93	566	404	445	472	0.19



In the first case, the third molecule has the characteristics of promethazine. In the second case, the plasmid DNA-like compounds reduces the antiplasmid effect of promethazine. It was observed that phenothiazines could also form some complexes with xanthine dyes, or some nucleotides.

High performance liquid chromatography (HPLC) measurement has shown the formation of a charge transfer (CT) complex in a chlorpromazine–GMP system [16,17]. The chlorpromazine–GMP system has non-Newtonian properties in viscosity [16,17]. The difference between Raman spectra of a chlorpromazine–GMP system predicts an interaction between the dimethylaminopropyl group of chlorpromazine and phosphate group of GMP [16,17]. It can therefore be suggested that the stacking interaction is present on a complex formation between promethazine and caffeine or indole. The interaction of a complex formation between promethazine and caffeine is stronger than that of a complex formation between promethazine and indole. If two interaction types including stacking exist, it means that at least two types of interactions are present for the complex formation between phenothiazines and plasmid DNA. One is an ionic interaction between the linker of phenothiazine and phosphate group of DNA. The second is a stacking interaction between aromatic sites of phenothiazines and nucleic acid bases such as guanosine, with a higher affinity than that of adenosine. On the basis of the weaker stacking interaction, the weaker antiplasmid competition effect of adenosine monophosphate (AMP) can be understood.

We concluded that promethazine might form a complex with two guanine–cytosine (G–C) rich regions of DNA. The native uncomplexed sites of the G–C rich regions are necessary for normal plasmid replication. Their phenothiazine complexes, however, should not maintain the physiological functions of the G–C rich regions in the ccc form of plasmid DNA.

To confirm this hypothesis, we plan to study the interaction of phenothiazines and some xanthine dyes, which also reduce the antiplasmid effect of phenothiazines to varying extents.

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Biological Activity of Kiwifruit Peel Extracts

Noboru Motohashi[1]*, Yoshiaki Shirataki[2], Masami Kawase[2], Satoru Tani[2], Hiroshi Sakagami[3], Kazue Satoh[4], Teruo Kurihara[5], Hideki Nakashima[6], Kristina Wolfard[7], Csilla Miskolci[7] and Joseph Molnár[7]

[1]Meiji Pharmaceutical University, Kiyose, Tokyo, Japan
[2]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama, Japan
[3]Department of Dental Pharmacology, Meikai University School of Dentistry, Saitama, Japan
[4]Analysis Center, School of Pharmaceutical Sciences, Showa University, Shinagawa-ku, Tokyo, Japan
[5]Faculty of Sciences, Josai University, Sakado, Saitama, Japan
[6]Department of Microbiology and Immunology, Kagoshima University Dental School, Kagoshima-shi, Kagoshima, Japan
[7]Institute of Microbiology, Albert Szent-Györgyi Medical University, Dóm tér 10, H-6720 Szeged, Hungary

Various bioactive substances in kiwifruit extracts were fractionated by organic solvent extractions, followed by silica gel and ODS chromatographies. Both cytotoxic activity and multi-drug resistance reversal activity were found in the less polar fractions. Cytotoxic activity was not always parallel the radical intensity. Antibacterial activity was distributed into various fractions and all fractions were inactive against *Candida albicans* and *H. pylori*. Only 70% methanol extracts showed anti-human immunodeficiency virus activity, and produced a broad ESR signal under alkaline conditions, in a fashion similar to lignin. These fractions also effectively scavenged O_2^- produced by the xanthine–xanthine oxidase reaction, suggesting a bimodal (pro-oxidant and antioxidant) action. These data suggest a medicinal efficacy of kiwifruit peel extracts.

Keywords: *Actinidia deliciosa*; Actinidiaceae; kiwifruit peel extracts; fractionation; cytotoxic activity; anti-HIV activity; radical intensity.

INTRODUCTION

Kiwifruit, *Actinidia deliciosa* (Actinidiaceae) has been mainly cultured in the tropics and subtropics such as New Zealand, Chile, France and Japan. Kiwifruits have been eaten stewed, in jams, jelly, juice and others. To assess the *in vitro* antioxidant potential of kiwifruit extracts, nucleoids are treated in the gel with H_2O_2 (Collins, 1999). Consequently, the extracts effectively reduced the H_2O_2-induced DNA breaks, suggesting its antioxidant activity (Collins, 1999). Kiwifruit extracts inhibited the adenosine diphosphate (ADP) and collagen-induced platelet activation/aggregation (Dutta-Roy, 1999). Modulation of platelet reactivity towards collagen and ADP by kiwifruit extracts may have prophylactic and therapeutic benefits in preventing and halting platelet-involved pathological processes (Dutta-Roy, 1999). Kiwifruit juice efficiently inhibits nitrosation, both *in vitro* and *in vivo*, possibly by various components such as ascorbic acid and other unidentified nitrile scavengers (Puju *et al.*, 1984). Additionally, kiwifruit is rich in ascorbic acid, which might affect cell proliferation and DNA synthesis (Bishun *et al.*, 1978).

We have investigated the biological activities of both exhausted and edible components of summerfruits (tropical and subtropical fruits), cacao, grape, feijoa and other fruits (Motohashi *et al.*, 1999; Shirataki *et al.*, 2000; Motohashi *et al.*, 2000). We investigated here the diverse biological activity of kiwifruit peel extracts.

* Correspondence to: Dr Noboru Motohashi, Meiji Pharmaceutical University, 2-522-1 Noshio, Kiyose, Tokyo 204-8588, Japan.
E-mail: motohasi@my-pharm.ac.jp
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MATERIALS AND METHODS

Materials. Kiwifruit peels were supplied from New Zealand Kiwifruit Marketing Board (ZESPRI), Auckland. The following chemicals and reagents were obtained from the companies indicated: RPMI1640 medium, Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY); fetal bovine serum (FBS) (JRH Bioscience); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Wako Pure Chemical Industries Ltd, Osaka); 3'-azido-2',3'-dideoxythymidine (AZT), dideoxycytidine (ddC) (Sigma); dextran sulphate (8 kD) (Kowa, Tokyo); curdlan sulphate (300 kD) (Ajinomoto, Tokyo); diethylenetriaminepentaacetic acid (DETAPAC) (Sigma Chemical Co., St Louis, MO); 3,4-dihydro-2,2-dimethyl-2*H*-pyrrole-1-oxide (DMPO) (a spin trap agent) (Aldrich Chemical Co. IN, USA); superoxide dismutase (SOD) from bovine erythrocytes (Dojin, Kumamoto, Japan). A strain of *Helicobacter pylori* (ATCC43504) was purchased from the American Type Culture Collection (Rockville, MD). Human red blood cells (RBCs) were collected from the heparinized human peripheral blood of normal volunteers. The *E. coli* [illegible] and *Staphylococcus epidermidis* ATCC 12228 laboratory test strains were used and *Candida albicans* was a clinical isolate.

Preparation and fractionation of kiwifruit peel extracts. Dried peels of *Actinidia deliciosa* (2.3 kg) were

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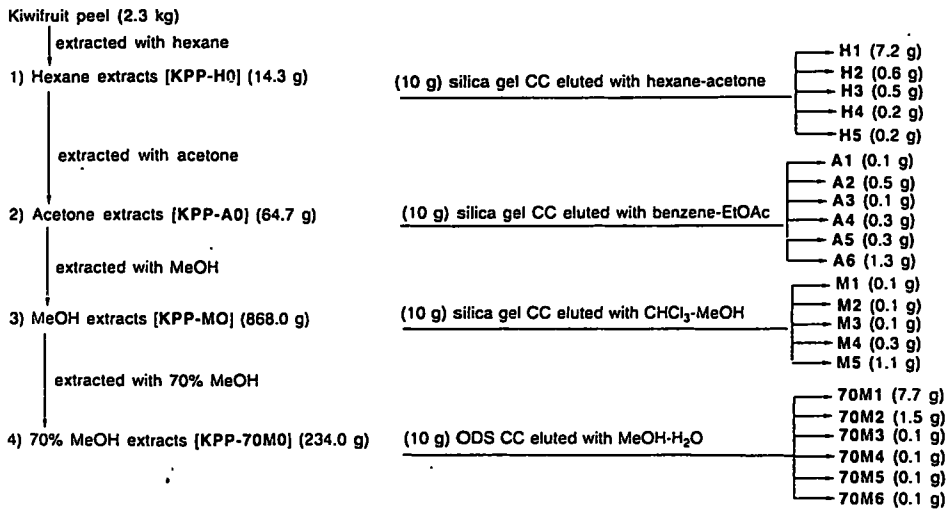

Figure 1. Fractional separation of kiwifruit peel extracts. CC: column chromatography.

successively extracted with hexane, acetone, MeOH and 70% MeOH at room temperature and the solvent was concentrated *in vacuo*, and the hexane extract (**HO**) (14.3 g), acetone extract (**AO**) (64.7 g), MeOH extract (**MO**) (868.0 g) and 70% MeOH extract (**70MO**) (234.0 g) were obtained, respectively (Fig. 1). First, an aliquot of the hexane extract (**HO**) (10 g) was applied to silica gel column chromatography, which was then eluted with a hexane–acetone gradient. The hexane fraction (**H1**) (7.2 g), hexane–acetone (9:1) fraction (**H2**) (0.6 g), (**H3**) (0.5 g), hexane–acetone (4:1) fraction (**H4**) (0.2 g), and hexane–acetone (1:1) fraction (**H5**) (0.2 g) were stepwise eluted. Second, the acetone extract (10 g) was applied to silica gel column chromatography, which was then eluted with a benzene–EtOAc gradient. The benzene fraction (**A1**) (0.1 g), benzene–EtOAc (9:1) fraction (**A2**) (0.5 g), (**A3**) (0.1 g) benzene–EtOAc (4:1) fraction (**A4**) (0.3 g), benzene–EtOAc (3:2) fraction (**A5**) (0.3 g), and benzene:EtOAc (1:1) fraction (**A6**) (1.3 g) were stepwise eluted. Third, the MeOH extract (10 g) was applied to silica gel column chromatography, which was then eluted with a $CHCl_3$–MeOH gradient. The $CHCl_3$ fraction (**M1**) (0.1 g), $CHCl_3$–MeOH (49:1) fraction (**M2**) (0.1 g), $CHCl_3$–MeOH (24:1) fraction (**M3**) (0.1 g), $CHCl_3$–MeOH (9:1) fraction (**M4**) (0.3 g) and $CHCl_3$–MeOH (4:1) fraction (**M5**) (1.1 g) were stepwise eluted. Finally, the 70% MeOH extract (10 g) was applied to ODS column chromatography, which was then eluted with a H_2O–MeOH gradient. The H_2O–MeOH (2:1) fractions (**70M1**) (7.7 g), (**70M2**) (1.5 g), (**70M3**) (0.1 g), H_2O–MeOH (1:1) fraction (**70M4**) (0.1 g), (**70M5**) (0.1 g), and (**70M6**) (0.1 g) were stepwise eluted (Fig. 1).

Assay for cytotoxic activity. Human oral squamous cell carcinoma cells (HSC-2), human salivary gland tumour cells (HSG) and human gingival fibroblasts (HGF) (5–7 population doubling levels) were cultured in DMEM medium supplemented with 10% FBS. These cells were [illegible] with the concentrations of test samples indicated, and the relative viable cell number was then determined by the MTT method and expressed as the absorbance at 540 nm (A_{540}). The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

Assay for anti-human immunodeficiency virus (HIV) activity. Human T cell leukaemia virus 1 (HTLV1)-bearing CD4 positive human T cell lines, MT-4 cells, were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV-, or as a control, mock-infected MT-4 cells (1.5×10^5/mL, 200 μL), were placed into 96-well microtitre plates and incubated in the presence of various concentrations of the test compounds. After incubation for 5 days at 37°C in a 5% CO_2 incubator, the cell viability was quantified by a colorimetric assay (at 540 nm and 690 nm), monitoring the ability of viable cells to reduce MTT to a blue formazan product (Nakashima *et al.*, 1992). All data represent the mean values of triplicate measurements (Table 1). The CC_{50} and 50% effective concentration (EC_{50}) were determined from the dose-response curve with mock-infected or HIV-infected cells, respectively. The selectivity index (SI) was defined as follows: $SI=CC_{50}/EC_{50}$.

Antibacterial activity. The experiments were done by adding 10 μL aliquots of the original solutions as a droplet to the minimal medium supplemented with 1% trypton and 0.5% yeast extract (MTY) broth and blood agar plates inoculated with 10^5 cells of the tested strains (Molnár *et al.*, 1998a). After incubation for 24 h at 37°C, the inhibitory zones in the plates were measured. As a control, 10 μL of DMSO was added to each strain. *Candida albicans* was sensitive to DMSO, whereas *E. coli* and *Staphylococcus epidermidis* were insensitive (Table 3).

Anti-*Helicobacter pylori* (*H. pylori*) activity. Mueller–Hinton broth containing 5% fetal bovine serum (FBS) was used as the medium, and was cultured in a jar conditioned with Campylo Pack (Dia Iatron) for 48 h. Briefly, *H. pylori* strains were inoculated on a Brucella agar plate containing 10% horse serum, and cultured at 37°C for 48 h. The bacterial colonies collected were diluted to 10^7 colony forming unit (CFU)/mL with 0.9% [illegible] solutions were dissolved in DMSO, and then diluted with Mueller–Hinton broth. To the solution of the fractions, a suspension of each bacterium species was added to make 10^6 CFU/100 mL/well. The mixture was incubated at 37°C for 48 h. The MIC (minimum inhibitory concentration) values of the fractions tested

Table 1. Cytotoxic and anti-HIV activity of kiwifruit peel extracts and fractions

Extract and fraction	Cytotoxic activity[a] (CC_{50}: μg/mL)				Anti-HIV activity		
	Human oral tumour cell line		Human gingival fibroblast (HGF)	SI (HGF/HSC-2)	CC_{50} (μg/mL)	EC_{50} (μg/mL)	SI (CC_{50}/EC_{50})
	HSC-2	HSG					
H0	>500	385	>500	>1	108	>200	<1
H1	>500	446	>500	>1	112	>200	<1
H2	186	269	277	1	105	>200	<1
H3	188	128	150	1	32	>40	<1
H4	338	311	299	1	103	>200	<1
H5	214	188	207	1	31	>40	<1
A0	401	340	>500	>1	123	>200	<1
A1	>500	>500	>500	>1	>200	>200	>1
A2	284	336	306	1	104	>200	<1
A3	184	170	190	1	22	>40	<1
A4	52	63	141	3	22	>40	<1
A5	138	153	156	1	120	>200	<1
A6	355	245	>500	1	71	>200	<1
M0	>500	>500	>500	>1	>200	>200	>1
M1	425	487	>500	>1	192	>200	<1
M2	116	111	189	2	=70	>200	<1
M3	373	271	480	1	=94	>200	<1
M4	>500	>500	>500	>1	>200	>200	>1
M5	>500	>500	>500	>1	>200	>200	>1
70M0	>500	>500	>500	>1	>200	>200	>1
70M1	>500	>500	>500	>1	>200	>200	>1
70M2	>500	>500	>500	>1	>200	>200	>1
70M3	>500	>500	>500	>1	>200	57	>4
70M4	>500	>500	>500	>1	118	>200	<1
70M5	>500	>500	>500	>1	>200	34	>6
70M6	368	362	392	1	>200	18	>11
Cell no. (A_{540})	0.904	0.649	0.281				
Dextran sulphate					636	4.14	154
Curdlan sulphate					>1000	0.72	>1389
AZT (μM)					276	0.033	8364
ddC (μM)					475	0.16	2969

[a] Near confluent HSC-2, HSG and HGF cells were incubated for 24 h with various concentrations of KGFP extracts, and the relative viable cell number (A_{540}) was determined by the MTT method. The 50% cytotoxic concentration (CC_{50}) was determined by the dose-response curve. Each value represents the mean of duplicate determinations. Control A_{540} values of HSC-2, HSG and HGF cells were 1.138, 0.804 and 0.507, respectively.

were determined by eye and densitometry measured at 620 nm (Numao *et al.*, 1997; Kawase *et al.*, 1999) (Table 3).

Cell and fluorescence uptake. The L5178 mouse T cell lymphoma cell line was transfected with a MDR1/A retrovirus, as previously described (Pastan *et al.*, 1988). MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line, and the L5178Y parent cell line, were grown in McCoy's 5A medium with 10% heat-inactivated horse serum, L-glutamine and antibiotics. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium and 0.5 mL aliquots of the cells were distributed into the Eppendorf centrifuge tubes. Then, 2.0 μL of 2.0 mg/mL of the tested fractions were added and incubated for 10 min at room temperature. Then 50 μL Rhodamine 123 (R123) indicator (5.2 μM final concentration) was added and incubated for a further 20 min at 37 °C. After washing twice and resuspending in 0.5 mL phosphate-buffered saline (PBS), the fluorescence of the cell population was measured by flow cytometry, using a Beckton Dickinson FACScan instrument (cell sorter). Verapamil was used as a positive control in the R123 accumulation experiments (Weaver *et al.*, 1993). The R123 accumulation was calculated from the fluorescence intensity of the samples. The percentage of the control of the untreated mean fluorescence intensity was calculated for the parental and MDR cell lines and compared with the fluorescence intensity of the treated cells. An activity ratio was calculated by the following equation (Kessel, 1989; Weaver *et al.*, 1993) (Table 3)

$$\text{MDR reversal activity} = \frac{(\text{MDR treated/MDR control})}{(\text{parental treated/parental control})}$$

Assay for radical intensity. Radical intensity was determined at 25 °C using electron spin resonance

Table 2. Radical modulation effect of kiwifruit peel extracts and fractions

Extract and fraction	Radical intensity of sodium ascorbate (3 mM) (at pH 8.0)[a]					O_2^- scavenging activity (SOD unit/mg)
	0	0.003	0.03	0.33	3 mg/mL	
H0	0.46[b]	0.44	0.53	0.58	1.40	0.85
H1	0.40	0.44	0.66	0.78	1.60	0.70
H2	0.46	0.45	0.47	0.59	1.26	0.90
H3	0.42	0.44	0.51	0.50	1.16	0.73
H4	0.44	0.54	0.39	0.68	1.21	0.98
H5	0.44	0.42	0.39	0.55	1.25	0.99
A0	0.44	0.36	0.34	0.35	0.77	13.66
A1	0.48	0.46	0.50	0.49	0.59	0.20
A2	0.44	0.46	0.55	0.80	1.92	0.72
A3	0.44	0.47	0.44	0.50	1.20	0.99
A4	0.42	0.44	0.46	0.89	2.09	N.D.[c]
A5	0.44	0.39	0.41	0.55	1.34	8.28
A6	0.45	0.38	0.59	0.62	1.03	29.96
M0	0.50	0.46	0.44	0.33	0.49	4.14
M1	0.49	0.50	0.49	0.54	0.95	1.97
M2	0.45	0.42	0.65	1.04	2.44	1.15
M3	0.45	0.43	0.56	0.96	1.48	4.51
M4	0.47	0.46	0.43	0.69	1.20	25.94
M5	0.50	0.43	0.43	0.54	0.72	16.73
70M0	0.48	0.37	0.34	0.35	0.67	3.76
70M1	0.44	0.43	0.39	0.37	0.51	2.52
70M2	0.46	0.40	0.36	0.36	0.69	5.74
70M3	0.47	0.40	0.51	1.19	2.49	18.92
70M4	0.47	0.45	0.95	1.90	3.08	48.36
70M5	0.48	0.36	0.46	0.90	1.98	17.95
70M6	0.45	0.46	0.43	0.95	1.89	6.23

[a] Radical intensity of sodium ascorbate (3 mM) in the presence of 0–3 mg/mL each fraction in 0.1 M Tris-HCl, pH 8.0.
[b] Each value represents the mean of triplicate determinations.
[c] N.D. not detected.

(ESR) spectroscopy (JEOL JES RE1X, X-band, 100 kHz modulation frequency). Instrument settings: centre field, 335.6 ± 5.0 mT; microwave power, 8 mW; modulation amplitude, 0.1 mT; gain, 630; time constant, 0.1 s; scanning time, 4 min. Radical intensity was determined in 0.1M Tris-HCl (buffer pH 8.0) in the presence of 3 mM sodium ascorbate containing 50% DMSO and the radical intensity was defined as the ratio of peak heights of these radicals to that of MnO (Satoh *et al.*, 1997) (Fig. 2).

Radical scavenging activity against superoxide anion (O_2^-). O_2^- was generated by hypoxanthine (HX) and

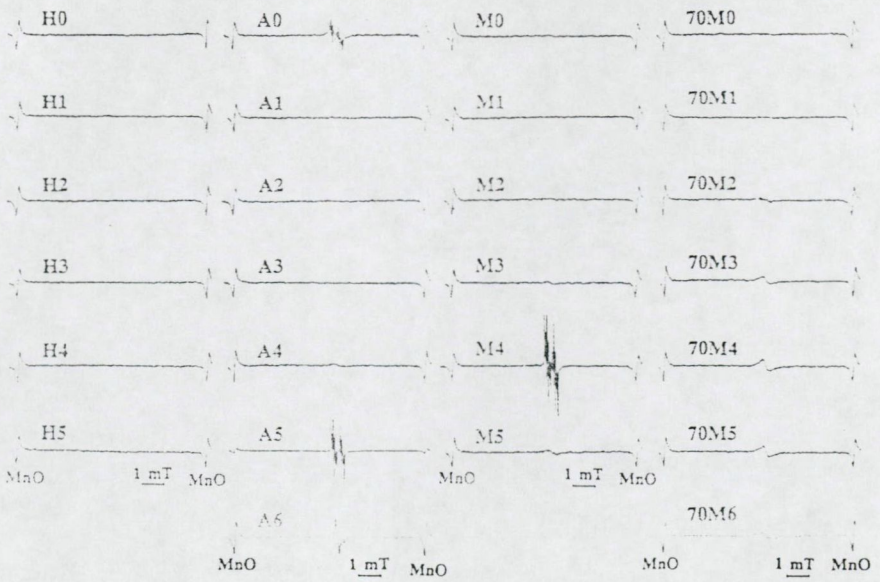

Figure 2. ESR spectra of 26 kiwifruit peel extracts and fractions (3 mg/mL) measured 1 min after dissolving in 0.1M $NaHCO_3/Na_2CO_3$, pH 10.5. The gain in A5 was changed to 100.

Table 3. Antimicrobial activity of kiwifruit peel extracts and fractions[a]

Extract and fraction	Gram-positive *Staphylococcus epidermidis* ATCC 12228	Gram-negative *Escherichia coli* K12LE140	Fungi (*Candida*) *Candida albicans* (cultivated on MTY agar)	*H. pylori* MIC (μg/mL)[e]
H0	–	–	–[f]	ND[b]
H1	–	±	–	>100
H2	+	+[c]	–	>100
H3	–	±	–	>100
H4	–	±	–	>100
H5	–	±	–	>100
A0	–	+	–	ND
A1	–	–	–	>100
A2	–	–	–	>100
A3	–	+	–	>100
A4	–	–	–	>100
A5	–	–	–	>100
A6	–	+	–	>100
M0	–	–	–	ND
M1	–	–	–	>100
M2	–[d]	–	–	>100
M3	–	–	–	>100
M4	–	+	–	>100
M5	–	–	–	>100
70M0	–	–	–	ND
70M1	–	–	–	>100
70M2	–	++	–	>100
70M3	–	–	–	>100
70M4	–	–	–	>100
70M5	–	±	–	>100
70M6	–	++	–	>100
Metronidazole	–	–	–	74.0
Clarithromycin	S[g]	R[h]	–	1.9
Erythromycin	S	R	–	1.8
DMSO (control)	–	–	–	

[a] ++, Complete growth inhibition on the surface of inoculated agar plates; +, growth inhibition but a few resistant colonies started to develop; ±, some growth inhibitions but majority of the cells were not sensitive; –, ineffective.
[b] ND: not detected.
[c] Brown precipitation.
[d] Inhibition of haemolysis.
[e] The MIC value was determined for each extract and fraction by a broth microdilution method using *H. pylori* (ATCC43504) (Numao *et al.*, 1997; Kawase *et al.*, 1999).
[f] Positive control fluconazol.
[g] S: Gram-positive *St. epidermidis* was sensitive to erythromycin and clarithromycin.
[h] R: Gram-negative *E. coli* was resistant to both erythromycin and clarithromycin.

xanthine oxidase (XOD) reaction (total volume: 200 μL) [2 mM HX in phosphate buffer (PB) (pH 7.8) 50 μL, 0.5 mM DETAPAC 20 μL, DMPO 10 μL, sample (in DMSO) 50 μL, H_2O or SOD 30 μL, XOD (0.5 U/mL in PB) 40 μL]. The scanning time was changed to 2 min. The radical intensity was determined by electron spin resonance (ESR) spectroscopy for 1 min. O_2^- scavenging activity was expressed as SOD unit/mg sample, by calibration with standard curve of SOD (Sakagami *et al.*, 1999).

RESULTS

Cytotoxic activity and radical intensity

Table 1 shows that the cytotoxic activity of kiwifruit peel extracts, fractionated as described in Fig. 1, decreased with the increase in polarity. The 70% methanol extract with the highest water-solubility showed the lowest cytotoxic activity.

The relative cytotoxic activity of these fractions was the same, regardless of the target cells, either HSC-2, HSG, HGF or MT-4 cells (Table 1). The cytotoxic activity was concentrated by silica gel chromatography, and fractions of hexane (**H2, H3**), acetone (**A3, A4, A5**) and methanol (**M2**) extracts showed high cytotoxic activity. However, these fractions except **A4** and **M 2** (Sl=2–3) did not show any specific cytotoxic activity against tumour cells (HSC-2, HSG) compared with normal (HGF) cells (Table 1).

Radical intensity

ESR spectroscopy showed that only selected fractions (**A5, A6, M4, M5**) produced radicals under alkaline conditions, whereas other fractions including all five hexane fractions produced no detectable amounts of radical. This indicates that the cytotoxic activity of kiwifruit extracts does not always parallel their radical intensity.

Table 4. MDR reversion by kiwifruit peel extracts and fractions in lymphoma-5178 cells

Extract and fraction	Concentration (μg/mL in DMSO)	Forward scatter count (FSC) (cell size ratio)	Side scatter count (SSC) (granulation of cell ratio)	Fluorescence one height (FL-1)[a]	Fluorescence activity ratio[b]
Par(control)[c]	10	394	131	4104.0	78.77
MDR+R123(mean)[d]	10	413	142	52.1	1.00
(±) Verapamil (positive control)	10	415	110	842.0	16.16
H0	200	532	174	128	2.09
H1	200	487	160	92	1.51
H2	200	584	177	199	3.25
H3	200	482	230	420	6.89
H4	200	435	304	977	16.01
	20	452	266	767	5.52
H5	200	430	295	1165	19.09
	20	398	217	473	3.40
A0	200	332	116	112	1.31
A1	200	334	106	68	0.79
A2	200	322	132	119	1.39
A3	200	380	84	536	6.24
A4	200	380	147	1193	13.90
	20	461	188	406	2.92
A5	200	269	166	75	0.89
A6	200	363	143	101	1.17
M0	200	458	156	95	1.56
M1	200	482	149	103	1.69
M2	200	503	204	1040	17.05
	20	511	205	427	3.07
M3	200	460	198	264	4.32
M4	200	498	142	77	1.26
M5	200	465	155	55	0.89
70M0	20	416	120	82	0.79
70M1	20	417	123	72	0.69
70M2	20	468	103	72	0.69
70M3	20	422	104	70	0.67
70M4	20	486	111	56	0.54
70M5	20	416	125	106	1.02
70M6	20	405	111	107	1.03

[a] Kessel 1989; Weaver *et al.*, 1993.
[b] The R-123 accumulation was calculated from fluorescence of one height value using equation: $\log y = \log_{10} \frac{x}{256}$ then the fluorescence activity ratios were calculated according to the formula given below

$$\text{MDR reversal activity} = \frac{\text{(MDR treated/MDR control)}}{\text{(parental treated/parental control)}}$$

[c] Par: a parental cell without MDR gene.
[d] MDR: a parental cell with MDR gene.

Table 2 shows that millimolar concentrations of all fractions enhanced the radical intensity of sodium ascorbate, suggesting its prooxidant action. These fractions also scavenged O_2^-, generated by the xanthine–xanthine oxidase reaction, and its scavenging activity was roughly increased with the increase in the water solubility (Table 2). It is interesting to note that radical producing fractions (A5, A6, M4, M5, 70M2, 70M3, 70M4, 70M5, 70M6) (Fig. 2) most efficiently scavenged O_2^- (Table 2).

Anti-HIV activity

All hexane (H1–H5), acetone (A1–A6) and methanol fractions (M1–M5) did not protect the MT-4 cells from HIV-induced cytopathic effects (SI < 1) (Table 1). On the other hand, 70% methanol fractions with increased water-solubility (70M3, 70M5, 70M6) showed some anti-HIV activity (SI = 4–11) (Table 1), and produced broad ESR peaks under alkaline conditions (Fig. 2).

Antimicrobial activity

Only one fraction of hexane extract (H2) partially inhibited the growth of *Staphylococcus epidermidis*. A MeOH fraction M2 inhibited the haemolysis of *Staphylococcus epidermidis*, possibly by the stabilization of red blood cell (RBC) membranes or due to other structural changes in RBCs, or the efflux of bacterial hemolysine could be inhibited (Table 2).

Two 70% methanol fractions (70M2, 70M6) marginally inhibited the growth of *E. coli* (Table 3). Four fractions A0, A3, A6 and M4 moderately inhibited the growth of *E. coli*. Of the antibiotics, clarythromycin and erythromycin were used as positive controls (Table 3). All 26 kiwifruit fractions were inactive against *Candida*

albicans, in contrast to diflucan as the positive control (Table 3). All 26 fractions were inactive against *H. pylori*, whereas metronidazole, clarithromycin and erythromycin were effective (Table 3).

Reversal of multi-drug resistance (MDR)

Four fractions, **H4** (ratio 16.01), **H5** (ratio 19.09), **A4** (ratio 13.90) and **M2** (ratio 17.05) comparably reversed the MDR activity of lymphoma-5178 cells. (±)Verapamil (ratio 16.16) (positive control), **H3** (ratio 6.89) and **A3** (ratio 6.24) were also effective (Table 4). These active fractions showed the dose-dependent effect, and their effects were still higher than that of 70% methanol extracts even at 20 μg/mL (Table 4).

DISCUSSION

The present study demonstrates that kiwifruit extracts contain various bioactive materials. Fractionation with organic solvents and silica gel or ODS column chromatographies separated these activities into various fractions (Table 1).

Both cytotoxic activity (Table 1) and MDR reversal activity (Table 4) were found in the same fractions of hexane (**H4**, **H5**), acetone (**A4**) and methanol fractions (**M4**). The biological activity of P glycoprotein (P gp) might be responsible for the multidrug resistance (MDR), therefore, the reversal effects of kiwifruit extracts and fractions can be based on the modification of this transmembrane efflux pump (Molnár *et al.*, 1998b). However, further purification of these substances is required to confirm that these are identical to each other.

Antibacterial activity was detected in various extracts and fractions, and all extracts and fractions were inactive against *Candida albicans* and *H. pylori*. Only 70% methanol fractions showed anti-HIV activity, and these fractions produced a broad ESR signal, enhanced the ascorbate radical intensity, and scavenged O_2^-, in a similar fashion to lignin (Satoh *et al.*, 1996), which showed a comparable magnitude in these activities (Sakagami *et al.*, 1999). We also found that the radical generation roughly paralleled the O_2^- scavenging activity, supporting the fact that many plant extracts have bimodal (prooxidant and antioxidant) actions. It remains to investigate further whether the same molecule can switch from prooxidant action to antioxidant action, and *vice versa*. The present study suggests the medicinal importance of kiwifruit extracts.

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Original article

Antimicrobial activity of trifluoromethyl ketones and their synergism with promethazine

Masami Kawase [a,*], Noboru Motohashi [b], Hiroshi Sakagami [c], Taisei Kanamoto [d], Hideki Nakashima [d], Lajos Ferenczy [e], Krystina Wolfard [f], Csilla Miskolci [f], Joseph Molnár [f]

[a] *Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0295, Japan*
[b] *Meiji Pharmaceutical University, Kiyose, Tokyo 204-8588, Japan*
[c] *Meikai University School of Dentistry, Sakado, Saitama 350-0283, Japan*
[d] *Department of Microbiology and Immunology, Kagoshima University Dental School, Kagoshima 890-8544, Japan*
[e] *Institute of Microbiology, Szeged, Hungary*
[f] *Faculty of Medicine, Institute of Medical Microbiology, Albert Szent-Györgyi Medical University, Szeged, Hungary*

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Abstract

The antimicrobial effects of 30 trifluoromethyl ketones [**1**–**30**] were studied on various representative bacteria. Of the ketones, 4,4,4-trifluoro-1-phenyl-1,3-butanedione [**10**], 1,1,1-trifluoro-3-(4,5-dimethyloxazol-2-yl)-2-propanone [**11**] and 1-(2-benzoxazolyl)-3,3,3-trifluoro-2-propanone [**18**] were found to exhibit potent antibacterial activity against the Gram-positive *Bacillus megaterium* and *Corynebacterium michiganese*, but not against Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Serratia marcescens*. Compounds **11** and **18** inhibited the *Escherichia coli*. Compound **18** was also effective against yeasts. The combination of promethazine with **18** was significantly synergistic against *E. coli* strains, especially the proton pump deficient mutant. The results suggest that membrane transporters are the target of trifluoromethyl ketones. The inhibition was more marked in the proton pump deficient *E. coli* mutant than in the wild type, which suggested that the antibacterial effect of trifluoromethyl ketones is partly prevented by the proton pump system.

Keywords: Trifluoromethyl ketone; Promethazine; Antibacterial; Synergism; Anti-HIV

1. Introduction

The widespread use of antibiotics has inevitably resulted in an increasing bacterial resistance to existing drugs which threatens public health [1,2]. There is an urgent need for the discovery of new antibacterial compounds, possibly acting through mechanisms different from those of existing drugs [3,4].

Trifluoromethyl ketones (TFMKs) are valuable compounds with both synthetic and biological interests because of the unique physical and biological properties imparted by fluorines. TFMKs are potent inhibitors of serine esterases, juvenile hormone esterase, mammalian carbonyl esterases and antennal esterases in insects [5]. Peptidyl trifluoromethyl ketones have antiviral activity through inhibition of HIV-1 protease [6] and human cytomegalovirus protease [7].

The development of new synthetic methods and the biological activity of TFMKs have been studied [8,9]. Here we report the antimicrobial activity of structurally related TFMKs against several Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi

* Corresponding author. Tel.: +81-492-862233x455; fax: +81-492-717984.
E-mail address: kawasema@josai.ac.jp (M. Kawase).

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and human immunodeficiency virus (HIV). Among thirty TFMKs, five active compounds [**5**, **10**, **11**, **18**, and **20**] were studied in detail for the interaction with the ATP-binding cassette (ABC) transporter inhibitor promethazine by the chequerboard method.

2. Materials and methods

2.1. Compounds

Compound **6** was obtained by the trifluoroacetylation of 2-lithiobenzoxazole, generated by lithiation of benzoxazole with lithium diisopropylamide (LDA), with ethyl trifluoroacetate. Five compounds [**11**, **14** and **18–20**] were synthesized by treatment of the corresponding methyl-substituted azines with trifluoroacetic anhydride in the presence of pyridine [10]. Compound **12** was obtained in a 71% yield by the oxidation of *N*-butoxycarbonyl-3-trifluoroacetyl-4,5-dihydropyrrole with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) [11]. Compound **13** was obtained from the reaction of mesoionic 4-trifluoroacetyl-3-methyl-2-phenyl-1,3-oxazolium-5-olate and formamidine [9]. Three compounds [**15–17**] were obtained by the trifluoroacetylation of the corresponding dimethylaminopyridine with trifluoroacetic anhydride [12]. Two compounds [**21** and **22**] were synthesized by the reactions of mandelic acid or 3-phenyllactic acid with trifluoroacetic anhydride [13]. Two compounds [**23** and **24**] were prepared by treatment of the corresponding *N*-alkyl-*N*-acyl-α-amino acids with trifluoroacetic anhydride [14]. Compound **25** (mp 81–84 °C) was obtained in 91% yield by the catalytic reduction of *N*-(2,6-dichlorobenzoyl)-2-trifluoroacetylpyrrolidine [15]. Compound **26** was obtained by the Dakin-West reaction of *N*-benzyloxycarbonyl-1,2,3,4-tetrahydroisoquinoline-1-carboxylic acid with trifluoroacetic anhydride [16]. Three compounds [**28–30**] were prepared by the reaction of *N*-alkoxycarbonylprolines with trifluoroacetic anhydride [11]. The other ten compounds [**1–5**, **7–10** and **27**] are commercially available. Details of other substances are: 3'-azido-2',3'-dideoxythymidine (AZT), dextran sulphate (DS) (8 kDa) (Kowa, Tokyo), curdlan sulphate (CRDS) (300 kDa) (Ajinomoto, Tokyo), dideoxycytidine (ddC) (Sigma, St. Louis, MO).

2.2. Organisms used

Bacillus megaterium (NCAIMB 01208), *Corynebacterium michiganese* 0016, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Saccharomyces cerevisiae*, *Candida albicans*, *Aspergillus niger* 0050, *Fusarium moniliforme* 0040 and *Escherichia coli* AG 100 (wild type) and AG 100A (its proton pump mutant) were obtained from Professor Nikaido (Dept. Molecular and Cell Biology, University of California) [17].

2.3. Measurement of antibacterial activity

The antibacterial activity was assayed by adding 10 μl of stock solutions as a droplet on a minimal medium supplemented with 1% tryptone and 0.5% yeast extract (MTE) broth and blood agar plates inoculated with 10^5 cells of the tested strains. The plates were incubated at 37 °C for 24 h after which the inhibitory zones were measured. As a control, 10 μl of DMSO was added to each strain. It was found that *P. aeruginosa* was moderately sensitive to DMSO. The growth of *E. coli* was not inhibited by DMSO. An ampicillin disc was used as positive control in the studies of antibacterial effects.

The combined effects of TFMKs were also determined by the chequerboard dilution technique for derivation of the fractional inhibitory concentration (FIC) indices and the MIC, according to Ellion et al. [18] and Eliopoulos et al. [19].

2.4. Measurement of antifungal activity

The agar medium (1% glucose, 0.25% peptone, 0.25% yeast extract, 0.5% maltose extract and 2.0% agar, pH 7.0) were supplemented with compounds (0–50 μg/ml) and poured into Petri dishes. After setting, the plates were inoculated with the suspension of microorganisms and the samples were incubated at 30 °C for 48 h.

2.5. Assay for anti-HIV activity

The inhibition of HIV-induced cytopathic effects by TFMKs was studied. Human T cell leukemia virus 1 (HTLV1)-bearing CD4 positive human T cell lines (MT-4 cells), were infected with HIV-1_{IIIB} at a multiplicity of infection (m.o.i.) of 0.01. HIV- or mock-infected MT-4 cells (1.5×10^5/ml, 200 μl) were placed into 96-well microtitre plates in RPMI 1640 medium supplemented with 10% heat-inactivated foetal calf serum (FCS) and incubated in the presence of varying concentrations of the compounds under test.

After incubation for 5 days at 37 °C in a humidified 5% CO_2 incubator, cell viability was quantified by a colorimetric assay (at 540 and 690 nm), by monitoring the ability of viable cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a blue formazan product [20]. All data represent the mean values of triplicate measurements.

3. Results and discussion

3.1. Antimicrobial activity

Thirty TFMKs were tested against the panel of microorganisms (Fig. 1). Three compounds [**10**, **11** and **18**] were found to exhibit potent antibacterial activity

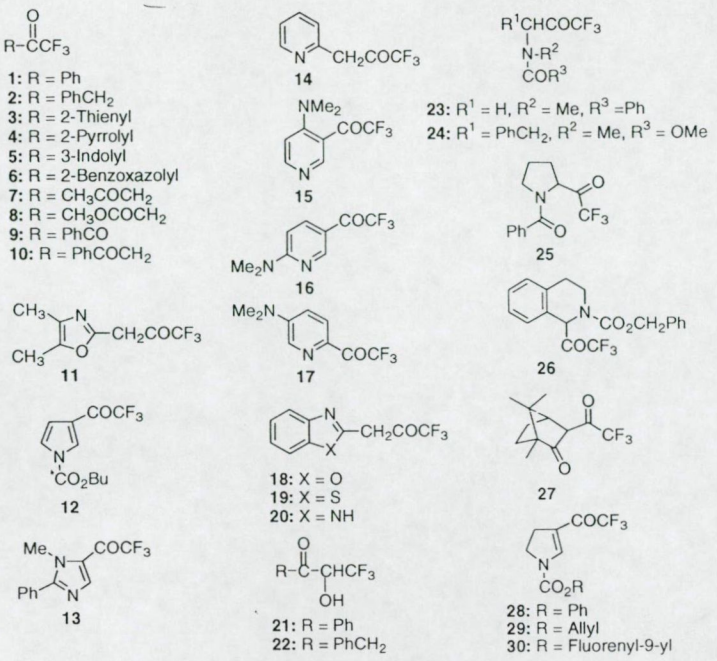

Fig. 1. Structure of trifluoromethyl ketones (TFMKs) [**1**–**30**].

against *B. megaterium* and *C. michiganese* in agar diffusion method. Compound **18** was also active against the yeast. Other TFMKs [**1**–**9**, **12**–**17** and **19**–**30**] were not active against any bacteria at 2 mg/ml. Compounds **10**, **11** and **18** that showed initial antibacterial activity were used to determine their minimum inhibitory concentration (MIC) values using an agar diffusion assay (Table 1). The MIC values of **10** and **11** (MIC: 12–50 μg/ml) for *B. megaterium* and *C. michiganase*, were much higher than that of **18** (MIC: 6 μg/ml). Similarly, compound **18** was also more active against the yeasts, compared with compounds **10** or **11** (Table 1). The MIC values of **5**, **10**, **11** and **18**–**20** against *E. coli* were also determined in a broth dilution assay (Table 2) and it was found that the proton pump deficient strain was more sensitive than the wild type.

3.2. Evaluation of combined effect

Because of the remarkable antimicrobial activity exhibited by some TFMKs, a further study was undertaken to determine the combined effects of TFMKs with promethazine which is a potent H_1-histamine receptor antagonist of long duration and with weak neuroleptic activity. Promethazine was active against various bacteria, especially Gram-positives [21–23]. The combination of promethazine with ampicillin, tetracycline and gentamicin was significantly synergistic both in vitro and in vivo [24–26]. A high degree of synergism was also found when promethazine was used in combination with methdilazine or bromodiphenhydramine [27,28].

The combined effects of each TFMK [**5**, **10**, **11**, **18** or **20**] with promethazine were determined by the agar diffusion and chequerboard technique for derivation of the fractional inhibitory concentration (FIC) indices [18]. For quantitation of synergism, the FIC indices for both combinations were calculated as described by Eliopoulos et al. [19]. Thus, the FIC index was interpreted as follows: ≤ 0.5, synergy; > 0.5–1.0, additive effects; > 1.0–4.0, indifference; and > 4.0, antagonism. The synergy in chequerboard testing results for some TFMKs and promethazine against *E. coli* are shown in Table 3. The most marked and clear-cut synergism was exhibited by the combination of **18** with promethazine in the proton pump expressing *E. coli* strain. Compound **11** also showed moderate synergy.

3.3. Anti-HIV activity

There was no significant inhibition by any of the 30 TFMKs of the cytopathic effects of HIV infection in

Table 1
MICs (mg/l) of some TFMKs for Gram-positive bacteria and yeasts

Compound	Microorganism[a]			
	BM	**CM**	**SC**	**CA**
10	25	12	>50	>50
11	>50	>50	>50	>50
18	6	6	6	12

[a] **BM**, *B. megaterium* NCAIM B 01208; **CM**, *C. michiganese* 0016; **SC**, *S. cerevisiae*; **CA**, *C. albicans*.

Table 2
MIC values of some TFMKs for *E. coli* AG 100 (wild type) and *E. coli* AG 100A (mutant) bacteria strains

Compound	MIC mg/l	
	E. coli AG100 (wild)	*E. coli* AG 100A (mutant)
5	1250	39.1
10	<9.8	<9.8
11	62.5	31.25
18	7.8	3.9
19	1250	312.5
20	1250	1250

The average count of bacteria were: *E. coli* AG 100: 1910 CFU/50 μl (3.8×10^4 CFU/ml); *E. coli* AG 100A: 1555 CFU/50 μl (3.1×10^4 CFU/ml).

MT 4 cells using effective concentrations of > 500 mg/l (Selectivity Index (SI) < 1), compared with the four positive controls — dextran sulphate (SI > 453), curdlam sulphate (SI > 7830), AZT (SI = 5118) and ddC (SI = 174).

3.4. Cytotoxic activities

The cytotoxic concentration (CC_{50}) against MT4 mock infected cells varied greatly (Table 4); values of **18**, **11**, or **10** were correlated to their antibacterial activity. The mechanism for this is not known.

4. Conclusion

The antibacterial activities of thirty TFMKs were tested to scan for possible lead structures bearing a trifluoroacetyl group. The compound exhibiting the highest potency was 2-trifluoroacetonylbenzoxazole [**18**]. Removal of the methylene of **18**, that is **6**, deleted the potency. The replacement of the benzoxazole residue of **18** with other aromatic or heteroaromatic rings resulted in significantly diminished potency, indicating that the benzazole derivatives were more active than either benzene or azole derivatives.

Table 3
MICs (mg/l) of the combination of **11** or **18** with promethazine (**PM**)

Compound	MIC mg/l	
	E. coli AG100 (wild)	*E. coli* AG 100A (mutant)
PM	156.25	78.15
11	62.5	31.25
PM+11	39.05+15.65	19.55+7.8
FIC index	0.5 (additive)	0.5 (additive)
18		0.8
PM+18	39.05+1.0	19.55+1.0
FIC index	0.4 (synergy)	0.5 (additive)

Table 4
Cytotoxic concentrations of TFMKs against mock-infected T4 cells

Compound	CC_{50} (mg/l)[a]
1	>500
2	7
3	>500
4	268
5	40
6	180
7	>500
8	3
9	2
10	4
11	0.4
12	>500
13	476
14	64
15	239
16	324
17	51
18	0.2
19	6
20	10
21	2
22	10
23	240
24	10
25	264
26	67
27	0.003
28	263
29	3
30	250
CRDS	>1000
DS	>1000
AZT (μM)	10.1
ddC (μM)	83.9

[a] 50% cytotoxic concentration (CC_{50}) against mock-infected cells.

These data suggest the benzoxazole moiety is of great importance for the antibacterial activity. Thus, compound **18** showed highly promising activity against bacteria. A further investigation of structure–activity relationship, including the killing mechanism of the bactericidal activity of compound **18**, are under way.

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S G Dastidar and N Motohashi

23

Effect of Some New 3-Benzazepines on Plasmid DNA, *mdr* P-glycoprotein and Reverse Transcriptase of Leukaemia

JOSEPH MOLNÁR[1]*, DIANA SZABO[1], CSILLA MISKOLCI[1], JÁNOS NACSA[1], MASAMI KAWASE[2], SETSUO SAITO[2] and NOBORU MOTOHASHI[3]

[1]Faculty of Medicine, Institute of Microbiology, Albert Szent-Györgyi Medical University, Dóm tér 10, H-6720, Szeged, Hungary;

[2]Faculty of Pharmaceutical Sciences, Josai University, Sakado, Saitama 350-0290, Japan;

[3]Department of Medicinal Chemistry, Meiji College of Pharmacy, Tanashi-shi, Tokyo 188-0001, Japan

Abstract

Two 3-benzazepines ([5], [10]) were able to form complex with replicative form of plasmid DNA. The multidrug resistance (*mdr*) P-glycoprotein efflux pump of mouse lymphoma cells was inhibited by three compounds ([5], [8], [10]). The inhibitory effects of compounds on reverse transcriptase (RT) of Moloney leukaemia had shown a great variety, however, the most effective compounds were [7], [8] and [9].

Introduction

Antibiotic resistance of bacteria is encoded by extrachromosomal DNA, called plasmids. There are plasmids encoding ATP binding cassette (ABC) transporter protein which have a great extent of similarity with a P-glycoprotein (P-gp) responsible for multidrug resistance (*mdr*) of cancer cells (Endicott and Ling, 1989). The emerging drug resistance is known

Abbreviations: 7,8-Dimethoxy-2-methyl-3-methanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [1]. 7,8-dimethoxy-2-methyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [2]. 3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [3]. 7,8-dimethoxy-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [4]. 7,8-dimethoxy-2-isopropyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [5]. 7,8-dimethoxy-2-phenyl-3-trifluoromethanesulfonyl-2,3,4,5-tetrahydro-3-benzazepin-1-one [6]. 7,8-dihydroxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [7]. 7,8-dihydroxy-3-methyl-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [8]. 7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine [9]. 7,8-dimethoxy-2-trifluoromethyl-2,3,4,5-tetrahydro-1H-3-benzazepine [10], dopamine [DA, 11]; norepinephrine [NE, 12].

among viruses, bacteria, protozoa and cancer cells including drug resistant retroviruses such as human immunodeficiency virus (HIV) (Hamada and Tsuruo, 1986; Zamora *et al.*, 1988; Kovács *et al.*, 1991; Schäfer *et al.*, 1993; Swartz, 1994; Taylor *et al.*, 1994; Artico *et al.*, 1996; Ecker *et al.*, 1996; Hewlett *et al.*, 1997; Suzuki *et al.*, 1997).

In addition, retroviruses can exist in an autonomous state and behave like plasmids-before the integration into host cell chromosomal DNA.

There are evidences that antiplasmid compounds can form a complex with plasmid DNA topoisomerase and *mdr* P-gp, resulting in the reversal of resistance. However, the majority of compounds do not block replication of HIV retrovirus although the reverse transcriptase (RT) and integrase were considered potential targets for inhibition of retrovirus replication. The search for novel agents has led to the synthesis of some rare phenothiazines (Motohashi *et al.*, 1996) and 3-benzazepines (Kawase *et al.*, 1997; Kawase *et al.*, 1998b).

To identify the standard features of 3-benzazepines required for the biological activities, the tetrahydro-3-benzazepines were subjected to interact with *E. coli* plasmid DNA, *mdr* P-gp of lymphoma cells and RT of Moloney leukaemia in model experiments.

Materials and Methods

Chemicals-The following six 2,3,4,5-tetrahydro-3-benzazepinones [**1-6**] and three 2,3,4,5-tetrahydro-1*H*-3-benzazepines [**7, 8, 10**] were newly synthesized as recently published (Kawase *et al.*, 1997; Kawase *et al.*, 1998b). Compound [**9**] was synthesized, vide literature (Pecherer *et al.*, 1971). [1] (m.w. 313.38, mp 165-166°C), [2] (m.w. 367.34, mp 102-103°C), [3] (m.w. 293.26, mp 76°C), [4] (m.w. 353.32, mp 158-160°C), [5] (m.w. 395.40, crystals), [6] (m.w. 429.42, mp 175-178°C), [7] (m.w. 328.13, mp 223°C (dec)), [8] (m.w. 297.70, mp 215°C (dec)), [9] (m.w. 206.12, mp 241°C (dec)), [**10**] (m.w. 275.24, oil).

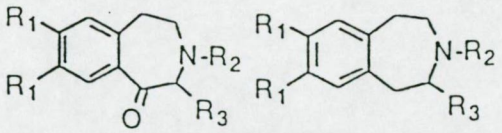
DA hydrochloride [**11**] was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NE [**12**] and rhodamine 123 hydrate (R123, ot#28394-0) were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) (Table 1).

DA [**11**]) and NE [**12**]) were used as controls and the compounds were issolved in 20% dimethylsulfoxide (DMSO) solution.

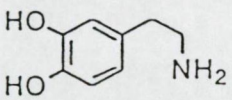
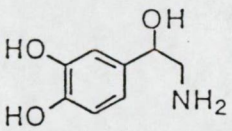
acterial strains.

E. coli K12 pBR 322 bacterial strain was applied for isolation of asmid DNA by agarose gel-electrophoresis (Fig. 1).

Table 1. Structures of 2,3,4,5-tetrahydro-1*H*-3-benzazepines [**1-10**] and two catechols [**11,12**] used in this study[a].



[**1-6**] [**7-10**]

Compound	R_1	R_2	R_3
[1]	MeO	Ms	Me
[2]	MeO	Tf	Me
[3]	H	Tf	H
[4]	MeO	Tf	H
[5]	MeO	Tf	Me_2CH
[6]	MeO	Tf	Ph
[7]	HO	H	CF_3
[8]	HO	Me	CF_3
[9]	HO	H	H
[10]	MeO	H	CF_3
DA [11]			
NE [12]			

a) abbreviations. Me: CH_3 Ms: SO_2CH_3; Tf: SO_2CF_3.

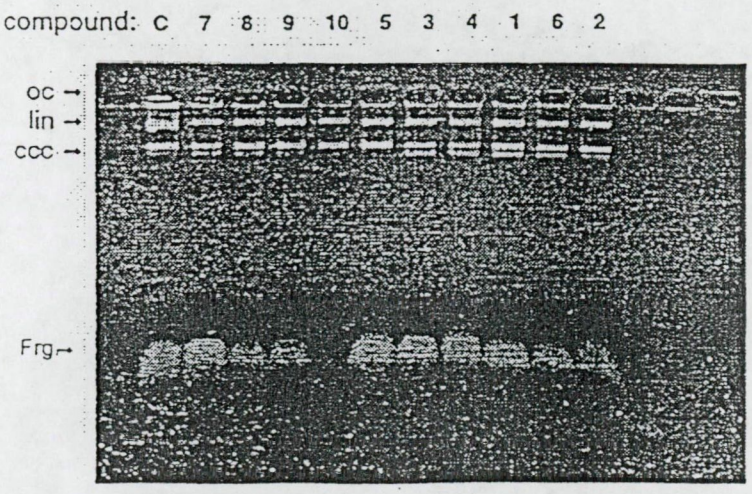


Fig. 1. Effect of tetrahydrobenzazepines on pBR322 plasmid DNA from *E. coli*. (C-control; ccc-covalently closed circular form; oc-open circular form; lin-linear form; and Frg-fragment of DNA

Antiretroviral effect. Moloney murine leukaemia virus (M-MuLV) RT assay.

Trifluoperazine-metal coordination complexes and 3′-azido-3′-deoxythymidine triphosphate (AZT-TP) as a control were assayed for their ability to inhibit M-MuLV RT (New England BioLabs). The assay is based on following: the poly-adenosine phosphate $(rA)_n$, oligo deoxythymidine $(dT)_{12\text{-}18}$ (New England BioLabs) directed incorporation of deoxythymidine triphosphate (dTTP) (Amsterdam) into complementary DNA (cDNA). The 10 × RT buffer contains 500 m*M* Tris-HCl (*p*H 8.3), 80 m*M* $MgCl_2$, 300 m*M* KCl, and 100 m*M* dithiothreitol (DTT). In all experiments, the final volume of the reaction assay was 20 μL. This contained water, 2 μL of 10 × RT buffer, 20 μg/mL template-primer, 5 μ*M* dTTP precursor (New England BioLabs), 0.2 μCi tritiated precursor, the compounds to be tested (administrated into the medium before adding the enzyme) and 5 units (U) RT initiating the reaction. This procedure was followed by incubation for 40 min at 37°C. 15 μL of the mixture was then transferred to Whatman DE81 filter paper disc, washed by 5% disodium-hydrophosphate buffer (3 × 3 min), water, then 96% ethanol, and after drying and putting into 5 mL scintillation cocktail (OptiPhase HiSafe 3′, Wallac), the radioactivity was measured by Packard Tri-Carb 2200 CE liquid scintillation counter. The residual enzymatic activities were compared to the control (no drug added). The IC_{50} of AZT-AT was 0.12 μ*M* in our experiments.

Cell and fluorescence uptake, mdr reversal effect.

The L5178 mouse T cell lymphoma cell line was infected with the pHa MDR1/A retrovirus as previosuly described (Aszalos *et al.*, 1995). MDR1 expressing cell lines were selected by culturing the infected cells with 60 ng/mL colchicine to maintain expression of the MDR phenotype. The L5178 MDR cell line and the L5178Y parent cell line were grown in McCoy's 5A tissue culture medium with 10% heat-inactivated horse serum, L-glutamine and 2,3,4,5-tetrahydro-1*H*-3-benzazepines [1-10]. The cells were adjusted to a concentration of 2×10^6/mL and resuspended in serum-free McCoy's 5A medium and the cells were distributed into 0.5 mL aliquote to Eppendorf centrifuge tubes. Then, the tested compounds were added in various concentrations (0.2-20.0 μL) of the 1.0 mg/mL stock solutions and the samples were incubated for 10 min at room temperature. Next, 10 μL (5.2 μ*M* final concentration) indicator of rhodamine 123 were added to the samples and the cells were incubated for further 20 min at 37°C, washed twice and resuspended on 0.5 mL phosphate-buffered saline

(PBS) for analysis. The fluorescence of cell population was measured by flow cytometry using Beckton Dickinson FACScan instrument. Verapamil was used as a positive control in the rhodamine 123 exclusion experiments (Weaver *et al.*, 1993). The percentage of control mean fluorescence intensity was calculated for the following equation on the basis of measured fluorescence values.

$$R = \frac{mdr \text{ treated}/mdr \text{ control}}{\text{parential treated/parental control}}$$

Complex formation between benzazepines and plasmid DNA

The *E. coli* pBR322 strain was grown in 5 mL yeast-extract-tryptone broth (YTB) in presence of tetracycline (100 µg/mL) and ampicillin (25 µg/mL) at 37°C for 16 hours. The overnight culture was distributed into 1 mL aliquote in Eppendorf tubes. Benzazepines were added to the plasmid containing bacterial suspension in 200 µg/mL final concentration, the samples were centrifuged for 5 min (3000 rpm) and the supernatant was discarded. The pellet was resuspended in 100 µL of Sol I (1*M* Tris-HCl, 0.5 *M* glucose, 0.25 *M* ethylenediaminetetraacetic acid (EDTA)), homogenized in vortex, and incubated at room temperature for 5 min. Then, 200 µL of Sol II (10 N NaOH, 20% sodium dodecyl sulfate (SDS)) was added to the bacterial suspensions, homogenized and incubated in water bath (0°C) for 5 min. Then, 150 µL of ice cold 5 *M* potassium acetate was added to the samples. After mixing, samples were incubated further for 5 min at 0°C, then centrifuged for 10 min (3000 rpm). The supernatant was treated with 200 µL phenol solution and 200 µL of chloroform: isoamyl alcohol (1:1). The samples were centrifuged for 1 min (3000 rpm) and the supernatants were precipitated with 96% ethanol and incubated at room temperature for 5 min and then precipitated plasmid DNA was centrifuged for 5 min (3000 rpm). The supernatant was discarded and the plasmid DNA was washed with 70% ethanol. Then, ethanol was discarded and the samples were dried at 37°C. The extracted plasmid DNA was dissolved in 20 µL Tris-EDTA (TE)-buffer containing RNAse (20 µL/mL, Sigma). 10 µL of the RNAse treated plasmid DNA solution were applied to 1% agarose gel (with 0.5 µg/mL ethidium bromide). Agarose gel electrophoresis was performed for 20 min (200 V). The various forms of plasmid DNA were detected under UV lamp (Fluo-link).

Results and Discussion

In the control sample [C], the covalently closed circular (ccc) form of plasmid DNA runs faster than the linear (lin) form and the two conformations can be seen near to each other (Fig. 1). The open circular

(oc) form ran slower, relatively far from linear (lin) form. In case of 2,3,4,5-tetrahydro-1*H*-3-benzazepine treated plasmid-containing cells, the oc form can be found in each sample. Similar situation exists with lin form. However, compounds ([5], [10]) were able to form complexes with the ccc form of plasmid DNA. The results showed an evidence for the selective complex formation of two active benzazepines ([5], [10]) with the superhelical form of plasmid DNA, meaning that this biological effect is dependent on the substituents of the benzazepine molecule.

The ccc form of plasmid DNA is maintained by bacterial gyrase (topoisomerase) which can be inhibited by benzazepines. Theoretically, it is also possible that a simple interaction of two benzazepines ([5], [10]) leads to the unwinding of ccc form into the oc form, which is due to a single nick introduced into one strand.

Two compounds ([5], [10]) selectively inactivate the replicative form of plasmid DNA that encode the antibiotic resistance (tetracycline (Tc) and ampicillin (Ap)) in bacteria. The DA [11] and NE [12] had no effects (data not shown).

When the substituted benzazepines were tested on the P-glycoprotein efflux-pump of tumour cells, only a few compounds were active (Table 2).

Mdr reversal on tumour cells

The *mdr* reversing effects of benzazepines [1-10] by their chemical structures were compared to that of verapamil, using a mouse leukaemia cell line (L-5178 cells). The effects were measured by fluorescence ratio between treated and untreated group cells. Compound [8] has the highest activity on *mdr* reversal (Fluorescence activity ratio 8.38) among 12 compounds used in this research and compound [8] was 2-fold more potent than verapamil (fluorescence activity ratio 4.18). Then, compound [8] might be an anti-*mdr* inducing agent of great interest, because the affinity of [8] to two dopamine D_1 and D_2 receptors was reduced by introduction of trifluoromethyl (CF_3) group at 2nd position of benzazepine ring (Kawase *et al.*, 1998b).

We have reported that benzazepines [7], [8] and [9] induced apoptic cell death in human promyelocytic leukaemia HL-60 cells and the cytotoxic activities of [7]-[9] were 1.3, 1.3 and 2.0 times higher than that of DA [11], respectively. Additionally, it is found that apoptosis induced by these benzazepines [7]-[9] is coupled with their radical generation (Kawase *et al.*, 1998a).

The retroviruses have a key enzyme, which called RT that is responsible for maintaining the virus multiplication, the transformation of

RNA encoded information into DNA. This intermediate stage of retroviral replication was effected by three compounds ([7], [8], [9],) (Table 3) (Figure 1).

The results obtained show a chemical structure-dependent effect on RT again. The active benzazepines inhibit the RNA-directed DNA synthesis, however, the majority of the derivatives did not show remarkable

Table 2. The effect of 2,3,4,5-tetrahydo-1*H*-3-benzazepines [1-10] and two analogues [11, 12] on lymphoma 5178 cells with multidrug resistance.

Compound [No.]	Concentration (μg/mL)	Forward scatter height [cell size ratio]	Side scatter height [granulation of cell ratio]	Fluorescence one height[a] value			Fluorescence activity ratio[c]
				x[b]	$y=\frac{x}{256}$	$\log(y)=\log_{10}\frac{x}{256}$	
par[e]	control	504.86	184.75	775.68	3.03	1060.98	49.98
mdr+R123[f]	control	552.95	211.52	340.48	1.33	21.23	1.00
(±) verapamil[d]	5	545.74	216.62	499.20	1.95	88.72	4.18
Benzazepines:							
[1]	5	473.30	188.91	253.44	0.99	9.85	0.46
[2]	5	425.16	205.05	325.12	1.27	18.69	0.88
[3]	5	494.57	193.54	284.16	1.11	12.97	0.61
[4]	5	467.29	188.79	309.76	1.21	16.39	0.77
[5]	5	491.72	190.79	422.40	1.65	44.77	2.11
[6]	5	507.50	197.30	291.84	1.14	13.89	0.65
[7]	5	469.78	187.09	314.88	1.23	17.01	0.80
[8]	5	447.77	191.53	576.00	2.25	177.91	8.38
[9]	5	477.28	189.43	309.76	1.21	16.12	0.76
[10]	5	507.71	198.41	373.76	1.46	28.68	1.35
Dopamines:							
DA [11]	2	579.02	251.38	343.04	1.34	21.77	0.84
	20	571.20	251.12	350.72	1.37	23.47	0.93
NE [12]	2	569.10	256.09	340.48	1.33	21.32	0.82
	20	564.14	254.11	330.24	1.29	19.53	0.80

a) Ref: Kessel,1989; Weber *et al.*, 1994.

b) x: Measured fluorescence value at linear scale [mg. uptake of R123].

c) The R-123 accumulation was calculated from fluorescence of one height value using 1st equation

$$\log(y)=\log_{10}\frac{x}{256}$$

then the fluorescence activity ratios were calculated according to the formula given below:

$$\text{Ratio} = \frac{(mdr \text{ treated}/mdr \text{ control})}{(\text{parental treated}/\text{parental control})}$$

d) (±)verapamil: a control for *mdr* reversal

e) par: parental without multidrug resistant gene.

f) *mdr*: parental with multidrug resistant gene.

Table 3. Inhibition of Moloney murine leukaemia virus rever transcriptase by 2,3,4,5-tetrahydro-1*H*-3-benzazepines [1-10] In concentration ($10^{-5}M$).

Compound (No.)	Concen-tration (µg/mL)	Activity of cDNA (DPM)[a]	Activity in percentage of DMSO control (%)
DMSO (control) 0.5 µL DMSO/ 20 µL assay		67401 54887 50297 64784	100
+ Control		83245 57408	-
[1]	50	39352 60832	88.2
[2]	50	49649 54104	89.1
[3]	50	48949 45591	80.4
[4]	50	63098 66862	112.6
[5]	50	56214 58153	98.7
[6]	50	31249 52950	72.1
[7]	50	23728 13754	32.6
[8]	50	17873 17305	29.5
[9]	50	15536 10126	21.8
[10]	50	60242 56805	102.6

[a]DPM: decomposition per minute (alternative to count per minute (cpm) values).

effects in the system. These derivatives possibly might modify the enzyme activity by binding to benzazepine binding sites on RT-enzyme.

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