MUTAGENIC AND CLASTOGENIC PROPERTIES OF
(3-[3-(6-BENZOYLOXY-3-CYANO-2-
PYRIDYLOXYCARBONYL)
BENZOYL]-1-ETHOXYMETHYL-
5-FLUOROURACIL)(BOF-A2),
A NEW 5-FLUOROURACIL
DERIVATIVE WITH ANTI-TUMOUR ACTIVITY

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Abstract—As part of the safety assessment of (3-[3-(6-benzoyloxy-3-cyano-2-pyridyloxycarbonyl)benzoyl]-1-ethoxymethyl-5-fluorouracil)(BOF-A2), a new 5-fluorouracil (5-FU) derivative with anti-tumour activity, its potential genotoxicity was studied in 3 different tests. BOF-A2 was negative in a reverse mutation (Ames) test in strains of *S. typhimurium* and *E. coli*. BOF-A2 induced chromosomal aberrations in Chinese hamster cells *in vitro* especially in the presence of exogenous metabolic activation, and was clastogenic *in vivo*, inducing micronuclei in mouse bone marrow. The clastogenic activity of BOF-A2 was similar to that of 5-FU.

Key words: (3-[3-(6-benzoyloxy-3-cyano-2-pyridyloxycarbonyl)benzoyl]-1-ethoxymethyl-5-fluorouracil), 5-fluorouracil, Ames test, *in vitro* chromosomal aberration test, CHO cells, mouse micronucleus test.

INTRODUCTION

(3-[3-(6-Benzoyloxy-3-cyano-2-pyridyloxycarbonyl)benzoyl]-1-ethoxymethyl-5-fluorouracil) (BOF-A2) as shown in Fig. 1, contains both CNDP (3-cyano-2, 6-dihydroxypyridine), an inhibitor of 5-fluorouracil (5-FU) degradation, and EM-FU (1-ethoxymethyl-5-fluorouracil), a masked form of 5-FU. Significant anti-tumour activity against mouse sarcoma 180 and rat Yoshida sarcoma has been observed for BOF-A2

(Fujii et al., 1989), and against sarcoma 180 it was even more potent than UFT (a 4 : 1 molar mixture of uracil and 1-{2-tetrahydrofuryl}-5-fluorouracil ; Fujii et al., 1978, 1979).

As 5-FU has been known for many years to induce chromosomal aberrations *in vitro* and *in vivo* (Yajima et al., 1981 ; Ishidate Jr., 1987 ; Yamamoto and Kikuchi, 1981) but not reverse mutations in bacteria (Seino et al., 1978), it was important to investigate the mutagenic and clastogenic properties of BOF-A2. Hence, we report here studies to investigate reverse muta-

Fig. 1 Chemical structure of BOF-A2

tion in *Salmonella typhimurium* and *Escherichia coli*, chromosomal aberrations in CHO cells and micronuclei in mouse bone marrow.

MATERIALS AND METHODS

Chemicals :

BOF-A2 was supplied by Otsuka Pharmaceutical Co., Ltd. It was dissolved by warming at 60°C in acetonitrile (ACN, Wako Pure Chemical Industries Co., Ltd., see Maron et al., 1981) for the bacterial study, was dissolved in ethylene glycol dimethyl ether (EGDME, HPLC grade) for the chromosomal aberration study, and suspended in 1% (w/v) hydroxy propyl methyl cellulose (HPMC) for the micronucleus test. The positive control chemicals were obtained as follows : N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG) from Nakarai Chemicals Ltd. ; ICR-191 from Polysciences, Inc. ; methyl methanesulfonate (MMS), 2-nitrofluorene (2NF) and 2-aminoanthracene (2AA) from Aldrich Chemical Co., Inc. ; cyclophosphamide (CPA) from Sigma Chemical Co.

Reverse Mutation (Ames) Test :

Salmonella typhimurium TA1535, TA1537, TA98 and TA100 were obtained from Professor B. N. Ames, University of California, USA and *Escherichia coli* WP2 *uvrA* was obtained from Professor T. Kada, National Institute of Genetics, Japan. Nutrient broth (NB, Oxoid) cultures were mixed with dimethyl sulfoxide (DMSO, 0.07 ml per 0.8 ml culture) as cryopreservative, and aliquots frozen at -80°C. Cultures were thawed for each experiment and grown overnight at 37°C for use.

For the experiments, viable bacteria were

estimated on plates containing NB base agar (1.5% agar, Wako Pure Chemical Industries Co., Ltd.) and NB top agar (0.6% Difco Bacto agar). Mutants were selected on Vogel-Bonner minimal medium E (VBM) base agar (1.5% agar, Wako Pure Chemical Industries Co., Ltd.) plates with top agar (0.6% Difco Bacto agar, 0.5% NaCl) containing 0.05 mM histidine-biotin for the *Salmonella* strains or 0.05 mM tryptophan for the *E. coli*. S-9 and cofactors were purchased from the Oriental Yeast Co., Ltd. Each 1 ml of S-9 mix contained 0.1 ml S-9 fraction, 8 μ mol $MgCl_2$, 33 μ mol KCl, 5 μ mol G-6-P, 4 μ mol NADPH, 4 μ mol NADH and 100 μ mol sodium phosphate.

Experiments were conducted according to Japanese Ministry of Health and Welfare guidelines (MOHW, 1989) and the pre-incubation method of Yahagi et al. (1975), namely to sterile tubes were added 0.05 ml of BOF-A2 solution or ACN (or 0.1 ml of positive control solution), 0.5 ml of S-9 mix or 0.1 M phosphate buffer (pH7.4), and 0.1 ml of overnight bacterial culture. The tubes were incubated, with shaking, at 37°C for 20 min, 2 ml of molten (45°C) top agar was added to each tube, the contents were mixed and poured onto the surface of VBM base agar plates. When the agar had set, plates were incubated at 37°C for 48 hr. Growth inhibition and precipitation on the plates were examined using a binocular microscope and colonies were counted manually. Sterility of the BOF-A2 solutions (highest concentration) and S-9 mix were confirmed by mixing these with agar in the absence of tester bacteria.

Concentrations of BOF-A2 to be used in the main experiments were determined in preliminary studies utilising concentrations ranging from 5000 to 0.5 μ g/plate. As BOF-A2 proved to be quite

toxic in the absence of S-9, a dose range of 50 to 1.56 $\mu\text{g}/\text{plate}$ was used in the main experiments. No toxicity was seen in the presence of S-9, so in the main experiments a dose range spanning 5000 to 156 $\mu\text{g}/\text{plate}$ was used. All platings were in triplicate, and 2 independent experiments were conducted.

ACN, DMSO and water formed the solvent controls. ENNG, ICR-191, MMS and 2NF were positive controls in the absence of S-9 and 2AA was positive control in the presence of S-9.

Positive results were concluded if numbers of revertant colonies increased in a dose-related fashion to exceed $2\times$ the solvent control values, and the findings were reproducible.

Chromosomal Aberration Test in CHO Cells In Vitro :

Chinese hamster ovary (CHO) cells were obtained from Dr. N. Danford, University of Swansea, UK and cultured in Hams F-12 medium containing 10% foetal calf serum (FCS) and 100 $\mu\text{g}/\text{ml}$ gentamycin (all obtained from Gibco). Stocks of cells were preserved at -196°C in medium containing 10% DMSO, and a fresh vial of cells thawed for each experiment. These stocks had been checked for karyotypic stability (modal number of 21 chromosomes) and absence of mycoplasma.

For the experiments, cells were sub-cultured at low density into 75 cm^2 tissue culture flasks, and incubated at 37°C in an atmosphere of 5% CO_2 in air so as to be in exponential growth (usually 2-3 days) at the start of treatment but unlikely to have reached confluence by the end of the experiment 48 hr later. S-9 was prepared as a standard homogenate (Ames et al., 1975) from the livers of male Wistar rats induced with sodium phenobarbitone (BDH Chemical) at 1 mg/ml in the drinking water over 7 days, and β -naphthoflavone (Sigma Chemical Co.) injected intraperitoneally at 80 mg/kg in corn oil 2 days before sacrifice. Acceptable S-9 preparations contained at least 21 mg/ml protein. Each 5 ml of S-9 mix contained 2 ml S-9 fraction, 1 ml KCl (150 mM), 1 ml NADP (25 mg/ml) and 1 ml G-6-P (180 mg/ml). S-9 mix was diluted 1/20 in the culture medium, giving a final concentration of 2% S-9 fraction in the treatment medium.

Experiments were conducted according to

Japanese Ministry of Health and Welfare guidelines (MOHW, 1989) which meant 3 treatment regimes were followed :-

Table 1.

Method	S-9	Duration of treatment (hr)	Harvesting time (hr after start of treatment)
1. Pulse	—	2	21
2. Continuous	—	24	24
		48	48
3. Metabolic activation	+	2	21

Treatment considered of the addition of 0.2 ml of a concentrated solution of BOF-A2 (or solvent or positive control solution) plus 1 ml of S-9 mix (or KCl for the non-S-9 treatments) to exponentially growing cultures to make a total of 20 ml. After the short (2 hr) treatments, media were removed, cell monolayers washed twice with sterile saline, and refed with Hams medium. Two hr prior to harvest, colchicine was added to all cultures at a final concentration of 1 $\mu\text{g}/\text{ml}$. Apart from the positive controls which were in duplicate, all treatments were in triplicate. The cells from one replicate of each of the latter were harvested by washing with sterile saline and detaching all cells with EDTA. These cultures were used for mitotic index (MI) determination. Mitotic cells for aberration analysis were harvested from all the other flasks by shake-off. After gentle swelling of the cells in hypotonic KCl, and fixing in fresh, ice-cold methanol/glacial acetic acid (3 : 1, v/v) (at least overnight), slides were made, dried and stained with Giemsa. Apart from the positive controls, 100 cells were analysed per culture (2 cultures per dose point) for aberrations, which were classified according to Scott et al. (1983). The cells with more than $2n+2$ chromosomes were scored as "numerical aberrations", which were categorised separately as hyperdiploid ($2n+2$ up to $3n$), polyploid and endoreduplicated.

Concentrations of BOF-A2 to be used in the main experiments were determined in a preliminary range-finding study. Cytotoxicity varied with length of treatment and presence or absence of S-9, so the following dose ranges were treated and scored :-

Table 2.

Method	S-9	Dose range treated (μ g/ml)	Doses scored (μ g/ml)
1. Pulse	—	100–5	60,40,20
2. Continuous (24)	—	100–10	70,55,40
Continuous (48)	—	160–20	80,60,40
3. Metabolic activation	+	120–20	100,80,60

Negative controls consisted of untreated and solvent (EGDME)-treated cultures. MMS was the positive control in the absence of S-9 and CPA was the positive control in its presence.

Positive results were concluded if numbers of aberrations in treated cultures were significantly higher (according to a chi-square test with Yates correction) than those in solvent control cultures, and also exceeded historical negative control ranges.

Micronucleus Test in Mouse Bone Marrow :

Out-bred CD-1 mice (21–33g at delivery) were purchased from Charles River UK Ltd., and housed in polypropylene cages with wire mesh lids and solid floors containing 1 cm depth of wood shavings. Animals were housed 2 or 3 per cage, and placed in an air-conditioned room at 21 (± 2)°C, 52–73% relative humidity, 15 fresh air changes per hr and 12 hr light/dark cycle. Bottled tap water and Charles River 20RF diet were provided *ad libitum*.

Animals were acclimatized for at least one week before the start of the study and were 7 weeks old at the time of treatment. They were allocated to the various experimental groups using a system of random numbers, and group body weights were checked on the day of treatment to ensure they did not differ from the overall mean by more than 5%. Because Yamamoto and Kikuchi (1981) reported that multiple treatments are suitable for detecting the micronuclei induction by base analogues, administration of BOF-A2 suspension was by oral gavage, daily for 4 days, and control mice received the vehicle, HPMC, the same way. Positive control mice received CPA in water orally by a single administration on day 4. All mice were sacrificed on day 5, one femur from each animal

collected, and bone marrow cells from the shank were aspirated into 1 ml of fresh FCS. After centrifuging at 1250 'g' for 2–3 min, most of the serum was decanted, the cell pellet was mixed into the remaining serum, and drops of cell suspension were smeared across clean microscope slides. After air-drying and storing for at least 2 days, slides were stained with Giemsa according to the modification of Gollapudi and Kamra (1979). Where possible, 1000 polychromatic erythrocytes (PCE) were scored per animal for micronuclei, and the ratio of PCE to NCE (normochromatic erythrocytes) was determined on at least 1000 cells total to give a measure of bone marrow toxicity. The frequencies of micronucleated NCE were also noted.

Dose levels for the main study were established in a range-finding study using small groups (3 males and 3 females) of mice. No mortality was seen up to 80 mg/kg/day, but there were slight reductions in bodyweight at 60 and 80 mg/kg/day, massive reduction in PCE/NCE ratio at 80 mg/kg/day and clear reductions in PCE/NCE ratio at 40 and 60 mg/kg/day. Doses of 10, 20 and 40 mg/kg/day were therefore chosen for the main study, and groups of 5 male and 5 female mice were used.

For data evaluation, numbers of micronucleated PCE and NCE in treated and control groups were compared using a chi-square test with Yates correction. Positive results were concluded if statistically significant increases in the incidence of micronucleated PCE were observed at one or more doses, and the incidence exceeded historical vehicle control levels.

RESULTS

Reverse Mutation Test :

As can be seen from the summarised results in Tables 3 and 4, some toxicity occurred at the highest dose of BOF-A2 in the absence of S-9, and precipitation occurred at all doses in the presence of S-9. The positive control chemical responses verified the sensitivity of the tester strains and the metabolising capability of the S-9 mix. There were no increases in revertant numbers following BOF-A2 treatment either up to toxic concentrations in the absence of S-9 or up to 5000 μ g/plate in its presence.

Table 3. Pre-incubation Ames Test with BOF-A2 without S-9 mix.

Test Substance	Dose (μ g/plate)	Revertant colonies per plate ^a									
		TA1535		TA1537		TA98		TA100		WP2uvrA	
		1 ^b	2 ^b	1	2	1	2	1	2	1	2
ACN ^c	-----	6	9	8	6	38	28	111	119	11	13
Water	-----							104	115		
DMSO	-----	8	7	6	6	25	27			10	14
BOF-A2 ^c	1.56	9	9	9	6	36	31	107	113	9	14
	3.13	7	8	8	7	33	32	104	112	9	14
	6.25	6	7	5	5	40	26	89	106	11	15
	12.5	8	9	7	6	34	30	95	108	10	11
	25	7	8	7	6	36	23	95	81	9	9
	50	5 ^d	8 ^d	4 ^d	5 ^d	32	21 ^d	90 ^d	83 ^d	11	9
ENNG	5	185	736								
ICR-191	1			520	762						
2NF	1					117	124				
MMS	200							564	715		
ENNG	2									81	267

^a mean of 3 replicate plates^b experiment number^c 50 μ l per plate^d thin background lawn**Table 4.** Pre-incubation Ames Test with BOF-A2 with S-9 mix.

Test Substance	Dose (μ g/plate)	Revertant colonies per plate ^a									
		TA1535		TA1537		TA98		TA100		WP2uvrA	
		1 ^b	2 ^b	1	2	1	2	1	2	1	2
ACN ^c	-----	10	10	10	9	29	25	103	114	11	15
DMSO	-----	8	9	10	9	24	28	92	102	11	15
BOF-A2 ^{c,d}	156	11	9	8	8	27	25	85	114	12	15
	313	10	7	9	7	27	25	93	105	11	16
	625	10	8	9	7	28	22	96	111	9	15
	1250	9	7	7	7	23	24	90	99	8	15
	2500	9	7	8	7	25	23	89	101	11	15
	5000	9	8	9	8	25	26	90	103	10	13
2AA	2	171	158	331	312						
2AA	0.5					507	554				
2AA	1							1177	1078		
2AA	20									714	736

^a mean of 3 replicate plates^b experiment number^c 50 μ l per plate^d all dose levels gave precipitate on the plates of most strains

Chromosomal Aberration Test in CHO Cells In Vitro :

As can be seen from Table 5, untreated and solvent-treated negative control cultures produced frequencies of chromosomal aberrations that were normal and within the historical control range. It was therefore acceptable to compare all treated cultures with the respective solvent control cultures. The positive control chemicals

MMS and CPA induced significant numbers of structural aberrations (chromatid and chromosome deletions and exchanges), verifying the sensitivity of the system and metabolising capability of the S-9 mix.

BOF-A2 treatments at 80 $\mu\text{g/ml}$ continuously for 48 hr in the absence of S-9, and at 80 and 100 $\mu\text{g/ml}$ for 2 hr in the presence of S-9, resulted in frequencies of structurally aberrant

Table 5. CHO Chromosomal Aberration Test with BOF-A2.

Method (time of exposure)	S-9	Test substance	Conc. ($\mu\text{g/ml}$)	% mitotic inhibition ^a	% cells with structural aberrations ^b		Tid. abs ^e /cell	Some. abs ^f /cell	% cells with numerical aberrations
					inc gaps ^{*c}	exc gaps ^d			
Pulse (2 hours)	-	Untreated	-	0	3	2.5	0.01	0.015	2.5
		EGDME	-	0	0.5	0.5	0	0.005	7.5
		BOF-A2	20	0	3	1.5	0.005	0.01	2.5
			40	13	3	2	0.01	0.01	5
			60	56	4.5	3.5	0.04	0.005	4
		MMS	100	ND ^g	36	32	0.62	0	4
Continuous (24 hours)	-	Untreated	-	0	5.5	3	0.02	0.01	1
		EGDME	-	0	8	3.5	0.02	0.015	1.5
		BOF-A2	40	0	6.5	3.5	0.025	0.02	3.5
			55	31	5.5	2.5	0.03	0	0.5
			70	49	9	4.5	0.065	0	2.5
		MMS	25	ND	74	74	1.12	0.1	0
Continuous (48 hours)	-	Untreated	-	0	1.5	0	0	0	0
		EGDME	-	0	3	1	0	0.015	2.5
		BOF-A2	40	0	5	3	0.015	0.01	3
			60	7	8	3	0.03	0.005	4.5
			80	67	16	9	0.09	0.065	5
		MMS	25	ND	44	38	0.54	0.18	12
Metabolic activation (2 hours)	+	Untreated	-	0	3.5	1.5	0.015	0.005	5.5
		EGDME	-	0	4	1.5	0.015	0.005	6.5
		BOF-A2	60	35	8	4.5	0.035	0.025	8.5
			80	45	21.5	11	0.135	0.035	4
			100	67	28	20.5	0.25	0.085	2
		CPA	12.5	ND	46	40	0.36	0.24	8

^a mitotic index as % of solvent control

^b scoring 200 cells per dose for untreated, EGDME and BOF-A2, and 50 cells per dose for MMS and CPA

^c including gaps

^d excluding gaps

^e chromatid-type aberrations (deletions + exchanges)

^f chromosome-type aberrations (deletions + exchanges)

^g ND = not determined

^h significantly different from solvent control, $p < 0.001$

ⁱ significantly different from solvent control, $p < 0.05$

^{*} exceeds historical solvent control range

^{*} a gap is defined as a non-staining region not greater than the width of a chromatid

cells that exceeded historical control ranges and were significantly different from controls. Large numbers of chromatid exchanges, as well as chromatid and chromosome deletions were induced, particularly in the presence of S-9. BOF-A2 is clearly clastogenic to CHO cells both in the absence and presence of S-9.

Micronucleus Test in Mouse Bone Marrow

As can be seen from Table 6, untreated and HPMC-treated mice showed normal PCE/NCE ratios and normal frequencies of micronucleated PCE and NCE. It was therefore acceptable to compare all treated groups with the HPMC (vehicle) control group. The positive control

chemical CPA induced significant increases in the incidence of micronucleated PCE, in the absence of any obvious bone marrow toxicity, verifying the sensitivity of the test system.

BOF-A2 treatments at all dose levels resulted in frequencies of micronucleated PCE that exceeded historical control ranges and were significantly different from controls. At 40 mg/kg/day these increases were accompanied by evidence of suppression of bone marrow proliferation, as the PCE/NCE ratio fell markedly. BOF-A2 was clearly able to induce micronuclei in mouse bone marrow.

Table 6. Mouse Micronucleus Test with BOF-A2.

Test Substance	Dose (mg/kg/day)	Sex	Mean PCE/NCE ratio	Micronucleated cells /1000 scored	
				PCE	NCE
Untreated	-	M	1.05	1.39	0.99
		F	1.36	1.18	0.77
		Both	1.21	1.29	0.89
HPMC	- ^a	M	0.92	1.18	0.51
		F	1.07	1.59	0.78
		Both	1.00	1.38	0.64
BOF-A2	10	M	1.19	3.36 ⁺	1.15
		F	0.87	5.48 ⁺	0.42
		Both	1.03	4.42 ^{c+}	0.70
	20	M	0.80	7.88 ⁺	1.89
		F	1.36	23.35 ⁺	3.38 ⁺
		Both	1.08	15.64 ^{c+}	2.36 ^d
	40	M	0.51	16.54 ⁺	1.49
		F	0.13	30.68 ⁺	1.37
		Both	0.32	23.20 ^{c+}	1.40
CPA	80 ^b	M	1.21	42.51 ⁺	0.77
		F	0.82	38.61 ⁺	0.42
		Both	1.02	40.57 ^{c+}	0.47

^a dose volume 10 ml/kg

^b single administration 1 day before sacrifice

^c total group value significantly different from vehicle control, $p < 0.001$

^d total group value significantly different from vehicle control, $p < 0.01$

⁺ exceeds historical vehicle control range

DISCUSSION

BOF-A2 is being developed as an anti-cancer drug which is potentially more effective than 5-FU because it contains CNDP which is very effective at inhibiting the enzyme (dihydrouracil dehydrogenase) that rapidly degrades 5-FU, plus EM-FU which is a masked form of 5-FU and should therefore be less readily degraded. Based on these properties BOF-A2 is a more effective anti-tumour agent in experimental models than other 5-FU derivatives (see Fujii et al., 1989).

From the genotoxicity data presented here, BOF-A2, resembling 5-FU (Seino et al., 1978), did not induce gene mutations in a bacterial reverse mutation test.

On the other hand, BOF-A2 clearly induced chromosomal aberrations in CHO cells at doses greater than 80 μ g/ml both after 48 hr in the absence of S-9 and after 2 hr in the presence of S-9. Induction of significant numbers of aberrations in the presence of S-9 may be indicative of metabolism mainly in the liver where EM-FU is converted to an active form, 5-FU (Fujii et al., 1989). Yajima et al. (1981) and Ishidate Jr. (1987) also reported a positive result for 5-FU *in vitro*.

BOF-A2 induced chromosomal aberrations in mouse bone marrow after 4 daily oral doses of 10, 20 and 40 mg/kg. This finding also confirms involvement of 5-FU which induced chromosomal aberrations in mouse bone marrow after 5 daily oral doses of 6.25, 12.5 and 25 mg/kg (Yamamoto and Kikuchi, 1981).

It has been demonstrated, therefore, that BOF-A2 resembles 5-FU in being unable to induce reverse mutations in bacteria but being clastogenic both *in vivo* and *in vitro*.

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