Table 39.

Mutagenicity study of OPC-13213 in WP2 *uvr*A.

Compound	Dose (μg/plate)	S-9	Revertant colonies per plate [a]							
			Expt.-1				Expt.-2			
			Plate-1	Plate-2	Plate-3	Mean	Plate-1	Plate-2	Plate-3	Mean
DMSO	–	–	11	12	12	12	9	10	12	10
OPC-13213	156	–	14	14	15	14	12	12	14	13
	313	–	8	11	15	11	8	10	10	9
	625	–	P 8	P 9	P 9	9	P 14	P 17	P 17	16
	1250	–	P 6	P 7	P 10	8	P 9	P 10	P 13	11
	2500	–	P 5	P 7	P 8	7	P 8	P 8	P 8	8
	5000	–	P 4	P 6	P 9	6	P 8	P 9	P 9	9
ENNG	2	–	146	158	181	163	171	181	194	182
DMSO	–	+	10	11	14	12	9	9	10	9
OPC-13213	156	+	14	15	17	15	9	13	15	12
	313	+	10	12	13	12	7	14	14	12
	625	+	P 6	P 9	P 9	8	P 11	P 18	P 18	16
	1250	+	P 9	P 9	P 10	9	P 9	P 13	P 14	12
	2500	+	P 6	P 9	P 13	9	P 10	P 11	P 14	12
	5000	+	P 6	P 7	P 8	7	P 7	P 10	P 11	9
2AA	20	+	713	724	756	731	623	631	675	643

a, Mean value of three replicate plates; P, Precipitation

Bacterial DNA Repair Assay (Rec-Assay) with Cilostazol (OPC-13013)

[DNA damage is subjected to cellular repair system in normal cells (wild-type H17 Rec^+ strain of Bacillus subtilis), but in repair-deficient cells (M45 Rec^- strain of B.subtilis), which are highly sensitive to the lethal effect of DNA-damaging compounds, growth inhibition occurs in the presence of mutagens. No significant growth inhibition occurs in wild-type cells in the presence of mutagens.]

Testing Facility:

Study Number: 211108-0699

Study Dates: May 25 to June 10, 1981

GLP Compliance: The study was conducted in compliance with GLP regulations.

Lot No. of the Test Compound: 1C74M (purity 99.57%)

Doses Tested: 250, 500, 1000, 2000 and 4000 µg OPC-13013/plate. (It is stated that these doses were selected based on the results of a preliminary experiment.)

Solvent: Dimethylsulfoxide (DMSO)

Tester Strains: Bacillus subtilis, H17 Rec^+ (wild type) and M45 Rec^- (repair-deficient, rec 45 mutation) strains

Negative Control Substance: Kanamycin sulfate (a protein synthesis inhibitor)

Positive Control Substance: Mitomycin C (a DNA-damaging substance)

Control substances were dissolved in sterile distilled water.

Results: The results are presented in Table 40. The test drug did not cause any growth inhibition for either strain at concentrations tested in the study. Kanamycin sulfate, a protein synthesis inhibitor, inhibited the growth of both H17 and M45 strains almost to the same extent, while mitomycin C, a DNA damaging agent, caused a 2-3 fold higher growth inhibition in M45 strain than in H17 strain. Since the test compound did not produce any inhibition of growth in the repair deficient M45 strain, OPC-13013 is considered to be a non-mutagen by the Rec-assay system.

Table 40.

Rec-assay of OPC-13013

Method		Regular method		Cold incubation method	
Compound	Amount (μg/disc)	Growth inhibition (mm)		Growth inhibition (mm)	
		H17(Rec$^+$)	M45(Rec$^-$)	H17(Rec$^+$)	M45(Rec$^-$)
H_2O	-	0	0	0	0
DMSO	-	0	0	0	0
KM	20	8.8	11.1	17.8	19.9
MMC	0.2	5.8	13.6	7.0	20.6
OPC-13013	250	0	0	0	0
	500	0	0	0	0
	1000	0	0	0	0
	2000	0	0	0	0
	4000	0	0	0	0

H_2O, distilled water; KM, kanamycin sulfate; MMC, mitomycin C; DMSO, dimethyl sulfoxide.

KM and MMC were dissolved in sterile distilled water.

OPC-13013 was dissolved in DMSO.

Bacterial DNA Repair Assay (Rec-Assay) with Cilostazol Metabolite OPC-13015

Testing Facility:

Study Number: 003681

Study Dates: November 25, 1986 to February 23, 1987

GLP Compliance: Not addressed.

Lot No. of the Test Compound: 4F83M

Doses Tested: 50, 100, 200, 400, 800 and 1600 µg OPC-13015/disc

Solvent: DMSO

Tester Strains: Bacillus subtilis, H17 Rec^+ (wild type) and M45 Rec^- (repair deficient) strains

Negative Control Substance: Kanamycin Sulfate

Positive Control Substance: Mitomycin C

Both positive and negative control substances were dissolved in sterile distilled water.

Results: Results are presented in Table 41. The test drug showed no growth inhibition of the test strains at concentration tested in the study. Kanamycin sulfate, a protein synthesis inhibitor, inhibited both H17 and M45 strain growths to a similar extent, whereas Mitomycin C, a DNA-damaging substance, exhibited stronger inhibition of M45 growth than of H17. Since the test compound did not produce any inhibition of growth, especially in the repair deficient M45 strain, the test compound is considered to be a

nonmutagen in this test system.

Table 41.

Results of Rec-Assay of OPC-13015

Test substance	Dose (μg/disc)	Growth inhibition (mm)* H17 (Rec+)	M45 (Rec-)
H_2O	-	0	0
DMSO	-	0	0
KM	20	16.0	16.4
MMC	0.2	7.9	20.8
OPC-13015	50	0	0
	100	0	0
	200	0	0
	400	0	0
	800	0	0
	1600	0	0

*Mean in duplicate

Bacterial DNA Repair Assay (Rec-Assay) with Cilostazol Metabolite OPC-13213

Testing Facility:

Study Number: 003683

Study Dates: November 25, 1986 to February 23, 1987

GLP Compliance: Not addressed.

Lot No. of the Test Compound: 4A73

Doses Tested: 62.5, 125, 250, 500, 1000 and 2000 µg OPC-13213/disc.

Solvent: DMSO

Tester Strains: Bacillus subtilis, H17 Rec^+ (wild type) and M45 Rec^- (repair deficient strains)

Negative Control Substance: Kanamycin sulfate

Positive Control Substance: Mitomycin C

Both control substances were dissolved in sterile distilled water.

Results: Results are presented in Table 42. The test drug showed no growth inhibition of the tester strains at concentrations tested. kanamycin sulfate, a protein synthesis inhibitor, inhibited both H17 and M45 growths to a similar extent, where as mitomycin C, a DNA damaging substance, showed stronger inhibition of M45 growth than of H17. Since the test compound did not produce any inhibition of growth, especially in the repair deficient M45 strain, the test compound is considered to be a non-mutagen in this test system.

Table 42.

Results of Rec-assay of OPC-13213

Test substance	Dose (μg/disc)	Growth inhibition (mm)* H17 (Rec+)	M45 (Rec-)
H_2O	-	0	0
DMSO	-	0	0
KM	20	16.0	16.4
MMC	0.2	7.9	20.8
OPC-13213	62.5	0	0
	125	0	0
	250	0	0
	500	0	0
	1000	0	0
	2000	0	0

*Mean in duplicate

Biochemical Induction Assay with Cilostazol (OPC-13013)

(Mutagenic compounds are known to induce an adaptive function called SOS function in E.coli. The biochemical induction assay is a test system for detecting chemical mutagens using E.coli incorporated with λ-lac z gene. Since the expression of λ-lac z gene is known to be induced through the SOS function, DNA damaging potential of chemicals can be detected, both qualitatively and quantitatively, by measuring the activity of β-galactosidase, a product of the λ-lac z gene.)

Testing Facility:

Study Number: 002578

Study Dates: November 19 to December 13, 1984

GLP Compliance: Not addressed

Lot No. of the Test Compound: 3C73M

Concentrations Tested: 2, 20 and 200 µg OPC-13013/ml (precipitation noted at 200 µg/ml concentration)

Solvent: DMSO

Tester Strain: Escherichia coli BR513 envA uvrB

Positive Control Substances: Aflatoxin B1 (with metabolic activation) and bleomycin sulfate (without metabolic activation)

Test Procedure: Cultures of tester strain were incubated with varying concentrations of test substance for 3 hours at 37° C. The β-galactosidase activity of the reaction mixture was determined after incubation.

Results: The results are presented in Table 43. The test compound did not produce any significant increase in β-galactosidase activity, compared to the vehicle control, at 2 or 20 µg/ml (precipitation occurred at 200 µg/ml). Positive control compounds markedly increased the enzyme activity with or without metabolic activation.

Table 43.

Biochemical Induction Assay

Compound	Dose[a] (μg/ml)	Enzyme units without S-9[b]	Enzyme units with S-9[b]
H_2O	-	333	292
DMSO	-	417	403
OPC-13013	2	389	299
	20	361	320
	200	0 P	125 P
BLM	500	9223	
AFB	500		10972

a: Final concentration

b: Mean in duplicate

P: Precipitation

BLM=bleomycin
AFB=aflatoxin B1

Effect of Cilostazol (OPC-13013) on Intercellular Communication in Chinese Hamster V79 Cells

[The cell-cell communication assay has been employed for the detection of potential tumor promoters. In this *in vitro* test system, the wild-type Chinese hamster V79 cells, containing the marker enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT), are able to metabolize 6-thioguanine (6TG) to 6-thioguanosine monophosphate (6TGMP), the latter compound being lethal to these cells. HGPRT-deficient cells, on the other hand, are resistant to 6TG and, hence, these cells can grow well in the presence of 6TG. (6TGMP is lethal also to HGPRT-deficient cells.)

When cells are cultured in close contact with each other, they develop gap-junctions between them forming a system of intercellular communication.

When the wild-type and HGPRT-deficient cells are cultured together in the presence of 6TG, they develop gap-junctions, enabling 6TGMP produced by wild-type cells to be transferred to HGPRT-deficient cells, thus killing the latter cells. Treatment of wild-type cells with tumor promoters blocks the formation of gap-junctions or impairs its function, thus blocking the transfer of 6TGMP to HGPRT-deficient cells resulting in its survival. Thus, in this assay system, the potential to block cell-cell communication, a characteristic of tumor promoters (determined by the assessment of the survival rate of resistant HGPRT-deficient cells), is used for the detection of tumor promoters.]

Testing Facility:

Study Number: 002090

Study Dates: March 12 to June 12, 1984

GLP Compliance: Not addressed

Lot No. of the Test Compound: 4A81M

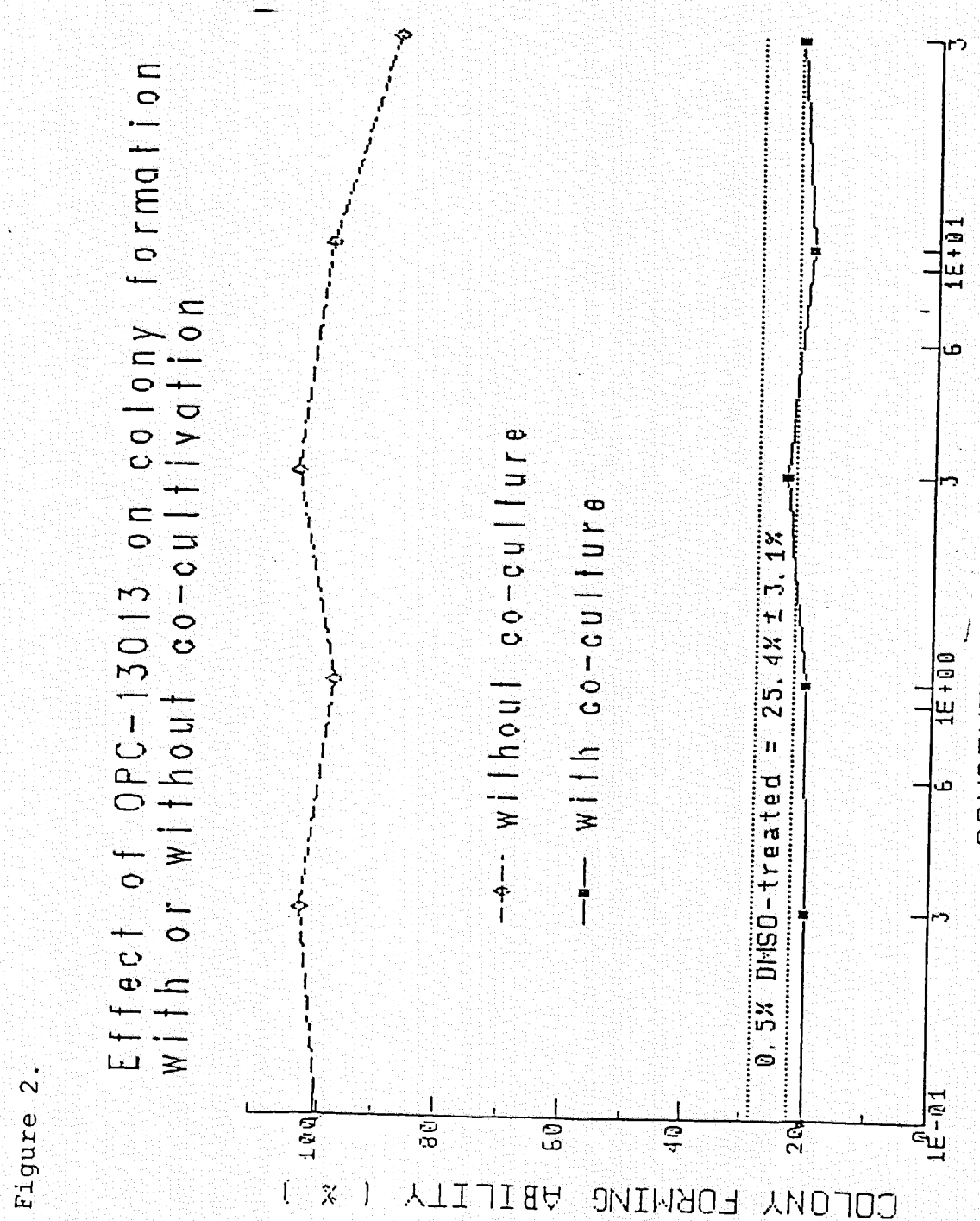
Doses Tested: 0.1, 0.3, 1.0, 3.0, 10.0 and 30.0 µg OPC-13013/ml. (Dose range finding studies showed that concentrations above 30 µg/ml were cytotoxic.)

Solvent: DMSO

Positive Control: 12-0-tetradecanoyl phorbol-13-acetate

Results: The effect of the test drug on colony formation, with and without co-culture, is presented graphically in Figure 2. Without coculture, the colony formation was about 90-100%, while with co-culture, it was only 20-25%, indicating that the test drug did not inhibit cell-cell communication of the V79 cells at concentrations tested in the study. On the other hand, the positive control substance produced 88-100% colony formation. Based on the above results, it is suggested that the test drug has no tumor promoting potential at the doses tested.

Figure 2.


Effect of OPC-13013 on colony formation
With or without co-cultivation
COLONY FORMING ABILITY (%)
100
80
60
40
20
0
1E-01
3
6
1E+00
3
6
1E+01
3
CONCENTRATION (μg/ml)
0.5% DMSO-treated = 25.4% ± 3.1%
without co-culture
with co-culture

Mouse Lymphoma L5178Y Cell Assay with Cilostazol (OPC-13013)

[L5178Y line of mouse lymphoma cells are heterozygous for the enzyme thymidine kinase ($TK^{+/-}$). Chemical-induced mutations at the TK locus result in the loss of thymidine kinase activity with the formation of a homozygous strain ($TK^{-/-}$). Both $TK^{+/-}$ and $TK^{-/-}$ strains can grow in normal medium, but the incorporation of 5-trifluorothymidine (TFT) into the medium results in cytotoxicity to the $TK^{+/-}$ cells, with growth and replication occurring only in mutant $TK^{-/-}$ cells. Thus, resistance to TFT indicates mutation, induced by the test compound, at the TK locus.

In this assay system, both gene mutations (large colonies) as well as chromosome aberrations (small colonies) can be detected.]

Testing Facility:

Study Number: 011765 (Sponsor's Number)
275/84 (Contract Lab.'s Number)

Study Dates: January 19 to June 6, 1996

GLP Compliance: The study was conducted in compliance with GLP regulations.

Lot No of the Test Compound: 4196K-4

Concentrations Tested: 10, 20, 30, 50 and 100 µg OPC-13013/ml for the first experiment and 20, 30, 50, 100 and 200 µg/ml for the second experiment. [In a cytotoxicity dose range finding study (10, 20, 50, 100, 200, 500 and 1000 µg/ml), precipitation was observed at the top 4 concentrations (heavy precipitation at 500 and 1000 µg/ml). Based on these results, 100 and 200 µg/ml concentrations were selected as top doses for the present study.]

Solvent: DMSO

Metabolic Activation System: Aroclor 1254 induced rat liver S-9 fraction

Positive Control Compounds: 4-nitroquinoline (NQO, without S-9) and benzo(a)pyrene (BP, with S-9)

The assay was considered valid if the following criteria were met:

1. the mutant frequencies in the solvent control cultures fell within the normal range and
2. at least one concentration of each of the positive control chemicals induced a clear increase in mutant frequency.

The test compound was considered to be mutagenic if:

1. the assay was valid
2. the mutant frequency at one or more doses was significantly greater than that of the negative control
3. there was a significant dose-relationship as indicated by the linear trend analysis and
4. the findings were reproducible.

Results: Two independent experiments were performed and the results are summarized in Table 44. Precipitation was observed at the top two doses (100 and 200 µg/ml) in Experiment 2, but no precipitation was seen in Experiment 1.

No statistically significant dose-related increases in mutant frequencies were observed after treatment with the test compound with or without metabolic activation. Mutant frequencies with the negative control were within the normal range, and significant increases in mutant frequencies were induced by the positive

control compounds. Small and large colony mutant frequencies for negative and positive controls are presented in Table 45. (Note: No small and large colony sizing data is given for the test compound.)

Table 44. **Summarized Data on Relative Survival and Mutant Frequencies**

Experiment 1

Treatment (µg/mL)	-S-9 %RS	-S-9 Mutant frequency#	Treatment (µg/mL)	+S-9 %RS	+S-9 Mutant frequency#
0	100.0	125.19	0	100.0	126.34
10	116.6	104.75 NS	10	78.6	112.19 NS
20	97.2	124.23 NS	20	92.6	112.61 NS
30	86.6	107.02 NS	30	79.6	135.14 NS
50	105.9	121.28 NS	50	83.9	144.95 NS
100	93.6	109.88 NS	100	86.8	104.70 NS
Linear trend		NS	Linear trend		NS
NQO			BP		
0.05	119.4	513.13	2	73.1	727.55
0.1	53.9	678.73	3	51.8	1930.24

Experiment 2

Treatment (µg/mL)	-S-9 %RS	-S-9 Mutant frequency#	Treatment (µg/mL)	+S-9 %RS	+S-9 Mutant frequency#
0	100.0	365.24	0	100.0	227.59
20	97.2	338.00 NS	20	79.6	210.28 NS
30	94.5	319.70 NS	30	91.7	165.72 NS
50	97.2	340.38 NS	50	93.1	264.93 NS
100 P	109.7	234.17 NS	100 P	83.1	121.32 NS
200 P	88.7	255.39 NS	200 P	99.3	157.42 NS
Linear trend		NS	Linear trend		NS
NQO			BP		
0.05	90.6	783.15	2	89.8	1349.72
0.1	54.7	1078.40	3	56.7	2080.23

\# Per 10^6 viable cells
NS Not significant
P Precipitate observed

RS=relative survival

Table 45.

Small and large colony mutant frequencies for negative and positive controls

Experiment	Concentration (μg/mL)	S-9	Mutant frequency*		Proportion small colony mutants
			Small colony	Large colony	
1	0	-	54.2	66.5	0.45
	NQO 0.05		255.1	192.0	0.57
	NQO 0.1		361.7	202.8	0.64
	0	+	54.7	64.8	0.46
	BP 2		344.9	232.9	0.60
	BP 3		1111.0	476.7	0.70
2	0	-	194.7	134.9	0.59
	NQO 0.05		378.6	249.3	0.60
	NQO 0.1		543.4	326.6	0.62
	0	+	110.7	104.6	0.51
	BP 2		639.2	371.0	0.63
	BP 3		1223.6	457.6	0.73

* Per 10^6 viable cells