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Synergism and Antagonism Induced by Three Carrier Solvents with t-Retinoic Acid and 6-Aminonicotinamide Using FETAX

J. R. Rayburn,[1] D. J. Fort,[1] R. McNew,[2] and J. A. Bantle[1]

[1]Department of Zoology, Oklahoma State University, Stillwater, Oklahoma 74078, USA and [2]Agricultural Statistics Laboratory, University of Arkansas, Fayetteville, Arkansas 72701, USA

The large number of water-insoluble chemicals requiring toxicity testing necessitates the development, validation and use of chemical cosolvents. Carrier solvents (cosolvents), such as dimethylsulfoxide (DMSO), acetone, and triethylene glycol (TG), are commonly used to solubilize hydrophobic compounds (Yalkowsky 1981). However, the use of solvents with in vitro bioassays may alter the developmental toxicity of test materials. Solvents interact with other compounds to change rates of reactions, membrane potentials, mutagenic activity, and many other cell processes (Coetzee and Ritchie 1969; Nemethy 1986; Demey et al. 1983; Gichner and Veleminsky 1987). For this reason solvent-compound interaction studies were performed to determine if the developmental toxicity of test materials was altered.

The Frog Embryo Teratogenesis Assay-*Xenopus* (FETAX), formally described by Dumont et al. (1983), is a 96-hr bioassay which determines relative teratogenic hazard. Several labs have evaluated compounds as well as environmental mixtures with FETAX (Courchesne and Bantle 1985; Dawson and Bantle 1987a; Dumont et al. 1983; Sabourin and Faulk 1987; Dawson et al. 1985). Fort et al. (1989) have also developed and evaluated an exogenous metabolic activation system for FETAX. The purpose of these experiments was to determine whether carrier solvents interacted with the teratogens t-retinoic acid and 6-aminonicotinamide to affect survival, development and growth of *Xenopus laevis* embryos.

MATERIALS AND METHODS

Animal care and breeding were performed according to Bantle et al. (1989). FETAX solution (Dawson and Bantle 1987b) which is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4$*$2H_2O$, and 75 mg $MgSO_4$ per L was used as the diluent for all experiments. For each concentration-response test, two groups of 25 embryos each were placed in 60 X 15 mm glass Petri dishes containing a total of 10 mL of solution. Four groups of 25 embryos each were used as controls for each test. Each experiment followed standard methods of test operation and embryo evaluation according to Bantle et al. (1989).

One range and three definitive experiments determined the 96-hr LC50, 96-hr EC50 (malformation), Teratogenic Index (TI) (96-hr LC50/ 96-hr EC50) and Minimum Concentration to Inhibit Growth (MCIG) for three solvents and two teratogens. The solvents were dimethylsulfoxide (DMSO) (CAS# 67-68-5; Sigma Chemical Co., St. Louis, Missouri)

Send correspondence to Dr. J.A. Bantle at address above.

acetone (CAS# 67-64-1; Fisher Scientific, Houston, Texas), and triethylene glycol (TG) (CAS# 112-27-6; Aldrich Chemical Co., Milwaukee, Wisconsin). The teratogens obtained from Sigma were trans-retinoic acid (RA) (CAS# 302-79-4) and 6-aminonicotinamide (6-AN) (CAS# 329-89-5). The 96-hr LC25 and LC50, and 96-hr EC25 and EC50 (malformation) were determined using the Litchfield-Wilcoxon probit analysis computer program part of the Manual of Pharmacological Calculations (Tallarida and Murray 1980). Dunnett's test was used to determine the MCIG and No Observable Effect Concentrations (NOEC). This data was used in determining test concentrations for the solvent-compound interaction study.

Two concentrations for each solvent and test material were selected. The two solvent concentrations were the NOEC and 96-hr EC25. The NOEC is the highest cosolvent concentration used in FETAX. The 96-hr EC25 was chosen in order to positively identify any interactions that might be taking place that were not observable at lower concentrations. The 6-AN and RA concentrations were the 96-hr EC25 and 96-hr LC25. These concentrations allowed for examination of the effects on both malformation and mortality. Both 6-AN and RA were soluble at these concentrations without the solvents.

The interaction experiments contained individual treatments for all concentrations and the interaction treatments for one solvent and one teratogen per experiment. The nine treatments were: 1) FETAX solution controls, 2) EC25 of teratogen, 3) LC25 of teratogen, 4) NOEC level of solvent, 5) EC25 of solvent, 6) EC25 of teratogen & NOEC of solvent, 7) EC25 of teratogen & EC25 of solvent, 8) LC25 of teratogen & NOEC of solvent, and 9) LC25 of teratogen & EC25 of solvent. All experiments contained four replicates of 25 embryos each per treatment. The analysis of solvent-teratogen interactions represented three pooled experiments using three different breeding pairs. Every 24 hrs dead embryos were removed and solutions changed. After 96 hrs of exposure, embryos were anesthetized with 3-Aminobenzoic Acid Ethyl Ester (MS-222) (CAS# E1-052-1; Sigma) and the number malformed recorded. The larvae were then killed with 0.7% (w/v) formalin so that head-tail length (growth) could be measured.

ANOVA was used to determine differences from theoretical additive values for mortality, malformation and growth according to Steel and Torrie (1980). Values were determined by partitioning the sum of squares for the interactions on mortality and malformation using the statistical software systat (Wilkinson 1989). Growth interactions were determined in the same manner with the Statistical Analysis System (SAS). Effects on the TI were inferred from the shifts of the mortality and malformation curves.

RESULTS AND DISCUSSION

Compounds were considered to pose teratogenic hazard when the TI > 1.5 (Bantle et al. 1989; Dawson et al. 1989). The TI represents the separation between the mortality and malformation curves. If a synergistic or antagonistic response altered a TI, false conclusions regarding teratogenic hazard could result.

Preliminary work revealed that all solvents caused effects at 2.0% (v/v) concentrations. Acetone had the highest TI of the solvents followed by DMSO and then TG (Table 1). NOEC levels for DMSO, Acetone and TG were 1% (v/v) for malformation, 0.9% (v/v) for malformation, and 1.7% (v/v) for growth, respectively. The 96-hr EC25 (malformation) for DMSO, Acetone and TG were 1.2% (v/v), 1.0% (v/v), and 2.0% (v/v).

Both 6-AN and RA caused teratogenic effects in FETAX and 6-AN had a much higher TI (Table 1). Mammalian literature supports the classification of RA and 6-AN as teratogens (Schardein et al. 1967; Chamberlain 1966; Kochhar 1975; Lammer et al. 1985). The 96-hr

EC25 (malformation), and 96-hr LC25 for each teratogen were as follows: Retinoic acid was 0.02 mg/L and 0.25 mg/L; 6-aminonicotinamide was 2 mg/L and 2500 mg/L.

Table 1. 96-hr LC50, 96-hr EC50(malformation), Teratogenic Index (TI)[a], and Minimum Concentration to Inhibit Growth (MCIG) for Dimethylsulfoxide (DMSO), Acetone, Triethylene glycol (TG), Retinoic Acid (RA) and 6-aminonicotinamide (6-AN).

Compound	Trial	96-hr LC50	96hr EC50	TI	MCIG
DMSO[b]	1	1.81(1.75-1.87[c])	1.4 (1.32-1.48)	1.3	1.3
	2	1.77(1.61-1.95)	1.29(1.25-1.33)	1.4	1.7
	3	1.86(1.4-2.3)	1.24(0.83-1.8)	1.5	1.5
Acetone[b]	1	2.16(2.07-2.25)	1.4 (1.29-1.43)	1.6	1.8
	2	2.49(2.10-2.95)	1.4 (1.04-1.36)	1.8	1.5
	3	1.92(1.90-2.14)	1.06(0.91-1.17)	1.83	1.0
TG[b]	1	2.4 (2.02-2.85)	2.0 (2.01-2.13)	1.2	1.8
	2	2.75(2.70-2.82)	2.4 (2.37-2.45)	1.1	1.8
	3	2.19(2.19-2.32)	2.05(1.99-2.11)	1.07	1.7
RA[d]	1	0.25(0.22-0.28)	0.024(0.018-0.031)	10.4	0.06
	2	0.50(0.46-0.61)	0.044(0.032-0.060)	11.4	0.08
6-AN[d e]	1	3190(3000 3400)	5.3(2.5-7.5)	602	100
	2	2950(2800-3100)	5.7(5.3-6.2)	518	NA

[a] TI = 96-hr LC50/96-hr EC50 (malformation).
[b] Concentrations expressed as % (v/v).
[c] 95% confidence limits.
[d] Concentrations expressed as mg/L.
[e] Data from Dawson et al. (1989).

Tables 2 and 3 show the treatment effects for each of the six solvent interaction experiments. Control malformation and mortality generally were less than 8% except for two experiments (Tables 2 and 3). Table 3 revealed that 2500 mg/L 6-AN was not the LC25 but was actually closer to the LC50. The reason for these discrepancies may be attributed to an antibiotic used by Dawson et al. (1989) to control bacterial growth and in the present study no antibiotic treatments were used. Because the values for solvent and compounds often varied due to different breeding pairs, the values were kept discrete for each experiment. For example, DMSO at 1.2% (v/v) should have caused 25 percent malformation. The retinoic acid experiment (Table 2) showed 20.9% malformation for 1.2% (v/v) DMSO, and the 6-aminonicotinamide (Table 3) showed 28.3% malformation for 1.2% (v/v) DMSO.

Effects on length were not different from additive values at $p = 0.05$. Therefore, none of the cosolvents affected embryo growth in a synergistic or antagonistic manner.

The interaction results for RA combined with DMSO (Figure 1) showed that mortality increased significantly at $p = 0.001$. DMSO at 1% (v/v) and 1.2% (v/v) concentrations with 0.25 mg/L RA increased mortality by 34.3% and 47.3%, respectively. Because

malformation of DMSO with RA was not significantly different at either concentration, the synergistic effect on mortality should cause the mortality curve to shift to the left (reducing the 96-hr LC50). DMSO addition would reduce the TI for RA.

Table 2. Interactive effects on mortality, malformation, and growth inhibition caused by the solvents and retinoic acid (RA).

Treatment[a]	Mortality No. (%)	Malformation No. (%)	Mean Length (mm)
FETAX soln.			
Control	11 (3.7 ± 1.43[b])	12 (4.2 ± 0.77)	9.61 ± 0.064
DMSO[c] RA[d]			
1.0%	11 (3.7 ± 0.77)	29 (10.1 ± 1.53)	9.44 ± 0.092
1.2%	22 (7.3 ± 1.76)	58 (20.9 ± 0.94)	9.17 ± 0.064
0.02	18 (6.0 ± 1.15)	65 (23.0 ± 3.58)	9.58 ± 0.081
0.25	64 (21.3 ± 6.86)	236 (100.0)	7.06 ± 0.381
1.0% 0.02	13 (4.3 ± 1.25)	104 (36.3 ± 6.06)	9.15 ± 0.098
1.2% 0.02	15 (5.0 ± 1.31)	111 (38.9 ± 3.61)	9.10 ± 0.069
1.0% 0.25	167 (55.7 ± 7.95)	133 (100.0)	6.55 ± 0.305
1.2% 0.25	217 (72.3 ± 9.84)	83 (100.0)	6.25 ± 0.460
FETAX soln.			
Control	6 (2.0 ± 0.78)	14 (4.7 ± 1.54)	9.54 ± 0.046
Acetone[c] RA[d]			
0.9%	9 (3.0 ± 1.22)	31 (10.6 ± 1.62)	9.04 ± 0.061
1.0%	13 (4.3 ± 1.25)	65 (22.7 ± 1.12)	8.94 ± 0.087
0.02	12 (4.0 ± 1.21)	93 (32.2 ± 5.49)	9.17 ± 0.087
0.25	87 (29.0 ± 7.45)	213 (100.0)	6.33 ± 0.352
0.9% 0.02	17 (5.7 ± 1.04)	145 (51.1 ± 5.18)	8.89 ± 0.066
1.0% 0.02	18 (6.0 ± 2.25)	251 (89.2 ± 4.94)	8.61 ± 0.098
0.9% 0.25	132 (44.0 ± 9.86)	168 (100.0)	6.20 ± 0.211
1.0% 0.25	120 (40.0 ± 11.79)	180 (100.0)	6.11 ± 0.140
FETAX soln.			
Control	23 (7.7 ± 2.9)	22 (7.9 ± 0.67)	9.36 ± 0.061
TG[c] RA[d]			
1.7%	35 (11.7 ± 2.53)	58 (22.1 ± 2.68)	8.28 ± 0.144
2.0%	27 (9.0 ± 2.04)	94 (34.5 ± 2.76)	8.31 ± 0.234
0.02	20 (6.7 ± 2.22)	68 (24.5 ± 1.62)	9.17 ± 0.061
0.25	54 (18.0 ± 4.89)	246 (100.0)	7.32 ± 0.237
1.7% 0.02	26 (8.7 ± 2.78)	100 (36.4 ± 3.72)	8.43 ± 0.199
2.0% 0.02	25 (8.3 ± 2.78)	131 (47.7 ± 4.76)	8.32 ± 0.210
1.7% 0.25	115 (38.3 ± 9.99)	185 (100.0)	6.20 ± 0.390
2.0% 0.25	139 (46.3 ± 11.11)	161 (100.0)	6.48 ± 0.498

[a] N for all treatments equaled 300 embryos, 12 dishes of 25 embryos each.
[b] Standard Error
[c] Concentrations are v/v%.
[d] Concentrations are mg/L
TG = Triethylene Glycol
DMSO = Dimethylsulfoxide

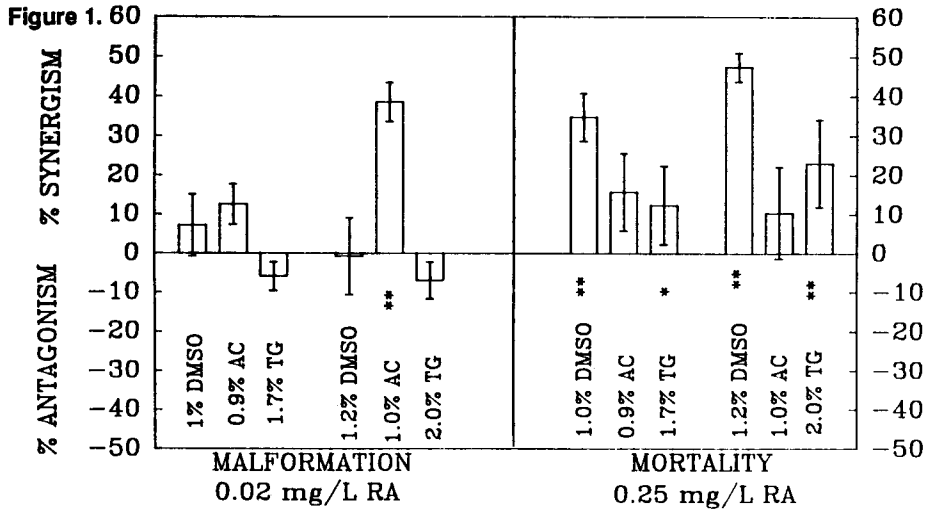
RA in the presence of acetone (Figure 1) significantly increased malformation by 38.5%

with 1% (v/v) acetone and 0.02 mg/L RA while not changing the mortality at 1% acetone and 0.25 mg/L RA. The synergistic shift of the malformation concentration-response curve to the left (reducing the 96-hr EC50) would increase the TI with acetone at the 1% (v/v) level. There were no significant effects at the 0.9% (v/v) level (NOEC) of acetone.

Table 3. Interactive effects on mortality, malformation and growth inhibition caused by the solvents and 6-aminonicotinamide (6-AN).

Treatment[a]	Mortality No. (%)	Malformation No. (%)	Mean Length (mm)
FETAX soln.			
Control	12 (4.0 ± 1.39[b])	26 (8.9 ± 2.10)	9.51 ± 0.115
DMSO[c] 6-AN[d]			
1.0%	14 (4.7 ± 1.69)	43 (15.0 ± 1.70)	9.55 ± 0.124
1.2%	25 (8.3 ± 2.97)	78 (28.3 ± 3.11)	9.60 ± 0.144
2.0	12 (4.0 ± 1.30)	71 (24.8 ± 1.80)	9.77 ± 0.064
2500	150 (50.0 ± 5.34)	150 (100.0)	6.70 ± 0.063
1.0% 2.0	18 (6.0 ± 1.94)	94 (33.4 ± 4.41)	9.71 ± 0.167
1.2% 2.0	19 (6.3 ± 2.43)	132 (46.9 ± 6.84)	9.56 ± 0.124
1.0% 2500	300(100.0)		
1.2% 2500	300(100.0)		
FETAX soln.			
Control	7 (2.3 ± 1.25)	15 (5.1 ± 0.74)	9.72 ± 0.084
Acetone[c] 6-AN[d]			
0.9%	11 (3.7 ± 1.04)	43 (15.0 ± 1.26)	9.43 ± 0.072
1.0%	20 (6.7 ± 2.16)	74 (26.6 ± 1.61)	9.17 ± 0.061
2.0	10 (3.3 ± 1.46)	70 (24.2 ± 1.23)	9.76 ± 0.075
2500	167 (55.7 ± 8.76)	133 (100.0)	6.06 ± 0.182
0.9% 2.0	25 (8.3 ± 2.89)	75 (27.2 ± 1.06)	9.29 ± 0.098
1.0% 2500	11 (3.7 ± 1.04)	135 (46.8 ± 2.87)	9.10 ± 0.058
0.9% 2.0	288 (96.0 ± 1.21)	12 (100.0)	6.08 ± 0.084
1.0% 2500	294 (98.0 ± 1.15)	6 (100.0)	5.84 ± 0.055
FETAX soln.			
Control	29 (9.7 ± 2.97)	17 (6.5 ± 1.45)	9.44 ± 0.136
TG[c] 6-AN[d]			
1.7%	52 (17.3 ± 6.35)	40 (16.0 ± 2.42)	9.00 ± 0.156
2.0%	57 (19.0 ± 6.44)	113 (46.6 ± 4.11)	8.60 ± 0.202
2.0	26 (8.7 ± 3.18)	57 (20.9 ± 1.36)	9.50 ± 0.181
2500	209 (69.7 ± 9.98)	91 (100.0)	6.69 ± 0.147
1.7% 2.0	64 (21.3 ± 6.27)	168 (71.4 ± 7.47)	8.60 ± 0.240
2.0% 2.0	33 (11.0 ± 5.10)	218 (81.6 ± 4.14)	8.40 ± 0.240
1.7% 2500	299 (99.7 ± 0.33)	1 (100.0)	7.74
2.0% 2500	300(100.0)		

a N for all treatments equaled 300 embryos, 12 dishes of 25 embryos per dish.
b Standard Error
c Concentrations expressed as % (v/v).
d Concentrations expressed as mg/L.
TG = Triethylene Glycol
DMSO = Dimethylsulfoxide



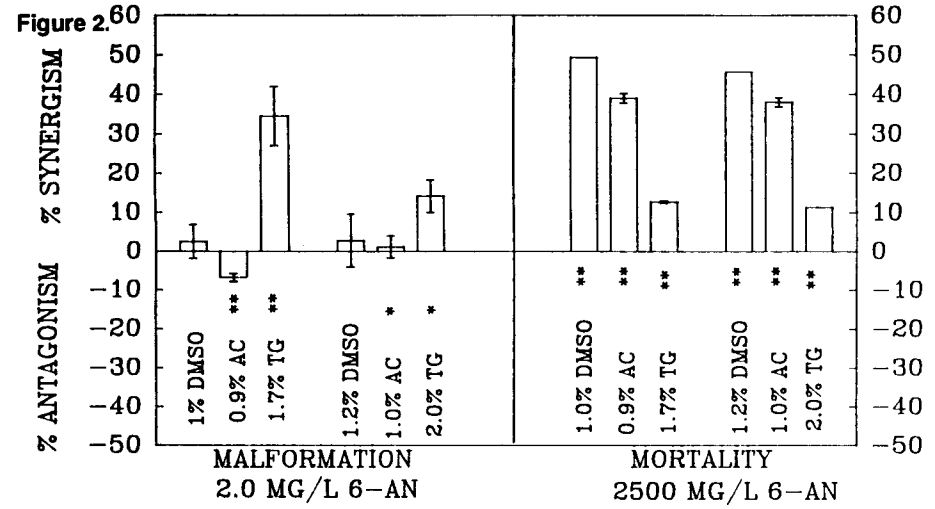


Figure 1. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for retinoic acid (RA).

Figure 2. Cosolvent-induced synergism or antagonism in FETAX mortality and malformation endpoints for 6-aminonicotinamide (6-AN).

Zero value represents the expected additive effect for the solvent and test compound. Error bars are the standard error of the actual mean effect for the solvent and test compound. * = significantly different at the p=0.05 and ** = significantly different at p=0.001. Solvents are Dimethyl Sulfoxide (DMSO), Acetone (AC) and Triethylene Glycol (TG). Significant interaction accounts for variation between experiments and is why two close values are significantly different.

TG with RA had effects on both malformation and mortality (Figure 1). Malformation with 2.0% (v/v) TG and 0.02 mg/L RA reduced the additive value by 7% at p = 0.059. Mortality rates of 1.7% (v/v) TG and 2.0% (v/v) TG with 0.25 mg/L RA increased the additive values

by 12.3% and 23% respectively. The antagonistic shift of the malformation curve to the right (increasing the 96-hr EC50) and the synergistic shift of the mortality curve to the left (decreasing 96-hr LC50) would produce a reduced TI.

DMSO combined with 6-AN synergistically increased mortality rates at both levels of the solvent at p = 0.001 (Figure 2). The increase in mortality for 1% (v/v) DMSO and 1.2% (v/v) with 2500 mg/L 6-AN was 49.3% and 45.7%, respectively. The mortality curve would shift to the left, decreasing the 96-hr LC50 and reducing the TI.

Acetone tested with 6-AN had significant effects at p <= 0.05 for both malformation and mortality (Figure 2). The malformation decreased with 0.9% (v/v) acetone and 2 mg/L 6-AN by 6.8%. However, the 1.0% (v/v) acetone with 2 mg/L 6-AN increased malformation by only 1.2%. The mortality caused by acetone with 6-AN increased for 0.9% (v/v) acetone and 1% (v/v) acetone with 2500 mg/l 6-AN by 39% and 38% respectively. Because the synergistic increase in mortality (reducing the 96-hr LC50) outweighed the antagonistic effects on malformation, the TI should be reduced.

TG in the presence of 6-AN significantly increased both malformation and mortality at p <=0.05 (Figure 2). TG at 1.7% (v/v) and 2% (v/v) combined with 2 mg/L 6-AN increased malformation by 34.5% and 14.2% respectively. Mortality increased for 1.7% (v/v) TG and 2.0% (v/v) with 2500 mg/L 6-AN by 12.7% and 11.3% respectively. Both mortality and malformation were both increased by approximately (reducing both the 96-hr LC50 and 96-hr EC50) the same amount thus TI should not change appreciably.

Malformation caused by all interaction treatments did not produce new or different types of malformation. All malformations were the same type as seen in the individual control treatments of each teratogen and solvent. The differences were in the magnitude of the response and number of the malformations.

The solvents tested generally caused synergistic effects with RA or 6-AN in FETAX. Mortality exhibited the greatest response to combined treatments while malformation was less affected and growth was unaffected. Interestingly, Malformation and growth inhibition are more sensitive endpoints than mortality. These results point out the need to consider the possibility of interactions for each endpoint separately and the necessity of using minimal solvent concentration. These interactions would probably cause problems with all in vitro aquatic bioassays. Therefore other methods of solubilizing insoluble material needs investigation.

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