Concerns with the NTP Technical Report 494 on the Toxicology and Carcinogenesis Studies of Anthraquinone in F344/N Rats and B6C3F$_1$ Mice

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The National Toxicology Program
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Primary Issue

Substantial peer-reviewed evidence has been generated that indicates that the preparation of anthraquinone used in the National Toxicology Program (NTP) bioassay was contaminated with biologically significant amounts of mutagenic contaminants, primarily 9-nitroanthracene (9-NA) (NTP, 2004). Estimates based on mutagenic potency indicate that it is plausible that the 9-NA and other contaminants could have been responsible for a portion, if not all, of the tumor induction observed in the NTP study (Butterworth *et al.*, 2001) (attached). In the absence of reliable cancer data, therefore, no conclusion as to the carcinogenic activity of anthraquinone (AQ) can be made at this time. While the contamination problem is discussed in the body of the revised NTP Draft Report, this critical issue is not presented in either the Title or the Abstract and could well be missed by those not reading the Technical Report in detail.

Contamination of the AQ Bioassay Material with 9-NA

Arkion Life Sciences utilizes anthraquinone for bird repellency that includes airport applications to minimize bird-strikes to improve flight safety. We have extensive experience on the manufacture, issues of purity, and biological activities associated with the compound. Commercial AQ is produced by three different production methods

worldwide including the oxidation of anthracene. The oxidation method begins with anthracene produced from coal tar and different lots can contain various contaminants, particularly the mutagenic isomers of nitroanthracene. AQ from this source is rarely used in the United States, but was the material utilized in the NTP cancer bioassay (NTP, 2004). A sample of the actual material used in the NTP cancer bioassay was mutagenic without S9 in the Ames test bacterial strains TA98, TA100, and TA1537 (Butterworth *et al.*, 2001). Removal of the 9-NA and other contaminants by recrystalization resulted in the complete loss of mutagenic activity (Butterworth *et al.*, 2001). AQ commonly used in the United States produced by other production methods was also shown to lack mutagenic activity in the Ames assay (Butterworth *et al.*, 2001). The weight of evidence also indicates that AQ is negative in various *in vitro* and *in vivo* mammalian cell genotoxicity assays (Butterworth *et al.*, 2001).

Effect of the Mutagenic Contaminant on Results of the Bioassay

All of the mutagenic activity could be attributed to 9-NA and other contaminants. In the mutagenicity assay of the bioassay test material, the lowest observed effect level (LOEL) in TA98 for the 9-NA component was at only 0.15 μg/plate, indicating that these contaminants are exceptionally potent mutagens. On the basis of revertants/microgram, 9-NA was more potent than benzo(a)pyrene and was about equally as potent as the 2-nitrofluorene run concurrently as positive controls (Butterworth *et al.*, 2001). TD_{50} quantitative carcinogenicity potency estimates indicate that a carcinogen of a potency in the range between benzo(a)pyrene and dimethylnitrosamine would be required to produce the observed carcinogenic response reported for AQ at the levels of the contaminants found in the test sample. While recognizing the limitation in extrapolating mutagenic potency to potential carcinogenic potency, these estimates do confirm that it is plausible that the 9-NA contaminant alone could have been responsible for a portion, if not all, of the tumor induction observed in the NTP study.

Mutagenic Potency of 9-NA

The mutagenic potency of 9-NA and other contaminants become critical in estimating the potential contribution to the carcinogenic response. For example, based on mutagenic potency, the authors of the NTP study conclude that 9-NA is unlikely to have made a significant contribution to the carcinogenic responses observed in the study (NTP, 2004 - p. 120). The mutagenicity data on which that conclusion is based is questionable because it is inconsistent with the observed potent activity of 9-NA reported in other studies as noted below.

A valuable measure of potency is the lowest dose at which mutagenic activity can be measured - the LOEL. The following LOEL values have been observed for 9-NA in tester strain TA 98 without metabolic activation.

LOEL Values for 9-NA

0.15 µg/plate	9-NA in the NTP bioassay material (Butterworth *et al.*, 2001).
0.17 µg/plate	NTP studies with contaminated AQ (NTP, 2004 - Table E1)*.
0.30 µg/plate	Covance study with pure 9-NA (Covance, 2001) (attached).
100.00 µg/plate	NTP study with pure 9-NA (NTP, 2004 - Table E7).

* The original draft of the NTP carcinogenesis report with AQ concluded that AQ was mutagenic in the Ames test (NTP, 2004 - Table E1). In fact, the pattern of activity and dose responses were remarkably similar to the Butterworth et al., 2001 study. The LOEL for TA98 was 33 µg/plate. The percentage of 9-NA in the preparation was not determined. However, assuming a concentration of 0.5% of 9-NA and attributing the mutagenic activity to this component yields a LOEL of 0.17 µg/plate. In subsequent studies, the NTP confirmed that upon purification AQ was without activity in the Ames test (NTP, 2004 - Table E2).

Note that the last LOEL value (NTP, 2004 - Table E7) is dramatically out of line with the other studies. Clearly the 9-NA dose-response issue needs to be resolved before the NTP report is released because of the pivotal role of the data in potency calculations. Interestingly, the LOEL for both 1-NA and 2-NA is 0.33 µg/plate (NTP, 2004 - Tables E5 and E6), which is more in line with the high mutagenic potency observed for the bioassay contaminants.

Conclusion - Recommended Course of Action

We have insufficient knowledge at this time to conclude that AQ has carcinogenic activity. The purpose of a cancer bioassay is not just to determine whether a compound can induce cancer, but rather to determine the conditions and shape of the dose response curve so that a meaningful quantitative risk assessment can be conducted. No such conclusions may be drawn for AQ from the revised NTP studies because of the contamination issue. One set of assumptions indicates that it is plausible that all of the carcinogenic activity came from the 9-NA contaminant (Butterworth *et al.*, 2001). In contrast, the authors of the NTP study entertain an alternate set of assumptions based on the biological activity of the 1- and 2-hydroxyanthraquinone metabolites and conclude that 9-NA was unlikely to contribute to the carcinogenic response (NTP, 2004). Clearly, no definite conclusion as to the carcinogenic activity of AQ can be made at this time.

AQ is an important compound in commerce and it is vital that we have a quantitative understanding of its carcinogenic potential in order to make sound decisions on utilization and acceptable exposures. The only avenue to do this is to conduct a new bioassay using the uncontaminated AQ in common use today. We recommend that the current report not be released, particularly without noting the serious contamination issue in the Title and Abstract. We urge that the NTP consider conducting a new bioassay as soon as is practical.

References

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The preparation of anthraquinone used in the National Toxicology Program cancer bioassay was contaminated with the mutagen 9-nitroanthracene

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Commercial anthraquinone (AQ) (9,10-anthracenedione) is produced by at least three different production methods worldwide: oxidation of anthracene (AQ-OX), Friedel–Crafts technology (AQ-FC) and by Diels–Alder chemistry (AQ-DA), with the final product varying in color and purity. AQ-OX begins with anthracene produced from coal tar and different lots can contain various contaminants, particularly the mutagenic isomers of nitroanthracene. AQ has been reported to be negative in a variety of genotoxicity tests including numerous Ames *Salmonella* mutagenicity assays. In addition, we report that AQ-DA is negative in the *Salmonella–Escherichia coli* reverse mutation assays, the L5178Y mouse lymphoma forward mutation assay, for inducing chromosomal aberrations, polyploidy or endoreduplication in Chinese hamster ovary cells, and in the *in vivo* mouse micronucleus assay. Further, a previous 18 month bioassay conducted with AQ administered to male and female B6C3F$_1$ and (C57BL/6 × AKR)F$_1$ mice reported no induction of cancer. Thus, it was somewhat unexpected that in a long-term study conducted by the National Toxicology Program (NTP) AQ-OX induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a strong increase in the livers of male and female B6C3F$_1$ mice. In the studies reported here, a sample of the AQ-OX used in the NTP bioassay was shown to be mutagenic in the Ames tester strains TA98, TA100 and TA1537. Addition of an S9 metabolic activation system decreased or eliminated the mutagenic activity. In contrast, the purified NTP AQ-OX as well as the technical grade samples AQ-FC and AQ-DA were not mutagenic in the Ames test. The chemical structure of AQ does not suggest that the parent compound would be DNA reactive. Therefore, a mutagenic contaminant was present in the NTP bioassay sample that is either directly mutagenic or can be activated by bacterial metabolism. Analytical studies showed that the primary contaminant 9-nitroanthracene (9-NA) was present in the NTP AQ-OX at a concentration of 1200 p.p.m., but not in the purified material. The 9-NA and any other contaminants that might have been present in the NTP AQ-OX induced measurable mutagenicity at 9-NA concentrations as low as 0.15 μg/plate in tester strain TA98, indicating potent mutagenic activity. On the basis of revertants per microgram, 9-NA was more potent than benzo[*a*]pyrene (B[*a*]P) and was about equally as potent as the 2-nitrofluorene run concurrently as positive controls. TD$_{50}$ quantitative carcinogenicity potency estimates indicate that a carcinogen of a potency in the range between B[*a*]P and dimethylnitrosamine would be required to produce the observed carcinogenic response at the levels of the contaminants found in the test sample. While recognizing that there are limitations in extrapolating mutagenic potency to potential carcinogenic potency, these estimates do indicate that it is plausible that the 9-NA contaminant might have been responsible for all of the tumor induction observed in the NTP study. In fact, in the absence of reliable cancer data, the genetic toxicology profile indicates that AQ would not be a genotoxic carcinogen. Thus, no conclusion as to the carcinogenic activity of AQ can be made at this time.

Introduction

The importance of source and purity

Anthraquinone (AQ) (9,10-anthracenedione) is used to enhance the efficiency of the Kraft Process for the production of paper, thus reducing the demand for trees to be cut down (Cofrancesco, 1992). AQ is the active ingredient in the most effective and non-harmful bird repellent used, for example, for keeping birds from airport runways or areas where they would conflict with the human population (Ballinger and Price, 1996; Cummings *et al.*, 1997; Ballinger *et al.*, 1998; Dolbeer *et al.*, 1998).

In assessing the potential biological activity of preparations of AQ, it is critical to be aware of how the preparation of interest was manufactured and the potential contaminants inherent with the different synthesis processes. AQ is produced in large quantities by at least three different production methods in various parts of the world (Cofrancesco, 1992). The oxidation of anthracene to yield AQ is the oldest known production process and is now practiced primarily in Europe. AQ from the oxidation process (AQ-OX) involves the oxidation of anthracene derived from coal tar. The quality of the AQ-OX produced is dependent on the number of contaminating high-boiling mutagenic and carcinogenic polycyclic aromatic hydrocarbons (PAHs), which co-distill with anthracene, found in the starting material. Profiles of contaminants from this process can differ substantially. Of particular concern is the observation that the mutagenic nitroanthracenes are often seen in AQ-OX preparations, sometimes at concentrations >2500 p.p.m. (US EPA, 1977; ICI, 1978a, 1978b).

Benzene and phthalic anhydride undergo the Friedel–Crafts reaction to yield *o*-benzoylbenzoic acid, which is treated with concentrated sulfuric acid to yield AQ. This is the most prevalent production method employed in China and India. AQ produced by the Friedel–Crafts process (AQ-FC) is substantially free of the PAH contaminants and nitroanthracenes that can be found in AQ-OX.

Production of AQ by the Diels–Alder reaction (AQ-DA) between 1,4-naphthoquinone and 1,3-butadiene is practiced primarily in Japan. Because this process involves shifts between

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the aqueous and organic phases, contaminants are easily removed and AQ-DA is particularly clean and free of contaminants.

To our knowledge, all AQ used commercially in the US is either AQ-FC or AQ-DA, rather than AQ-OX. Interestingly, the reagent grade material supplied to research laboratories is often AQ-OX. The National Toxicology Program (NTP) recently completed a cancer bioassay with AQ showing that it exhibited weak to modest carcinogenic activity (NTP, 1999). The material employed in that bioassay was from the oxidation process, AQ-OX, and contained an unidentified peak by GC analysis at a level of 0.12% (Battelle, 1993; NTP, 1999). One purpose of the studies presented here was to identify and quantify that and other potential contaminants in the material used by the NTP.

Genotoxicity

A large number of mutagenicity assays have reported that neither AQ nor its metabolites exhibit genotoxic activity. Negative results in the Ames *Salmonella* bacterial mutagenicity assay have been reported by seven independent laboratories (Brown and Brown, 1976; Anderson and Styles, 1978; Gibson *et al.*, 1978; Salamone *et al.*, 1979; Sakai *et al.*, 1985; Tikkanen *et al.*, 1983; National Cancer Institute, 1987). AQ is negative in the Syrian hamster embryo (SHE) cell transformation assay (Kerckaert *et al.*, 1996). AQ is also not mutagenic in a line of human B-lymphoblastoid cells that constitutively express cytochrome P4501A1 (Durant *et al.*, 1996). An 18 month bioassay conducted with AQ administered to male and female $B6C3F_1$ and $(C57BL/6\times AKR)F_1$ mice reported no induction of cancer (Innes *et al.*, 1969). In that study, AQ composition was confirmed by infrared spectroscopy, gas chromatography and thin-layer chromatography. No contaminants were reported (Innes *et al.*, 1969).

Thus, it was somewhat unexpected when the NTP reported that AQ induced a weak to modest increase in tumors in the kidney and bladder of male and female F344/N rats and a strong increase in the livers of male and female $B6C3F_1$ mice (NTP, 1999). In contrast to the numerous papers documenting a lack of AQ mutagenic activity noted above, two papers reported that AQ was mutagenic in the Ames *Salmonella* mutagenicity assay. The pattern of activity of AQ reported was, however, unusual in that mutagenic activity was seen without metabolic activation, and that addition of an S9 metabolic activation system reduced or eliminated the response (Liberman *et al.*, 1982; Zeiger *et al.*, 1988). The chemical structure of AQ does not suggest that the parent compound would be a DNA reactive mutagen. Therefore, it appeared that a mutagenic contaminant was present in the positive Ames test samples that was either directly mutagenic or could be activated by bacterial metabolism. Similarly, there is one report of weak induction of micronuclei in SHE cells, but the material used was the NTP AQ-OX (Gibson *et al.*, 1997) and, as noted below, several other micronuclei assays are negative.

In fact, the problem of contamination of AQ-OX with nitroanthracenes producing a mutagenic preparation has been well documented (US EPA, 1977; ICI, 1978a, 1978b). For example, for a TSCA submission, six samples of AQ were submitted to the Ames *Salmonella* mutagenicity assay (US EPA, 1977). Only one was positive, and that was without metabolic activation. It was concluded that the mutagenic activity came from contamination of the sample with 9-nitroanthracene (9-NA). When that sample was purified and retested, it showed no mutagenic activity.

Table I. Contaminants in preparations of AQ

NTP AQ-OX	
Component	Concentration[a]
AQ	99%
9-NA	1200 p.p.m.[b]
phenanthrene	200 p.p.m.
target toxic compounds[c]	ND[d]
Purified NTP AQ-OX	
Component	Concentration[a]
AQ	99%
9-NA	ND[e]
phenanthrene	ND[d]
target toxic compounds[c]	ND[d]
AQ-FC	
Component	Concentration[a]
AQ	99%
9-NA	ND[e]
target toxic compounds[c]	ND[d]
AQ-DA	
Component	Concentration[a]
AQ	99%
9-NA	ND[e]
target toxic compounds[c]	ND[d]

[a]GC-MS analyses were conducted to identify impurities using the conditions specified in US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40. Quantitation of contaminants was based on HPLC analysis.
[b]Noted in the NTP report at 0.12% but not identified at that time (NTP, 1999).
[c]US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40 identify 16 PAH and 72 additional toxic or carcinogenic substances of concern in environmental samples. Analyses were done using the conditions and looking for the target toxic compounds specified in these standard procedures.
[d]Not detected (ND) at a limit of detection of <50 p.p.m.
[e]Not detected (ND) at a limit of detection of <5 p.p.m.

It is clear that samples of technical grade AQ can vary widely in how they are produced, and the types and amounts of impurities. Another purpose of these studies was to determine the mutagenic activity of the actual material used in the NTP bioassay and to contrast that with other preparations of AQ. A sample of the archived NTP AQ-OX was generously provided by the NTP. The activity of this material was contrasted in the Ames *Salmonella* mutagenicity assay with purified NTP AQ-OX as well as samples of AQ-FC and AQ-DA. In addition, results from several additional genotoxicity assays are reported using the AQ-DA material.

Materials and methods

Chemicals

A sample of the AQ-OX powder employed in the NTP 2-year toxicology and carcinogenesis studies (NTP, 1999) was generously provided by Cynthia Smith of the NTP and Donna Browning, NTP Chemical Custodian (Battelle, Columbus, OH). This sample was designated as NTP AQ-OX. The bright yellow powder obtained from the NTP was labeled Anthraquinone, Battelle Task Identifier: 5-064-SHIP-211, lot: 5893, CAS: 84–65-1. The technical report stated that the sample had been analyzed by the NTP and was found to be ~99% AQ and noted an impurity at a concentration of 0.12% (Battelle, 1993; NTP, 1999).

A portion of the NTP AQ-OX was purified by 2× recrystalization from ethanol. This sample was designated as purified NTP AQ-OX.

A sample of technical grade AQ-FC typical of that in commercial use was obtained from Environmental Biocontrol, Intl. (Wilmington, DE). This sample was designated as AQ-FC.

Samples of technical grade AQ-DA typical of that in commercial use were obtained from Environmental Biocontrol, Intl (Wilmington, DE). These were used over a period of several months for the various genotoxicity assays

Table II. Activity of NTP AQ-OX in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) AQ-OX μg/plate	9-NA[a] μg/plate	Average revertants per plate ± SD[a]				
		TA98	TA100	TA1535	TA1537	WP2uvrA
0	0	18 ± 4	89 ± 9	12 ± 2	7 ± 3	15 ± 4
30	0.04	20 ± 3	107 ± 14	12 ± 6	10 ± 1	14 ± 2
60	0.07	25 ± 10	113 ± 15	12 ± 1	10 ± 3	13 ± 5
125	0.15	42 ± 6[b]	113 ± 18	10 ± 4	8 ± 4	10 ± 3
250	0.3	62 ± 5[b]	127 ± 18	16 ± 7	21 ± 10[b]	12 ± 6
500	0.6	116 ± 16[b]	142 ± 4	17 ± 9	26 ± 5[b]	20 ± 1
1000	1.2	213 ± 29[b]	131 ± 21	11 ± 1	40 ± 5[b]	10 ± 2
2000	2.4	433 ± 40[b]	220 ± 6[b]	18 ± 3	95 ± 6[b]	16 ± 6
pos. con.		193 ± 23[c]	617 ± 6[c]	589 ± 96[c]	503 ± 21[c]	145 ± 6[c]
With liver microsomes (S9)						
0	0	30 ± 8	149 ± 4	20 ± 1	10 ± 4	15 ± 3
30	0.04	33 ± 4	140 ± 19	19 ± 3	12 ± 2	14 ± 3
60	0.07	30 ± 9	138 ± 15	25 ± 4	11 ± 3	14 ± 1
125	0.15	32 ± 4	134 ± 3	19 ± 1	14 ± 1	20 ± 5
250	0.3	37 ± 4	127 ± 2	20 ± 4	11 ± 4	12 ± 5
500	0.6	52 ± 5	130 ± 9	18 ± 5	18 ± 2	12 ± 4
1000	1.2	102 ± 11	147 ± 21	20 ± 6	20 ± 4	16 ± 4
2000	2.4	162 ± 13	164 ± 22	21 ± 1	32 ± 8	15 ± 4
pos. con.		373 ± 17[d]	534 ± 81[d]	78 ± 5[d]	111 ± 12[d]	245 ± 21[d]

[a]NTP AQ-OX contains 9-NA at a level of 1200 p.p.m. This column shows the calculated amount of this contaminant on the plate. Since AQ is not mutagenic, the mutagenic activity can be ascribed to the 9-NA and any other contaminants that might have been present. Since 9-NA was the major contaminant identified with analytical studies, and as a first approximation, mutagenic activity has been assumed to arise from the 9-NA. Nevertheless, the same issues and conclusions hold even if mutagenic activity was to be shown to arise from several different contaminants.
[b]Judged as a positive response. Criteria for a positive response are an increasing dose–response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.
[c]Positive controls without S9: TA98 1.0 μg 2-nitrofluorene; TA100 and TA1535 2.0 μg sodium azide; TA1537 2.0 μg ICR-191; WPuvrA 1.0 μg 4-nitroquinoline-*N*-oxide.
[d]Positive controls with S9: TA98 2.5 μg B[*a*]P; TA100, TA1535 and TA1537 2.5 μg 2-aminoanthracene; WpuvrA 25 μg 2-aminoanthracene.

presented here. High quality control standards and analytical verification indicated that these samples were very uniform. These samples were designated as AQ-DA.

Analytical techniques including high-performance liquid chromatography (HPLC) and GC-MS were performed by DCV Group (Wilmington, DE) and Covance Laboratories (Leesburg, VA) to verify that the main component in all preparations was AQ, that purification did not alter the parent compound and to identify potential contaminants (Table I). GC-MS analysis was conducted to identify impurities. US EPA Methods 610, 625 and 8270 in the Code of Federal Register 40 identify 16 PAH and 72 additional toxic or carcinogenic substances of concern in environmental samples. Analyses were done using the conditions and looking for the target toxic compounds specified in these standard procedures. Quantitation of contaminants was based on HPLC analysis.

Bacterial mutagenicity assays

Samples NTP AQ-OX, purified NTP AQ-OX, AQ-FC and AQ-DA were submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the *Salmonella–Escherichia coli*/Mammalian-Microsome Reverse Mutation Assay with a confirmatory assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Ames *et al.*, 1975; Brusick *et al.*, 1980; Maron and Ames; 1983). The assay tested for the ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains (Ames test), and the tryptophan locus in an *E.coli* tester strain both in the presence and absence of an exogenous metabolic activation system (Ames *et al.*, 1975; Brusick *et al.*, 1980; Maron and Ames; 1983). The activation system was a microsomal enzyme preparation derived from Aroclor-induced rat liver (S9). Dose levels were based on a toxicity range-finding study. The top doses demonstrated test article precipitate on the plates. No appreciable toxicity was observed with any of the samples. The experiments were repeated independently to confirm initial results.

Criteria for a positive response were at least a 2-fold increase in the mean revertants per plate of at least one tester strain over the mean revertants per plate of the appropriate vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least a 3-fold increase for tester strains TA1535 and TA1537.

L5178Y thymidine kinase (TK)+/– mouse lymphoma forward mutation assay

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) to test for mutagenic activity in the L5178Y TK+/– mouse lymphoma forward mutation assay. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Amacher *et al.*, 1980; Clive *et al.*, 1987). The objective of the assay was to evaluate the ability of AQ to induce forward mutations at the TK locus in the mouse lymphoma L5178Y cell line. The test article formed a suspension in dimethylsulfoxide at concentrations >1.56 mg/kg and was insoluble in medium above ~25 μg/ml. Assays were run with and without a rat liver S9 metabolic activation system. Range-finding studies showed AQ to be non-toxic at nominal doses up to 500 μg/ml with and without metabolic activation. The testing limit for the mutation assays was set at 50 μg/ml, which is about twice the solubility limit in medium. Two independent assays were conducted each with and without metabolic activation. The criterion for a positive response is induction of a mutation frequency that is at least two times that of the control mutant frequency for that given experiment. Colony sizing was not done because no positive responses were observed with AQ.

Chromosomal aberration induction in Chinese hamster ovary (CHO) cells

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) to test for the ability to induce chromosomal aberrations in CHO cells with and without metabolic activation. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Evans, 1962). A dose range-finding study was conducted to select experimental doses. Solubility considerations determined the highest dose to be 50 μg/ml. Replicate cultures of CHO cells were incubated with up to 50 μg/ml AQ-DA with and without metabolic activation, with a 20.0 h harvest in an initial trial and with 20.0 and 44.0 harvests in the confirmatory trials. No visual signs of cytotoxicity were observed in the cultures analyzed. A test article was considered positive for inducing chromosomal aberrations if a significant increase was observed compared to controls at a level of $P < 0.01$

In vivo bone marrow mouse micronucleus assay

A sample of AQ-DA was submitted to Covance Laboratories (Vienna, VA) for evaluation in the *in vivo* mouse micronucleus assay. The objective of this whole animal assay was to evaluate the ability of AQ-DA to induce micronuclei

Table III. Activity of purified NTP AQ-OX in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-OX µg/plate	Average revertants per plate ± SD				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	14 ± 4	75 ± 4	10 ± 4	6 ± 2	15 ± 5
30	16 ± 3	87 ± 8	15 ± 7	9 ± 1	18 ± 4
60	20 ± 1	81 ± 11	6 ± 5	6 ± 3	15 ± 4
125	15 ± 4	80 ± 4	8 ± 3	8 ± 4	13 ± 3
250	10 ± 3	83 ± 12	9 ± 1	5 ± 4	10 ± 2
500	11 ± 2	80 ± 9	13 ± 4	6 ± 1	12 ± 1
1000	15 ± 2	95 ± 2	13 ± 3	7 ± 2	14 ± 5
2000	22 ± 3	86 ± 8	13 ± 5	6 ± 1	13 ± 3
pos. con.	121 ± 10[b]	533 ± 8[b]	418 ± 17[b]	657 ± 38[b]	132 ± 26[b]
With liver microsomes (S9)					
0	21 ± 3	93 ± 15	13 ± 1	8 ± 2	14 ± 3
30	23 ± 3	96 ± 14	13 ± 6	10 ± 5	18 ± 2
60	32 ± 3	88 ± 17	8 ± 2	10 ± 3	20 ± 5
125	31 ± 6	84 ± 10	11 ± 1	9 ± 2	19 ± 2
250	26 ± 5	75 ± 14	10 ± 4	9 ± 1	18 ± 4
500	28 ± 5	79 ± 6	6 ± 0	9 ± 3	19 ± 4
1000	28 ± 2	87 ± 10	9 ± 2	9 ± 5	17 ± 2
2000	29 ± 3	86 ± 7	14 ± 4	7 ± 2	22 ± 6
pos. con.	321 ± 18[c]	582 ± 22[c]	135 ± 12[c]	565 ± 105[c]	350 ± 23[c]

[a]Criteria for a positive response are an increasing dose–response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.
[b]Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.
[c]Positive controls with S9: TA98 2.5 µg B[*a*]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

Table IV. Activity of AQ-FC in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-FC µg/plate	Average revertants per plate ± SD[a]				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	14 ± 3	93 ± 9	13 ± 3	9 ± 1	18 ± 2
30	11 ± 4	89 ± 6	13 ± 1	6 ± 3	17 ± 6
60	12 ± 3	92 ± 11	9 ± 3	5 ± 2	19 ± 6
125	14 ± 3	94 ± 11	10 ± 3	5 ± 3	16 ± 3
250	17 ± 6	93 ± 8	12 ± 6	6 ± 1	17 ± 8
500	16 ± 5	107 ± 7	10 ± 1	9 ± 5	13 ± 3
1000	14 ± 6	96 ± 12	10 ± 2	7 ± 2	17 ± 2
2000	14 ± 4	98 ± 5	12 ± 2	6 ± 4	14 ± 6
pos. con.	156 ± 8[b]	547 ± 16[b]	482 ± 10[b]	421 ± 33[b]	152 ± 19[b]
With liver microsomes (S9)					
0	24 ± 6	123 ± 13	10 ± 6	5 ± 2	18 ± 4
30	16 ± 5	91 ± 8	9 ± 2	6 ± 4	18 ± 3
60	23 ± 9	95 ± 12	12 ± 1	8 ± 2	20 ± 3
125	19 ± 9	92 ± 8	14 ± 2	8 ± 3	18 ± 3
250	20 ± 5	107 ± 10	10 ± 4	7 ± 1	20 ± 2
500	19 ± 3	104 ± 5	10 ± 2	8 ± 3	21 ± 4
1000	22 ± 3	93 ± 7	12 ± 6	8 ± 4	18 ± 3
2000	21 ± 0	116 ± 10	12 ± 7	8 ± 2	20 ± 6
pos. con.	351 ± 20[c]	916 ± 20[c]	138 ± 10[c]	143 ± 9[c]	355 ± 41[c]

[a]Criteria for a positive response are an increasing dose–response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.
[b]Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.
[c]Positive controls with S9: TA98 2.5 µg B[*a*]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

in bone marrow polychromatic erythrocytes (PCE) of Crl:CD-1 (ICR) BR mice. Industry accepted standard protocols and published procedures were followed in compliance with Good Laboratory Practice regulations (Heddle *et al.*, 1991; Salamone and Mavournin, 1994; Schmid, 1976). In the dose selection study the test article was suspended in corn oil and dosed by oral gavage at up to 5000 mg/kg. Based on lack of significant toxicity, 5000 mg/kg was selected as the highest dose for the micronucleus studies. In the micronucleus assay, AQ-DA was suspended in corn oil and dosed by oral gavage at 1250, 2500 and 5000 mg/kg. Five males and five females were randomly assigned to each dose per harvest time group. Animals were killed

Table V. Activity of AQ-DA in the Ames and *E.coli* mutagenicity assays

Without liver microsomes (S9) Purified AQ-FC µg/plate	Average revertants per plate ± SD[a]				
	TA98	TA100	TA1535	TA1537	WP2uvrA
0	13 ± 5	96 ± 7	9 ± 3	7 ± 1	18 ± 2
30	15 ± 1	92 ± 6	11 ± 4	6 ± 1	17 ± 6
60	15 ± 3	93 ± 12	13 ± 1	11 ± 2	17 ± 2
125	13 ± 2	89 ± 8	11 ± 2	4 ± 3	18 ± 1
250	16 ± 3	94 ± 9	10 ± 2	7 ± 1	18 ± 2
500	12 ± 4	96 ± 13	9 ± 4	5 ± 1	19 ± 8
1000	15 ± 3	96 ± 4	11 ± 0	5 ± 2	18 ± 2
2000	14 ± 2	105 ± 8	11 ± 1	8 ± 2	20 ± 4
pos. con.	188 ± 9[b]	498 ± 65[b]	444 ± 31[b]	612 ± 83[b]	119 ± 14[b]
With liver microsomes (S9)					
0	13 ± 2	72 ± 4	9 ± 4	9 ± 1	20 ± 3
30	12 ± 5	73 ± 6	10 ± 2	9 ± 1	23 ± 3
60	12 ± 6	77 ± 8	6 ± 1	9 ± 1	19 ± 6
125	14 ± 5	85 ± 13	12 ± 2	13 ± 2	22 ± 8
250	11 ± 2	83 ± 7	12 ± 1	9 ± 3	19 ± 1
500	15 ± 7	77 ± 9	9 ± 6	8 ± 1	18 ± 4
1000	9 ± 1	82 ± 5	10 ± 4	9 ± 2	19 ± 6
2000	9 ± 1	82 ± 5	10 ± 4	11 ± 4	16 ± 8
pos. con.	191 ± 21[c]	582 ± 22[c]	519 ± 31[c]	126 ± 25[c]	301 ± 12[c]

[a]Criteria for a positive response are an increasing dose–response curve with at least one response equal to or greater than twice the mean vehicle control for tester strains TA98, TA100 and WP2uvrA and/or at least three times the tester strains TA1535 and TA1537.
[b]Positive controls without S9: TA98 1.0 µg 2-nitrofluorene; TA100 and TA1535 2.0 µg sodium azide; TA1537 2.0 µg ICR-191; WPuvrA 1.0 µg 4-nitroquinoline-*N*-oxide.
[c]Positive controls with S9: TA98 2.5 µg B[*a*]P; TA100, TA1535 and TA1537 2.5 µg 2-aminoanthracene; WpuvrA 25 µg 2-aminoanthracene.

~24, 48 and 72 h after dosing with the test article for extraction of the bone marrow. A response was judged positive if it was significantly greater than the corresponding vehicle control at a level of $P < 0.01$.

Results

Quantitative analysis

In every case the main component of the test material was confirmed to be AQ (Table I). Thus, purification of the NTP AQ-OX did not alter the main AQ constituent. The NTP report on AQ (Battelle, 1993; NTP, 1999) noted an extra peak on the GC trace in the AQ-OX at a level of 1200 p.p.m., but did not identify the contaminant at that time. The same peak was observed also at 1200 p.p.m. in the analytical studies reported here and was identified as 9-NA. The purification procedure removed the 9-NA from the NTP AQ-OX (Table I).

Bacterial mutation assays

All samples were run in the same laboratory under identical conditions. The NTP AQ-OX sample was mutagenic in a dose-dependent manner in strains TA98, TA100 and TA1537 (Table II). Mutagenic activity was reduced or eliminated by addition of an S9 rat liver microsome metabolic activation system. In contrast, no mutagenic activity was observed with the purified NTP AQ-OX (Table III). Because AQ is not mutagenic, all the mutagenic activity in the NTP AQ-OX sample can be ascribed to the 9-NA. Mutagenic activity of the 9-NA is seen at concentrations as low as 0.15 µg/plate, indicating potent activity (Table II). In tester strain TA98, the induced revertants over controls per microgram of chemical for the concurrently run positive controls are 137 revertants/µg for benzo[*a*]pyrene (B[*a*]P), and 175 revertants/µg for 2-nitrofluorene. The 9-NA with 173 revertants/µg is more potent than B[*a*]P and equal in potency to 2-nitrofluorene (Table II).

Neither AQ-FC nor AQ-DA showed any mutagenic activity

Table VI. Activity of AQ-DA in the L5178Y mutagenicity assay

Concentration	Relative growth (%)[a]	Mutant frequency (10^{-6} U)[b]
Technical 1 AQ-DA without metabolic activation (µg/ml)		
0.0	100.0	82.6
3.13	93.4	72.1
6.25	78.6	82.8
12.5	99.3	71.0
25.0	74.8	85.9
37.5	75.2	79.5
50.0	81.1	83.1
Methylmethanesulfonate positive control		
5 nl/ml	7.4	923.9[c]
Technical 1 AQ-DA with metabolic activation (µg/ml)		
0.0	100.0	114.3
1.57	108.7	180.5
3.13	101.2	158.6
6.25	85.9	139.8
12.50	104.6	129.5
25.0	122.1	127.0
37.5	97.9	121.1
50.0	94.9	116.0
Methylcholanthrene positive control		
2 µg/ml	7.4	923.9[d]

[a]Relative growth = (relative suspension growth×relative cloning efficiency)/100.
[b]Mutant frequency = (total mutant colonies/total viable colonies)×2×10^{-4}. Decimal is moved to express the frequency in units of 10^{-6}.
[c]Mutagenic as judged by exceeding the minimum criterion of 165.2 for this experiment (twice the control frequency).
[d]Mutagenic as judged by exceeding the minimum criterion of 228.6 for this experiment (twice the control frequency).

Table VII. Induction of chromosomal aberrations in CHO cells

Concentration	Aberrations per cell	Percent cells with aberrations[a]	Polyploid cells[a]	Endo-reduplicated cells[a]
20 h exposure without added metabolic activation				
AQ-DA (μg/ml)				
0.0	0.03	2.0	1.5	0.0
12.5	0.01	0.5	0.5	0.5
25.0	0.01	0.5	1.0	0.0
37.5	0.00	0.0	1.0	0.0
50.0	0.01	1.0	0.5	0.0
44 h exposure without added metabolic activation				
AQ-DA (μg/ml)				
0.0	0.01	0.5	1.0	0.0
12.5	0.00	0.0	2.5	0.0
25.0	0.01	1.0	6.5[a,b]	0.0
37.5	0.00	0.0	4.5[a,b]	0.0
50.0	0.03	2.5	6.0[a,b]	0.0
20 h exposure without added metabolic activation				
Mytomycin C positive control				
0.1 μg/ml	0.30	28.0[a]	1.5	0.0
20 h exposure with added metabolic activation				
AQ-DA (μg/ml)				
0.0	0.01	1.0	2.5	1.0
12.5	0.00	0.0	1.5	0.0
25.0	0.00	0.0	0.0	0.0
37.5	0.01	0.5	2.0	0.0
50.0	0.01	1.0	0.0	0.0
44 h exposure with added metabolic activation				
AQ-DA (μg/ml)				
0.0	0.00	0.0	2.0	0.0
12.5	0.00	0.0	1.0	0.0
25.0	0.01	1.0	1.0	0.0
37.5	0.00	0.0	1.0	0.0
50.0	0.00	0.0	1.5	0.0
20 h exposure with added metabolic activation				
Cyclophosphamide (CP) positive control				
5.0 μg/ml	0.46	32.0[a]	2.0	0.0

[a]A test article was considered positive for inducing chromosomal aberrations if a significant increase was observed compared to controls at a level of $P < 0.01$.
[b]The weak increase in polyploidy observed in these cultures was judged a statistical anomaly because the values observed are within the historical negative control data of 0–9.5% and the solvent control data of 0–10% and there was no indication of activity under any of the other exposure conditions or trials.

either with or without metabolic activation in the Ames tester strains or in WP2uvrA (Tables IV and V).

L5178Y TK +/– mouse lymphoma forward mutation assay

No cytotoxicity was observed in any of the trials in the L5178Y mutation assays. Mutant frequencies of treated cultures varied randomly with dose toxicity and no increases above the minimum criteria for a positive response were induced (Table VI). AQ-DA was therefore evaluated as negative with and without metabolic activation at the TK locus in L5178Y mouse lymphoma cells under the conditions used in this study.

Chromosomal aberration induction in CHO cells

CHO cells were incubated with up to 50.0 μg/ml of AQ-DA with harvest times of 20 and 44 h (Table VII). No significant increase in cells with chromosomal aberrations, polyploidy or endoreduplication was observed at the concentrations analyzed. AQ-DA was considered negative for inducing chromosomal aberrations in CHO cells with and without metabolic activation.

In vivo bone marrow mouse micronucleus assay

In the micronucleus assay, AQ-DA was suspended in corn oil and dosed by oral gavage at 1250, 2500 and 5000 mg/kg. No bone marrow toxicity was observed as a decrease in the polychromatic erythrocyte: normochromatic erythrocyte (PCE:NCE) ratio. AQ-DA did not induce a significant increase in micronuclei in bone marrow polychromatic erythrocytes under the conditions of this assay and is considered negative in the mouse bone marrow micronucleus test (Table VIII).

Discussion

Genetic toxicology

The lack of mutagenic or genotoxic activity in a variety of assays in numerous laboratories indicates that AQ is not a DNA-reactive genotoxic carcinogen (Brown and Brown, 1976; Anderson and Styles, 1978; Gibson *et al.*, 1978; Salamone *et al.*, 1979; Sakai *et al.*, 1985; Tikkanen *et al.*, 1983; National Cancer Institute, 1987; Kerckaert *et al.*, 1996; Durant *et al.*, 1996). Those assays are strengthened by the studies presented here in which we report that purified NTP AQ-OX, AQ-FC and AQ-DA are negative in the expanded Ames mutagenicity test, that AQ-DA is negative in the L5178Y mouse lymphoma forward mutation assay, that AQ-DA does not induce chromosomal aberrations, polyploidy or endoreduplication in CHO cells, and that it is negative in the *in vivo* mouse micronucleus assay (Tables III–VIII).

Table VIII. *In vivo* bone marrow micronucleus assay

Treatment	Dose (mg/kg)	Harvest time (h)	% Micronucleated PCEs Mean of 1000 per animal ± SE	
			Males	Females
Corn oil		24	0.02 ± 0.02	0.14 ± 0.07
		48	0.02 ± 0.02	0.14 ± 0.05
		72	0.16 ± 0.05	0.02 ± 0.02
AQ-DA	1250	24	0.06 ± 0.04	0.10 ± 0.03
	1250	48	0.06 ± 0.04	0.12 ± 0.05
	1250	72	0.04 ± 0.02	0.08 ± 0.06
	2500	24	0.02 ± 0.02	0.16 ± 0.07
	2500	48	0.06 ± 0.02	0.02 ± 0.02
	2500	72	0.06 ± 0.02	0.02 ± 0.02
	5000	24	0.02 ± 0.02	0.04 ± 0.04
	5000	48	0.06 ± 0.02	0.06 ± 0.04
	5000	72	0.00 ± 0.00	0.00 ± 0.00
Cyclo-phosphamide	80	24	4.94 ± 0.72[a]	3.00 ± 1.04[a]

[a]Significantly greater than the corresponding vehicle control at the $P < 0.01$ level.

The report of direct-acting genotoxic activity in two Ames tests (Liberman *et al.*, 1982; Zeiger *et al.*, 1988) was unexpected because the data were in conflict with so many other reports of negative mutagenic activity. In both of those reports the AQ was obtained from Aldrich Chemical Co. and the label purity was listed as only 97% (Liberman *et al.*, 1982; Zeiger *et al.*, 1988). No analysis of the identity of the remaining 3% of non-AQ material was done. The problem of contamination with nitroanthracenes producing genotoxic activity in preparations of AQ-OX has been described (US EPA, 1977; ICI, 1978a, 1978b). In fact, the NTP AQ-OX was contaminated with 1200 p.p.m. 9-NA (Table I). The NTP AQ-OX was mutagenic in strains TA98, TA100 and TA1537, while the purified NTP AQ-OX was not. The structure of AQ does not suggest direct DNA reactivity, yet the NTP AQ-OX was mutagenic without added metabolic activation. Taken together these data indicate that AQ is not genotoxic, rather that the NTP sample of AQ-OX contains the mutagenic contaminant 9-NA.

Cytogenetics

The NTP report described a mouse peripheral blood micronuclei assay test from the 14 week range-finding study that preceded the cancer bioassay (NTP, 1999). The tentative conclusion was that the data showed that AQ-OX exhibited weak activity in that assay. However, the doses used were up to four times the maximum tolerated dose used in the bioassay, no response was seen in the female animals, and the response in the males was judged as positive only with a highly non-conservative trend test. In contrast, AQ-OX was negative in a bone marrow micronucleus assay (NTP, 1999). AQ-DA was also negative in a mouse bone marrow micronucleus assay in the studies reported here (Table VIII) and a chromosomal aberration assay in CHO cells (Table VII). Taken together, the weight of evidence of the data indicates that AQ does not induce cytogenetic damage.

Cancer studies

The observation of a mutagenic contaminant confounds any interpretation of the NTP bioassay with AQ. A previous bioassay with males and females in two strains of mice had been conducted with AQ. That study was not done following contemporary bioassay standards and needed to be repeated. Nevertheless that bioassay did not show carcinogenic activity with AQ (Innes *et al.*, 1969). Looking at the data as a whole strongly indicates the possibility that the observed tumors in the NTP bioassay cancer were the result of a mutagenic contaminant.

Pathological evaluations of tissues from the NTP study suggest that there may have been some degree of cell death and regenerative cell proliferation in some target tissues. For example, in the rat kidneys from AQ-treated animals hyaline droplet accumulation, nephropathy, transitional epithelium hyperplasia and mineralization were observed. Centrilobular hypertrophy and focal necrosis were seen in the male $B6C3F_1$ mice livers, and centrilobular hypertrophy and focal fatty degeneration were seen in the female $B6C3F_1$ mice livers (NTP, 1999). In follow up studies in F-344 rats, AQ-induced cell proliferation was noted in the urinary bladder (National Institute of Environmental Health Sciences, 1999). If 9-NA or other mutagenic contaminants were present, even a small amount of regenerative cell proliferation would act synergistically to enhance the mutagenic and carcinogenic responses (Columbano *et al.*, 1981).

Contaminants as confounders of the cancer bioassay

The best way to evaluate the plausibility as to whether the contaminant in the NTP AQ-OX produced the tumor response in the NTP study would be to do a potency calculation based on the carcinogenic potency of the 9-NA contaminant. Unfortunately, no cancer data are available for 9-NA. One way to estimate potency is to extrapolate comparisons of genotoxic potency to potential carcinogenic potency. Although it is recognized that there are limitations to such comparisons, a reasonable estimate of plausibility can usually be made. Another way to address the plausibility question is to ask how potent a carcinogen the contaminant would have to be in order to produce the observed response.

Mutagenic potency comparisons

Both 9-NA and 2-nitroanthracene (2-NA) are mutagenic with the 2-NA isomer being substantially more potent (Fu *et al.*, 1986). 9-NA is present in the NTP AQ-OX at a level of 1200 p.p.m. Therefore, the amount of the contaminant present on each Ames test plate is easily calculated (shown in Table II). Since AQ is not mutagenic, all the mutagenic activity can be ascribed to the contaminants. Table II shows that mutagenic activity can be seen in strain TA98 without metabolic activation at a level of only 0.15 μg 9-NA per plate. As a first approximation it is reasonable to assign this mutagenic activity to the primary contaminant 9-NA. Accordingly, on the basis of induced revertants per microgram in tester strain TA98, 9-NA with 173 revertants/μg was more potent than B[*a*]P with 137 revertants/μg and was as potent as the 2-nitrofluorene with 175 revertants/μg run concurrently as positive controls (Table II).

Cancer potency calculations

A critical question is whether it is plausible that a contaminant could be a significant contributor to the carcinogenic activity observed in the NTP bioassay. The *Handbook of Carcinogenic Potency and Genotoxicity Databases* defines a valuable parameter to rank cancer potency, the Tumor Dose 50 (TD_{50}) (Gold and Zeiger, 1997). The TD_{50} is the tumorigenic dose-rate for 50% of experimental animals, or the dose-rate that will halve the probability of remaining tumor free at the end of a standard lifespan. For a given target site, if there are no

Table IX. TD_{50} values

Gender species tumor type	TD_{50} assuming only AQ induced the tumors (mg/kg/day)[a]	TD_{50} assuming only 9-NA induced the tumors (mg/kg/day)[b]
Male F344 rats	940	1.1
Renal tubule adenoma Male F344 rats	810	0.97
Bladder transitional epithelial papiloma Female F344 rats	310	0.37
Renal tubule transitional adenoma or carcinoma Male $B6C3F_1$ mice	380	0.45
Hepatocellular adenoma, hepatocellular carcinoma or hepatoblastoma Female $B6C3F_1$ mice Hepatocellular adenoma or hepatocellular carcinoma	390	0.47

[a]The TD_{50} for key target sites in the NTP AQ cancer study were estimated from a least squares linear fit of the tumor data dose–response curve (Gold and Zeiger, 1997; NTP, 1999).
[b]The theoretical TD_{50} for 9-NA was calculated knowing that the percentage of contaminating 9-NA was 0.12%, and assuming that the 9-NA was responsible for inducing the all the tumors.

tumors in control animals, then the TD_{50} is that chronic dose rate in mg/kg body weight/day, which would induce tumors in half the test animals at the end of a standard lifespan study. This parameter is useful because it is analogous to the LD_{50} and the units are understandable as mg/kg/day. The TD_{50} for key target sites in the NTP AQ cancer study were estimated from a least squares linear fit of the dose–response tumor data and are presented in Table IX. Knowing that the percentage of contaminating 9-NA was 0.12%, and with the assumption that the 9-NA was responsible for inducing all the tumors, the resulting theoretical TD_{50} for 9-NA was calculated and is also presented in Table IX. The TD_{50} for AQ is in the range 310–940 for rats and is about 380 for mice. The AQ TD_{50} potency in rats is in the range of butylated hydroxyanisole. If the 9-NA were responsible for all the tumorigenic activity, the TD_{50} values would be in the range 0.37–1.1 for rats and would be about 0.45 for mice. Such values indicate that 9-NA would have to be in the potency range of B[*a*]P or 2-acetylaminofluorene in rats or dimethylnitrosamine or 3-nitro–3-hexene in mice (Gold and Zeiger, 1997). In fact, 9-NA has a greater mutagenic potency than B[*a*]P (Table II). These data indicate that it is plausible that the 9-NA contaminant was responsible for all of the tumor induction observed in the NTP study.

In fact, in the absence of reliable cancer data, the genetic toxicology profile indicates that AQ would not be a genotoxic carcinogen. Thus, no definitive conclusion can be drawn at this time as to whether AQ itself might exhibit carcinogenic activity.

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Final Report

Study Title	*Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test) with 9-nitroanthracene
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Sponsor	American Forest and Paper Association 1111 19th Street, NW Washington D.C. 20036
Test Facility	Covance Laboratories Inc. 9200 Leesburg Pike Vienna, Virginia 22182-1699
Covance Study Number	22241-0-401SC
Report Issued	December 11, 2001
Page Number	1 of 6

STUDY INFORMATION

Sponsor
American Forest and Paper Association

Test Article

Sponsor's Identification: 9-nitroanthracene, 97%
Lot 11112BU
Date Received: February 16, 2001
Physical Description: Dark yellow crystalline powder
Storage Conditions: Ambient temperature

Assay Information

Type of Assay: *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test)
Protocol No.: 401SC, Edition 1
Covance Study No.: 22241-0-401SC

Study Dates

Initiation Date: July 19, 2001
Experimental Start Date: August 30, 2001
Experimental Termination Date: September 05, 2001

Study Supervisory Personnel

Study Director: Michael S. Mecchi, MS
Laboratory Supervisor: Magnus A. Evertson, BS

SUMMARY

Introduction

At the request of American Forest and Paper Association, Covance investigated 9-nitroanthracene for mutagenic activity in the *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test). This assay evaluated the test article and/or its metabolites for the ability to induce reverse mutations at the histidine locus in the genome of specific *Salmonella typhimurium* tester strains in the presence and absence of an exogenous metabolic activation system of mammalian microsomal enzymes derived from Aroclor™-induced rat liver (S9).

The tester strains used in the mutagenicity assay were *Salmonella typhimurium* tester strains TA98 and TA100. The assay was conducted with seven doses of test article in both the presence and absence of S9 mix along with the appropriate vehicle and positive controls. The S9 homogenate was purchased from Molecular Toxicology, Inc. (Lot 1257, 38.5 mg of protein per mL). The doses tested were 0.0100, 0.0300, 0.100, 0.300, 1.00, 3.00, and 10.0 µg per plate with and without S9 mix. All dose levels of test article and the positive controls were plated in duplicate and the vehicle controls were plated in triplicate.

Results

The mutagenicity screening assay results for 9-nitroanthracene are presented in Tables 1 and 2. These data were generated in Experiment 22241-B1. The data are presented as individual plate counts (Table 1) and as mean revertants per plate ± standard deviation (Table 2) for each treatment and control group.

In Experiment 22241-B1 (Tables 1 and 2), all data were acceptable and a 3.5-fold positive increase in the mean number of revertants per plate was observed with tester strain TA98 in the absence of S9 mix. The no observed effect level (NOEL)-lowest observed effect levels (LOEL) for 9-nitroanthracene in TA98 were 0.1-0.3 µg/plate. In addition, a 2.0-fold positive increase in the mean number of revertants per plate was observed with tester strain TA100 in the absence of S9 mix. The NOEL-LOEL levels for 9-nitroanthracene in TA100 were 3.0-10.0 µg/plate. No positive increases in the mean number of revertants per plate were observed with tester strain TA98 or TA100 in the presence of S9 mix.

All criteria for a valid study were met.

CONCLUSIONS

The results of the *Salmonella*/Mammalian-Microsome Reverse Mutation Screening Assay (Ames Test) indicate that, under the conditions of this study, the test article, 9-nitroanthracene, did cause a positive 3.5-fold increase with tester strain TA98 in the absence of S9 mix with NOEL-LOEL levels of 0.1-0.3 µg/plate. In addition, under the conditions of this study, the test article, 9-nitroanthracene, did cause a positive 2.0-fold increase with tester strain TA100 in the absence of S9 mix, with NOEL-LOEL levels of 3.0-10.0 µg/plate. The test article did not cause a positive increase in the mean number of revertants per plate with tester strain TA98 or TA100 in the presence of S9 mix.

Study Director:

Michael S. Mecchi, MS
Genetic and Molecular Toxicology
Covance-Vienna

12-11-01
Date

TABLE 1 : MUTAGENICITY ASSAY RESULTS – INDIVIDUAL PLATE COUNTS

Test Article ID: 9-nitroanthracene

Experiment ID: 22241-B1

Date Plated: 30-Aug-01 Vehicle: DMSO

Date Counted: 05-Aug-01 Plating Aliquot: 50 µL

	Dose/Plate	Revertants Per Plate TA98 1	TA98 2	TA98 3	TA100 1	TA100 2	TA100 3	Background Lawn[a]
Microsomes: Rat Liver								
Vehicle Control		27	23	21	98	63	88	N
Test Article	0.0100 µg	33	26		108	86		N
	0.0300 µg	25	21		87	77		N
	0.100 µg	27	26		95	105		N
	0.300 µg	24	38		94	93		N
	1.00 µg	24	30		123	88		N
	3.00 µg	29	34		78	117		N
	10.0 µg	37	38		119	116		N
Positive Control[b]		485	390		1468	1326		N
Microsomes: None								
Vehicle Control		9	24	12	84	101	102	N
Test Article	0.0100 µg	23	10		82	99		N
	0.0300 µg	18	21		111	93		N
	0.100 µg	27	22		117	115		N
	0.300 µg	28	33		98	86		N
	1.00 µg	36	23		111	120		N
	3.00 µg	42	32		140	134		N
	10.0 µg	53	51		183	195		N
Positive Control[c]		437	333		1371	1325		N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

[c] TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate

TABLE 2 : MUTAGENICITY ASSAY RESULTS – SUMMARY

Test Article ID: 9-nitroanthracene

Experiment ID: 22241-B1

Date Plated: 30-Aug-01 Vehicle: DMSO

Date Counted: 05-Aug-01 Plating Aliquot: 50 µL

Dose/Plate		Mean Revertants Per Plate with Standard Deviation TA98 Mean	TA98 S.D.	TA100 Mean	TA100 S.D.	Background Lawn[a]
Microsomes: Rat Liver						
Vehicle Control		24	3	83	18	N
Test Article	0.0100 µg	30	5	97	16	N
	0.0300 µg	23	3	82	7	N
	0.100 µg	27	1	100	7	N
	0.300 µg	31	10	94	1	N
	1.00 µg	27	4	106	25	N
	3.00 µg	32	4	98	28	N
	10.0 µg	38	1	118	2	N
Positive Control[b]		438	67	1397	100	N
Microsomes: None						
Vehicle Control		15	8	96	10	N
Test Article	0.0100 µg	17	9	91	12	N
	0.0300 µg	20	2	102	13	N
	0.100 µg	25	4	116	1	N
	0.300 µg	31	4	92	8	N
	1.00 µg	30	9	116	6	N
	3.00 µg	37	7	137	4	N
	10.0 µg	52	1	189	8	N
Positive Control[c]		385	74	1348	33	N

[a] Background Lawn Evaluation Codes:
N = normal R = reduced O = obscured A = absent P = precipitate

[b] TA98 benzo[a]pyrene 2.5 µg/plate
TA100 2-aminoanthracene 2.5 µg/plate

[c] TA98 2-nitrofluorene 1.0 µg/plate
TA100 sodium azide 2.0 µg/plate