
Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Gene Mutation (Bacterial)

Acrylamide Positive

YANG2005: Yang HJ;Lee SH;Jin Y;Choi JH;Han CH;Lee MH; (2005) Genotoxicity and toxicological effects of acrylamide on reproductive system in male rats, *J Vet Sci* 6(2):103-109

Methods:

Acrylamide (0.625, 1.25, 2.5 or 5 mg per plate, Sigma) in DMSO was tested in TA98, TA100, TA1535 and TA1537 ± Rat S9 (Aroclor 1254 induced). Positive Controls: TA98, 2-AF at 10 mg/plate; TA100 and TA1535, Sodium Azide at 1.5 µg/plate; and TA1537, ICR191 at 0.1 µg/plate.

Major Findings:

Significant increases in revertants in TA100 +S9 (2.5 and 5 mg per plate, $p < 0.01$) with a dose response and TA98 +S9 (only at 2.5 mg per plate, $p < 0.05$). Significant increases in revertants in TA98 -S9 at 2.5 ($p < 0.05$) and 5 mg per plate ($p < 0.01$).

Role of Metabolism:

Positive results were obtained in the presence and absence of metabolic activation.

Acrylamide Negative

EMMERT2006: Emmert B;Bunger J;Keuch K;Muller M;Emmert S;Hallier E;Westphal GA; (2006) Mutagenicity of cytochrome P450 2E1 substrates in the Ames test with the metabolic competent *S. typhimurium* strain YG7108pin3ERb5, *Toxicology* 228(1):66-76

Methods:

Acrylamide was tested in *S. typhimurium* YG7108pin3ERb5 (YG7108 is methytransferase deficient derived from TA1535 to which a plasmid carrying an electron transport chain, cyp450 reductase, cytochrome b5 and cytochrome P4502E1) at concentrations up to 10 mg/plate

Acrylamide was tested alongside a number of putative 2E1 substrates (including acrylonitrile, acetamide, ethylacrylate, ethylcarbamate, trichloroethylene, tetrachloroethylene and vinyl acetate). 3 independent tests were undertaken. Incubation time was increased to 72 h due to slow growth of the tester strain. Positive control: N-Nitrosodiethylamine.

Major Findings:

Acrylamide failed to produce a positive response. The authors reported that acrylamide was toxic to the recombinant strain, but not the parent strain.

Role of Metabolism:

These cells are metabolically competent for CYP 2E1; however, in addition to acrylamide, several known or proposed CYP 2E1 substrates tested negative (acetamide, acrylonitrile, ethyl acrylate, ethyl carbamate, methyl-methacrylate, vinyl acetate, and tri- and tetra-chloroethylene). The authors suggest that this may be due to the formation of metabolites that have greater toxic potency than genotoxic potency. It would have been interesting if the level of glycidamide formation had been measured in these metabolically competent bacteria. It would also have been interesting to know whether this strain was sensitive to glycidamide, if it were to have been produced.

Additional Comments:

A positive was also reported for 2-aminoanthracene and allyl chloride with YG7018ypin3ERb5.

Nitrosodiethylamine gave a dose related positive response. N-nitrosopyrrolidine was positive in YG7108 in presence of phenobarbital/β-naphthoflavone S9; which indicates the metabolic competence of the tester strain.

The authors suggest that the high protein content of S9 mix may help mask the cytotoxic effects by scavenging reactive cytotoxic metabolites.

Overall this study does not explain the lack of activation of acrylamide in standard Ames tests since there is inconsistent evidence for metabolic competence of the tester strain. The use of CYP2E1 pyrazole induced S-9 might be helpful in this regard

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In Vitro Gene Mutation (Mammalian)

Acrylamide Positive

BESARATINIA2003: Besaratinia A; Pfeifer GP; (2003) Weak yet distinct mutagenicity of acrylamide in mammalian cells, *J Natl Cancer Inst* 95(12):889-896

Methods:

Cultured Big Blue mouse embryonic fibroblasts, carrying the lambda phage cII transgene in a recoverable shuttle vector, were treated with acrylamide (4 h, 9 concentrations in order of magnitude increments between 32 nM and 320 mM, 99.9% purity, in distilled water). Cells were grown for a further 8 days. Experiments were conducted in triplicate.

Mutations were detected using the phage based Stratagene lambda Select-cII mutation detection system in *E. coli* G1250, where commitment to lysis or lysogeny is dependent on the effects of the inhibitory cII protein on the temperature sensitive mutant cI repressor. Genomic DNA was isolated by phenol-chloroform extraction, and shuttle vectors were recovered and packaged into viable phage particles. A minimum of 3×10^5 rescued phages/experiment. Plaques containing putative cII mutations were sequenced following PCR amplification from genomic DNA.

The mutation spectra were determined from 232 plaques treated at 320 μ M and compared to 173 plaques from control plates.

Major Findings:

cII mutation frequency reported to increase in a dose dependent manner at 3.2, 32 and 320 μ M, the maximum increase at 320 μ M was 2 fold over control ($P < 0.001$ ANOVA). Mutation frequency was similar to controls in the mM dose range. The authors suggest this lack of dose dependency at higher concentrations may be indicative of a saturable process or cytotoxicity.

After exclusion of "Jackpot" Mutations (formed during embryonic development), there was a statistically significant difference in mutation spectra between acrylamide and control cII mutant plaques. ($P = 0.024$, 95% CI 0.016-0.031, Adams Skopest test). These were predominantly single base substitutions (73% of spontaneous and 81% of acrylamide induced mutations). The percentage of combined T to C plus A to G transitions and G to C plus C to G transversions showed a 2.6 and 2.2 fold increase over spontaneous. Some mutation sites co-localised with DNA adduction sites, but was no direct relationship between pattern of induced mutations and mapping of DNA adducts (see separate report).

Mechanistic Data:

Authors concluded that acrylamide had distinct mutagenic effects in mouse embryonic fibroblasts. The mechanism of mutagenicity in this study was unclear. The authors comment on possible direct Michael type reactions and possibly glycidamide formation, but no conclusions can be drawn.

Role of Metabolism:

No metabolic activation although the cell line may have had some metabolic competency

Additional Comments:

Cell viability (assessed by trypan blue exclusion) was reduced at 3.2 mM and above, with ~10% cell viability at 32 mM. The positive control, benzo[a]pyrene diol epoxide (0.01, 0.1 & 1 μ M), gave a dose response.

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In Vitro Gene Mutation (Mammalian)

Acrylamide Positive **Glycidamide Positive**

BESARATINIA2004: Besaratinia A;Pfeifer GP; (2004) Genotoxicity of acrylamide and glycidamide, J Natl Cancer Inst 96(13):1023-1029

Methods:

Cultured Big Blue mouse embryonic fibroblasts, carrying the lambda phage cII transgene in a recoverable shuttle vector, were treated with acrylamide (320 µM, Borhringer Mannheim) and glycidamide (500 µM, LKT Laboratories Indianapolis), for 4 h in distilled water. Mutation frequency was also assessed with glycidamide (0.05, 0.5, 5, 50, 500 and 5000 µM). Cells were grown for a further 8 days post treatment. Number of replicates not reported.

Mutations were detected using the phage based Stratagene lambda Select-cII mutation detection system in *E. coli* G1250, where commitment to lysis or lysogeny is dependent on the effects of the inhibitory cII protein on the temperature sensitive mutant cI repressor. Genomic DNA was isolated by phenol-chloroform extraction, and shuttle vectors were recovered and packaged into viable phage particles. A minimum of 3x10⁽⁵⁾ rescued phages/experiment. Plaques containing putative cII mutations were sequenced following PCR amplification from genomic DNA.

The mutation spectra were determined from 134 plaques treated with glycidamide and compared to 173 plaques from control plates (data for acrylamide was taken from Besaratinia 2003).

Major Findings:

Glycidamide treatment dose-dependently increased the frequency of cII mutations relative to control treatment 4.1 fold over background at 5 µM (P<.001 ANOVA). The spectrum of glycidamide-induced cII mutations was statistically significantly different from the spectrum of spontaneously occurring mutations in the control-treated cells (P=.038). Compared with spontaneous mutations in control cells, cells treated with glycidamide or acrylamide had more A to G transitions and G to C transversions and glycidamide-treated cells had more G toT transversions (P<.001). Acrylamide induced a 2 fold increase at 320 µM

Mechanistic Data:

Many of the glycidamide induced adducts clustered at specific locations within the cII transgene also coincided with sites of acrylamide adduct formation.

Role of Metabolism:

No metabolic activation although the cell line may have had some metabolic competency. Authors concluded that the mutagenicity of acrylamide in human and mouse cells is based on the capacity of its epoxide metabolite glycidamide to form DNA adducts

Additional Comments:

Acrylamide and Glycidamide reduced cell viability in the mM range in both bronchial and fibroblast cell lines in a dose and time dependent manner. The authors noted an the excess of A to G and G to C transversions were characteristic of acrylamide mutagenesis, there was also an excess of G toT transversions in glycidamide-treated cells.

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In Vitro Gene Mutation (Mammalian)

Acrylamide Negative Glycidamide Positive

BAUM2005A: Baum M;Fauth E;Fritzen S;Herrmann A;Mertes P;Merz K;Rudolphi M;Zankl H;Eisenbrand G; (2005) Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood, Mutat Res 580(1-2):61-69

Methods:

Acrylamide obtained from Merck (Dramstock, Germany). Glycidamide synthesised. Both test materials reported to be reagent grade. V79 cell (1 x10⁶) with 24 hour exposure and 4-5 day expression period. hPRT mutation frequencies determined from triplicate trials. Cloning efficiency determined from duplicate trials. Cytotoxicity assessed by trypan blue exclusion. Positive control N-methyl-N'-nitro-N-nitroso-guanidine (MNNG).

Major Findings:

Cytotoxicity not reported at up to 5mM acrylamide. Cytotoxicity reported at 0.8mM glycidamide and above (50% reduction in viable cells at 2 mM). No significant increase in mutation frequency at up to 10 mM acrylamide. For glycidamide a significant increase in mutation frequency reported at 0.8 mM and above. (cf 14±8/10(6), cf 4±2/10(6) in solvent control).

Role of Metabolism:

Study did not include exogenous metabolic activation.

Additional Comments:

MNNG gave expected mutagenic response at 0.001mM.

Glycidamide Positive

JOHANSSON2005: Johansson F;Lundell T;Rydberg P;Erixon K;Jenssen D; (2005) Mutagenicity and DNA repair of glycidamide-induced adducts in mammalian cells, Mutat Res 580(1-2):81-89

Methods:

This study examined the effects of glycidamide (up to 4 mM h, >95% purity) on the hPRT locus in CHO wild-type and base excision repair (BER) deficient cell lines. Cells (5x10⁵ cells/treatment) were exposed to glycidamide for 1 h followed by a 24 h recovery. 3x10⁵ cells were seeded for expression. Selection plates were seeded with 10⁴ cells (in triplicate). Survival and cloning efficiency were undertaken with 500 cells/treatment.

Major Findings:

Glycidamide showed approximately five fold greater toxicity towards the BER deficient cell line and a mutagenic response was not observed. Statistically significant (linear regression P=0.05) mutagenicity was observed in wild-type cells.

The authors were not able to draw definite conclusions on the type of mutagenic effect of glycidamide from this study and considered the observed response was near the limit of detection for this assay.

Not Applicable

SILVARI2005: Silvari V;Haglund J;Jenssen D;Golding BT;Ehrenberg L;Tornqvist M; (2005) Reaction-kinetic parameters of glycidamide as determinants of mutagenic potency, Mutat Res 580(1-2):91-101

Methods:

Recalculation of data from Johanssen F (2005) Mutation Research, 580, 81-89.

Major Findings:

Mutagenic potency of glycidamide in hprt in CHO cells was 3.45±2.1 mutations/10(5) cells. Glycidamide reported to be approximately seven times more potent than ethylene oxide in this test system.

Additional Comments:

relative mutagenic potency reflected calculated nucleophilic potency.

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In Vitro Gene Mutation (Mammalian)

Acrylamide Equivocal Glycidamide Positive

KOYAMA2006: Koyama N; Sakamoto H; Sakuraba M; Koizumi T; Takashima Y; Hayashi M; Matsufuji H; Yamagata K; Masuda S; Kinae N; Honma M; (2006) Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells, *Mutat Res* 603(2):151-158

Methods:

Acrylamide and glycidamide mutagenicity was assessed by thymidine kinase (TK) assay. Acrylamide and glycidamide were purchased from Wako Pure Chemicals (Tokyo). TK6 cells (20 ml of 5×10^5 /ml, cf 10^7 cells) were incubated with acrylamide (0, 2.6 to 14 mM) or glycidamide (0, 0.6 to 2.4 mM) for 4 h (in absence of exogenous metabolic activation). Cultures were maintained for 3 days post treatment. Cells were reseeded in the presence of trifluorothymidine (TFT; 3 μ g/ml). Normal growing colonies were scored 14 days post plating. TFT medium was refreshed and slow growing colonies were scored after a further 14 days.

Genomic DNA was extracted from mutant colonies and multiple copies were prepared by PCR using primers for exons 4,7 and β -globin. Loss of Heterozygosity was determined for these sites and also for 10 microsatellite loci on chromosome 17q. Two independent experiments were undertaken with acrylamide (2.5-14mM) and single experiments for glycidamide (0.6-2.4mM). Relative survival was used as a measure of cytotoxicity.

Major Findings:

Acrylamide induced concentration dependent cytotoxicity (20% relative survival at 14 mM). Significant increase (pair wise and trend test $P < 0.05$) in mutation frequency was reported for acrylamide in one trial at 14 mM. (not repeated). Glycidamide gave a dose related increase in TK mutants at up to 20 fold the background at 2.4 mM. Glycidamide reduced the number of slow growing mutant colonies and increased the proportion of colonies with normal growth characteristics. (implying predominantly point mutations). Following examination of 48 colonies for LOH, it was reported that acrylamide increased the fraction of hemizygous LOH compared to spontaneous induced colonies indicating acrylamide predominantly induced deletions. For glycidamide 93/5 of mutant colonies examined ($n=44$) were non LOH indication point mutations or small intragenic mutations. Mutation spectra for acrylamide indicated intermediate sized (100-3000kb) deletions linked to the TK locus (exons 4 and 7) but not in the microsatellite loci.

Mechanistic Data:

For acrylamide, the authors considered the evidence for positive mutagenicity in the TK6 loci did not indicated a direct effect on DNA (particularly in view of negative comet assay results with acrylamide). For glycidamide the evidence was consistent with predominantly point mutations.

Role of Metabolism:

No exogenous metabolising fraction data reported

Additional Comments:

Authors discussed role of exogenous metabolism in in-vitro mutagenicity tests of acrylamide and concluded rat liver S-9 was unsuitable and transfection with CYP2E1 might represent a suitable way forward.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Gene Mutation (Mammalian)

Glycidamide Positive

THIELEN2006: Thielen S;Baum M;Hoffmann M;Loeppky RN;Eisenbrand G; (2006) Genotoxicity of glycidamide in comparison to (+/-)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide and alpha-acetoxy-N-nitroso-diethanolamine in human blood and in mammalian V79-cells, *Mol Nutr Food Res* 50(4-5):430-436

Methods:

Glycidamide mutagenicity was assessed in a hypoxanthine-phosphoribosyl-transferase (hPRT) gene mutation assay in V79 Chinese hamster lung fibroblasts. Cells (1×10^6 in triplicate) were cultured for 24h before treatment, then incubated with either glycidamide (400, 800, 1200 & 2000 μM synthesised according to Payne and Williams, *J.Org. Chem*, 1961, 26, 651-9; no purity data), alpha-acetoxy-N-nitroso-diethanolamine (a-A-NDELA; 0, 3, 10 & 30 μM), or (\pm)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE; 0, 3, 10 & 30 μM) for 24 h. Cells were cultured for a further 5 days, including 2 sub-culturing steps. An expression period of 5 days was used. Cloning efficiency was determined using 240 cells in duplicate. Cytotoxicity was assessed by trypan blue exclusion.

Major Findings:

All three compounds gave a concentration related increase in mutant frequency, this became significant at 800 μM in glycidamide treated cells. Glycidamide was considerably less effective than BPDE and a-A-NDELA, inducing significant increases in mutant frequency at approximately 80-300-fold higher concentrations; BPDE was significant at 3 μM and a-A-NDELA gained significance at 10 μM . Neither BPDE nor NDELA affected cell viability; however, glycidamide was found to be cytotoxic at 800 μM (no indication of the degree of cytotoxicity at this concentration, presumably not 100%).

Role of Metabolism:

This study used the proposed active metabolite glycidamide

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Gene Mutation (Mammalian)

Acrylamide Positive **Glycidamide Positive**

MEI2007; Mei N; Hu J; Churchwell MI; Guo L; Moore M;
Doerge DR; Chen T (2007) Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells, *Food Chem Toxicol E Pub*

Methods:

L5178Y/Tk+/- cells (6x10⁶ cells/plate) were treated with acrylamide (2-18 mM, Sigma, purity >99.9%) or glycidamide (0.125-4 mM; Toronto Research Chemicals, Ontario, Canada; purity >99.5%) for 4 hours in the absence of metabolic activation. Cells were then reseeded (3x10⁵ cells/ml, 25 cm² flask) for phenotypic expression (2 days). Prior to mutant selection, cell densities were standardised, trifluorothymidine (TFT; 2 µg/ml) was added, and cells seeded into 4 x 96 well plates (2000 cells/well). After 11 days incubation colonies were counted and classified as small or large. Mutant frequencies calculated by Poisson distribution. Plating efficiency and cytotoxicity (relative growth) were determined.

Acrylamide (16 mM) or glycidamide (4 mM) treated mutant clones from TFT selection plates (48 large and 48 small colonies) were analysed for LOH. DNA was extracted by digestion in lysis buffer containing proteinase K (90 min, 60°C). PCR was performed to assess LOH at Tk, D11Mit22, D11Mit59 and D11Mit74 loci (spanning chromosome 11). The amplification products were subjected to electrophoresis to assess the presence of single (LOH) or double (retained heterozygosity) bands. LOH analyses were performed in a computer program for Monte Carlo analysis. 4-nitroquinoline-1-oxide (0.53 µM) was used as a positive control

Major Findings:

Treatment with either compound caused dose-dependent increases in both cytotoxicity and Tk mutant frequency (both small and large colonies). LOH analysis indicated that 100% of the small and 94% of the large colonies in both treatment groups had lost heterozygosity at the TK locus. The most common mutation for both treatments was a small deletion limited to the Tk locus. Compared to glycidamide, acrylamide induced more mutants whose LOH extended to D11Mit22 and D11Mit74, an alteration of DNA larger than half of the chromosome. Statistical analysis of the mutational spectra revealed a significant difference between the types of mutations induced by acrylamide and glycidamide treatments (P=0.018), and also between treated and untreated cells (P<0.001).

Mechanistic Data:

The authors suggest that both compounds act through a clastogenic rather than point mutation mode of action in mouse lymphoma cells. Although both are clastogenic, glycidamide appears to induce mutations via a DNA adduct mechanism whereas acrylamide induces mutations by a mechanism not involving the formation of glycidamide adducts.

Role of Metabolism:

The authors suggest that acrylamide does not require metabolic activation to cause mutations. PCR could not detect CYP2E1 RNA in L5178Y/Tk+/- cells

Additional Comments:

The authors suggest that, when dose response curves are compared, glycidamide is more mutagenic than acrylamide. LOH results contrast Koyama et al. 2006, where 2.2 mM glycidamide induced primarily non-LOH mutations in human TK6 cells.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Cell Transformation

Acrylamide Positive

PARK2002: Park J;Kamendulis LM;Friedman MA;Klaunig JE; (2002) Acrylamide-induced cellular transformation, Toxicol Sci 65(2):177-183

Methods:

Acrylamide (>99.9% pure from Midwest Scientific Co) was used in a continuous exposure of SHE cells (consisting of feeder and target cell layers) for 6 days followed by morphological assessment for transformed colonies. Relative plating efficiency was used as a measure of cytotoxicity. Treatment concentrations of 0.1, 0.3, 0.5 and 0.7 mM (1 mM induce 50% reduction in relative plating efficiency).

Major Findings:

Dose dependent morphological transformation was reported at 0.5 and 0.7 mM ($P < 0.05$, Fisher). Co-treatment with 1-aminobenzotriazole (ABT), a non specific P450 inhibitor, produced no changes in number of transformed colonies compared to acrylamide alone. Co-treatment with DL-buthionone-[S,R]-sulfoximine (BSO), a selective inhibitor of gamma glutamylcysteine, increased the percent of morphologically transformed colonies (at 0.3 and 0.5 mM acrylamide) compared to acrylamide alone. There was no effect of BSO alone. Acrylamide reduced GSH levels in SHE cells, and co-treatment with N-acetyl-cysteine (a suphydril donor) reduced the number of morphologically transformed colonies compared to acrylamide alone. BSO enhance the depletion of GSH.

Mechanistic Data:

Authors suggested that acrylamide induced a clastogenic effect responsible for cell transformation and that GSH depletion was partly responsible for this effect.

Role of Metabolism:

No exogenous metabolising fraction data reported.

Additional Comments:

Authors reported acrylamide induced cell transformation in other cell lines (C3H10T1/2, NIH/3t3 and BALB/c3T3) in studies conducted prior to 1995. No positive control data reported.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Chromosomal Aberrations

Acrylamide Positive

GLATT2005: Glatt H;Schneider H;Liu Y; (2005) V79-hCYP2E1-hSULT1A1, a cell line for the sensitive detection of genotoxic effects induced by carbohydrate pyrolysis products and other food-borne chemicals, *Mutat Res* 580(1-2):41-52

Methods:

Acrylamide (research grade, Serva, Heidelberg Germany) was added to cultures of V-79 hCYP2E1-hSULT1A1 transfected or V79-Mz cells (1.67×10^5 cells) for 32h in presence of BrdU. Colcemid was used to arrest cell division and chromosomes processed for SCE evaluation. Proliferation index used as measure of cytotoxicity.

Major Findings:

A dose-related trend in increasing SCEs was reported for acrylamide (between 200-1600 μ M, concentrations abstracted from graph) in V-79 hCYP2E1-hSULT1A1 transfected cells (Mann Whitney $p=0.05$ or $0.01-0.001$). Authors report a weaker, but statistically significant increase in SCEs in V79Mz cells, although there is no dose-response apparent. An apparent increase is reported at 200 μ M in V79 Mz cells but not at 400 μ M with significant but not dose related increases at 800 μ M and 1600 μ M. The magnitude of effect was small compared to V-79 hCYP2E1-hSULT1A1 transfected cells.

Mechanistic Data:

Authors consider there is evidence for acrylamide effects mediated by glycidamide.

Role of Metabolism:

Authors consider CYP2E1 was active in the cell line used. Con current positive control data with DMN provided. No exogenous metabolising fraction data reported.

Additional Comments:

COM has previously considered that results of SCE tests have little relevance to mutagenicity assessment since mechanism of SCE formation is not fully understood. One aspect of this study is providing evidence that a V79 cell line can be successfully transfected with CYP2E1. The authors noted that addition of SULT1A1 apparently increased CYP2E1 activity, although the mechanism for this observation was unknown.

Acrylamide Positive

YANG2005: Yang HJ;Lee SH;Jin Y;Choi JH;Han CH;Lee MH; (2005) Genotoxicity and toxicological effects of acrylamide on reproductive system in male rats, *J Vet Sci* 6(2):103-109

Methods:

Chromosomal aberrations were examined in Chinese hamster lung (CHL) fibroblasts following treatment with acrylamide (1.25 2.5 5 10 and 50 mM, Sigma) \pm Rat S9 (Aroclor 1254 induced). Positive controls: Mitomycin C (0.05 μ g/ml) or BaP (0.02 μ g/ml). Cells were incubated with \pm S9 (22h) then treated with colcemid (0.2 μ g/ml, 2 h) before metaphase analysis.

Cytotoxicity was determined by microscopical examination following incubation of 10^4 cells for 24 h with examination 24 h later. Details of number of cells evaluated, and results excluding gaps were not reported

Major Findings:

Dose related increase in chromosomal aberrations at 5, 10 and 50 mM \pm S9.

Role of Metabolism:

Positive results were obtained in the presence and absence of metabolic activation. This would suggest that acrylamide can cause chromosomal aberrations in mammalian cells without metabolic activation, however there are limitations in the methodology (non standard S9 incubation time) in this study.

Additional Comments:

B[a]P +S9 and Mitomycin C (-S9) gave expected results. Results appear similar \pm S9

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Chromosomal Aberrations

Acrylamide Positive Glycidamide Positive

MARTINS2007: Martins C;Oliveira NG;Pingarilho M;Gamboa dC;Martins V;Marques MM;Beland FA;Churchwell MI;Doerge DR;Rueff J;Gaspar JF; (2007) Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts, Toxicol Sci 95(2):383-390

Methods:

Acrylamide (>99.5% pure, Fluka (Buchs Switzerland)) and glycidamide (>98.5%, with 1% acrylamide, Toronto Research Chemicals, Ontario, Canada). Approximately 5x 10⁵ V79 Mz cells were exposed to 1-2000 µM acrylamide and glycidamide for 16h, and treated with colchicine for .5 h prior to harvest, hypotonic treatment and processing for chromosome examination. MMC was used as positive control. Percent aberrant cells excluding gaps was used as a measure of clastogenicity %ACEG. SCEs were investigated following a 27h culture of exposed cells.

Major Findings:

Both acrylamide and glycidamide produced apparent dose related increases in chromosome aberrations with the maximum effect occurring at 2000 µM acrylamide and 1000 µM glycidamide. There was a clear cytotoxic effect at the highest concentrations producing chromosome aberrations. Glycidamide produced a more pronounced effect on cytotoxicity than acrylamide at equimolar concentrations. It was reported that doses of up to 1mM acrylamide or glycidamide produced 50% cell survival. For both compounds predominant findings were chromatid breaks with some rearrangements for glycidamide. Both compounds produced a significant increase in chromatid gaps. Glycidamide induced SCEs at = 10 µM whilst acrylamide produced SCEs at 2000 µM (where the effect reported 1.6x was relatively small). There was a strong correlation between DNA adducts and SCEs reported (see in vitro DNA adducts).

Mechanistic Data:

Clastogenic effect of acrylamide in this test system not related to formation of glycidamide. Authors suggest acrylamide Michael-type reaction or free radical formation might explain the clastogenicity of acrylamide to some extent. It was noted that there was only a moderate increase in clastogenicity of glycidamide compared to acrylamide but DNA adducts were formed at 2-3 orders greater magnitude. The mechanism of glycidamide induce clastogenicity was thus unclear and the authors considered further evaluation was warranted. The formation of SCEs were consistent with depurination of DNA adducts.

Role of Metabolism:

Authors cite Glatt 2005 and report that V79Mz cells have no CYP2E1 activity.

Additional Comments:

Overall authors considered clastogenic effect of acrylamide was moderate at the highest concentration tested. The authors considered that serum concentrations of acrylamide and glycidamide would be equivalent to doses reporting positive effects in this study. (The authors cite Twaddle 2004, Cancer Lett, 207, 9-17. cf 450 µM acrylamide and 250 µM glycidamide following a single oral dose of 50 mg/kg acrylamide to b6c3F1 mice.) Mitomycin C gave expected response.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Micronucleus

Acrylamide Positive

JIE2001: Jie YM;Jia C; (2001) Chromosomal composition of micronuclei in mouse NIH 3T3 cells treated with acrylamide, extract of *Tripterygium hypoglaucum* (level) hutch, mitomycin C and colchicine, detected by multicolor FISH with centromeric and telomeric DNA probes, *Mutagenesis* 16(2):145-149

Methods:

Chromosomal composition of micronuclei (MN) induced in NIH 3T3 cells was analysed by multicolour fluorescence in situ hybridisation (FISH). NIH3T3 (48 h cultures) were exposed to Acrylamide (100, 200 and 400 µg/ml; Sigma) and 24 h cell samples taken trypsinised and fixed (no hypotonic treatment). DNA probes (biotinylated pmKB6, and telomeric repeat labelled with digoxigenin) were used for FISH (37°C overnight). Signal detection used Cy3 for biotin probes and FITC with anti sheep digoxigenin. 3000 cells/dose level were analysed.

Major Findings:

Acrylamide induced a clear dose-response in MN total (r^2 0.9859 $P < 0.01$), MN with centromeric and telomeric signals (r^2 0.9939 $P < 0.01$) and MN with acentric fragments (r^2 0.8728 $P < 0.05$). At 100 µg acentric fragments predominated whilst at 400 µg centromeric MN made up 69% of total MN.

Mechanistic Data:

Acrylamide induces a variety of mechanisms of Mn formation. This study suggested aneugenic mechanism occurred at higher concentrations than mechanisms of centric fragment induction.

Role of Metabolism:

No exogenous metabolic activation used in this study.

Additional Comments:

Overall authors considered acrylamide is clastogen and aneugen.

Acrylamide Negative

Glycidamide Negative

BAUM2005A: Baum M;Fauth E;Fritzen S;Herrmann A;Mertes P;Merz K;Rudolphi M;Zankl H;Eisenbrand G; (2005) Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood, *Mutat Res* 580(1-2):61-69

Methods:

Acrylamide obtained from Merck (Dramstock, Germany). Glycidamide synthesised. Both test materials reported to be reagent grade. Whole blood cultures ($n=14$) from 15 unrelated healthy donors (23-33 y). After 48 h of culture, cells were treated with test materials (Acrylamide 0.5-5mM, and glycidamide 0.05-1mM). Positive control bleomycin (0.004 mM). Cells harvested at 71 h culture. CBMN undertaken according to Fenech (*Mutat Res*, 534, 65-75, 2003). 1000 BN cells scored. Nuclear Division Index used as measure of cytotoxicity.

Major Findings:

Cytotoxicity identified by reduction of NDI following acrylamide treatments at 2.5 and 5.0 mM. No cytotoxic effects reported in glycidamide trials. A doubling of mutation frequency was reported for acrylamide at 5 mM in 7/15 donors, no effect in other donors. An approximated 2.4 fold increase in mutation frequency was reported at 1mM glycidamide (statistically significant in only two donors). Overall no increases from acrylamide or glycidamide trials were reported to be statistically significant.

Role of Metabolism:

Study did not include exogenous metabolic activation.

Additional Comments:

Bleomycin gave appropriate positive response in all trials. Higher doses of glycidamide were not used. Authors reported 1mM to be several orders of magnitude above anticipated systemic levels following dietary exposure to acrylamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro Micronucleus

Acrylamide Equivocal Glycidamide Positive

KOYAMA2006: Koyama N; Sakamoto H; Sakuraba M; Koizumi T; Takashima Y; Hayashi M; Matsufuji H; Yamagata K; Masuda S; Kinae N; Honma M; (2006) Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells, *Mutat Res* 603(2):151-158

Methods:

Acrylamide and glycidamide were purchased from Wako Pure Chemicals (Tokyo). TK6 cells (20 ml of 5×10^5 cells/ml, cf 10^7 cells) were incubated with test materials for 4 h (in absence of exogenous metabolic activation). 48 hours after treatment, 10^6 cells were subject to hypotonic treatment (10 min), fixed and stained with acridine orange. 1000 interphase cells were examined for MN formation and data analysed by Fisher exact (pair wise) and Cochran-Armitage (for dose-response). Relative survival was used as a measure of cytotoxicity.

Major Findings:

Acrylamide induced an increase in MN formation at 12.5 mM (in the presence of significant reduction of relative survival, (approximately 10-30%). Glycidamide induced an increase in MN formation at dose levels which equated to minimal or no effect on relative cell survival. The authors reported a dose-dependent increase, with a four-fold increase at the highest concentration of glycidamide used.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Positive (human) Acrylamide Negative (rat)

BJORGE1996: Bjorge C; Brunborg G; Wiger R; Holme JA; Scholz T; Dybing E; Soderlund EJ; (1996) A comparative study of chemically induced DNA damage in isolated human and rat testicular cells, Reprod Toxicol 10(6):509-519

Methods:

Acrylamide (analytical grade from commercial supplier). Rat and human testicle cells prepared. Human testis were obtained at death of organ transplant donors (n=7, aged 29-71y). Testes decapsulated and tissue minced and kept at 4°C for 8-12h where treated with collagenases and cells separated by centrifugation. 15×10^6 cells/donor, 95% cell viability (trypan blue). Wistar rat testes were decapsulated and incubated with collagenase (20 min) and trypsin (further 12-15 min). Fetal calf serum added and cells isolated by centrifugation (70×10^6 cells/animal, cell viability 95%). The percent haploid/diploid and tetraploid cells estimated. Cell morphology of stained smears evaluated. Crude cell suspensions (4×10^6 in 2ml) were exposed to 30-1000 μ M (human) and 100-1000 μ M (rat) acrylamide in DMSO for 30 min (32°C) in HEPES plus 1% BSA. Cells were centrifuged and cell viability determined and samples loaded onto polycarbonate tubes. Cells were lysed, deproteinised and DNA eluted. DNA damage was assessed using Normalised Area Above Curve (Brunborg in Chem Biol Interact, 1996, 101, 33-48). Data analysed by Wilcoxon two sample distribution test.

Major Findings:

The composition of human testicular cell preparation varied considerably (ratio of haploid/diploid/tetraploid cells) between donors. The rat testicular preparations were more consistent. Acrylamide induced a low level but statistically significant increase in ssDNA at 1000 μ M in human testicular cells but not in rats cells. The authors reported that further studies using ARA-c or hydroxyurea did not increase ssDNA formation in these assays.

Mechanistic Data:

The authors suggest the low level ssDNA formation is consistent with the low level DNA alkylation previously reported in testicular cells for acrylamide.

Additional Comments:

The authors investigated the effects of delaying cell preparation with investigations using rat testicular preparations and DBCP and reported no effect. The authors noted that although human testicular cell preparations varied considerably there was little inter donor variation in ssDNA results. Some negative results in both human and rat tissue were reported for cisplatin, thipeta, benomyl and cadmium. The authors suggest a longer exposure time was required for cisplatin. Positive results in both species were reported for a number of chemicals including styrene oxide, 1,2bromomethan (EDB) and thiram.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Positive

MA2003: Ma XX; Yao GD; Cheng H; Zeng QL; Chen Q; (2003) [Effects of acrylamide on DNA damage in human keratinocytes], *Zhonghua Lao Dong Wei Sheng Zhi Ye Bing Za Zhi* 21(2):96-98

Methods:

Keratinocyte cell line HaCaT cells treated with acrylamide for 44 hours, cell survival rate was detected by MTT method. DNA damage assessed by comet assay. Cells were treated with acrylamide (2.00 mmol/L) plus 1-aminobenzotriazole (1-ABT, 0.50 mmol/L), an inhibitor of cytochrome P-450 enzymes (CYP-450), for 4 hours.

Major Findings:

Cytotoxicity was not detected after 4-hour acrylamide treatment, but significant DNA damage was observed in all treatment groups with a dose response in tail length.

In cells treated with 1-ABT and 2 mmol/L acrylamide, comet rate and tail length were significantly reduced 15.4% and $8.2 \pm 2.0 \mu\text{m}$ ($P < 0.01$) when compared with cells treated with acrylamide (2 mmol/L), 80.6% and $44.3 \pm 4.0 \mu\text{m}$.

Mechanistic Data:

Authors consider there is evidence for acrylamide effects mediated by metabolism.

Role of Metabolism:

Authors consider that 1-ABT inhibition of metabolism in this cell line occurred (data not provided). Authors suggest acrylamide may have mediated DNA damage via oxidative metabolites.

Additional Comments:

Data available as abstract only

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Positive

BLASIAK2004: Blasiak J;Gloc E;Wozniak K;Czechowska A; (2004) Genotoxicity of acrylamide in human lymphocytes, *Chem Biol Interact* 149(2-3):137-149

Methods:

PBLs (from male healthy donors age 20-31 y, 99% cell viability) using the alkaline-, neutral- and 12.1 versions of the comet assay and pulse-field gel electrophoresis using exposure for 1 h to acrylamide (Sigma). The comet assay was undertaken according to Singh 1988. Conditions of electrophoresis included neutral (pH9), pH 12.1 and pH>13. Slides examined by fluorescence microscopy. Tail length, %DNA in tail, and tail moment recorded. (Pulse approach used 1-500s for 59h and 1500-1600s for 31 h ad 14oC.) data assessed using Students' t-test or Mann Whitney U-test. Cell viability assessed after 3 day exposure of 0.1-50 µM using MTT assay.

Major Findings:

Acrylamide induced a concentration dependent increase in viability (87.5% at 50 µM). Acrylamide induced a dose related increase in all three parameters of DNA damage assessed in the alkaline assay at between 0.5-50 µM. Statistically significant effects on tail moment in the alkaline assay were recorded at 0.1 µM (P<0.05). An increase in DNA damage in the neutral assay at 50 µM indicated the formation of double strand breaks in addition to single strand breaks. The authors reported that DNA damage induced by acrylamide at 0.5 and 5 µM was repaired during a 60 minute repair incubation (and after 120 minutes at 50 µM).

Post treatment of damaged DNA with repair enzymes thymine glycol DNA N glycosylase (Nth) and formamidopyrimidine-DNA glysoylase (Fpg), recognising oxidised DNA bases, as well as 3-methyladenine-DNA glycosylase II (Alk A), recognising alkylated bases, caused an increase in the extent of DNA damage, indicating the induction of oxidative and alkylative DNA base modifications by acrylamide. Effects in the presence of Nth and ALkA were seen at 0.5 µM acrylamide and in the presence of Fpg at 50 µM acrylamide.

Pretreatment of the lymphocytes with compounds/vitamins which inhibit free radicals/oxygen species such as N-tert-butly-alpha-phenylnitronone (PBN), a spin trap (50 µM inhibited acrylamide at 5 µM), as well as vitamins C (50 µM inhibited 50 µM acrylamide) and vitamin E (10 µM inhibited 5 µM acrylamide) caused a decrease in DNA damage mediated by acrylamide

Mechanistic Data:

The authors noted that in addition to evidence for the formation of reactive oxygen species, there was also evidence for formation alkylated DNA bases and oxidative DNA damage. It was unclear as to what extent the effects were mediated by acrylamide and or glycidamide formed by metabolism of exogenously applied acrylamide

Role of Metabolism:

It was unclear to what extent PBLs mediated the metabolism of acrylamide to glycidamide. No exogenous metabolising fraction data reported.

Additional Comments:

Acrylamide was shown to reduce the repair of DNA damage induced by hydrogen peroxide and increased caspase 3 activity (DEVDp-nitroaniline substrate at 50 µM acrylamide. The authors suggest a wide range of acrylamide effects including base modification and apoptosis.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Negative Glycidamide Positive

BAUM2005A: Baum M;Fauth E;Fritzen S;Herrmann A;Mertes P;Merz K;Rudolphi M;Zankl H;Eisenbrand G; (2005) Acrylamide and glycidamide: genotoxic effects in V79-cells and human blood, Mutat Res 580(1-2):61-69

Methods:

Acrylamide obtained from Merck (Dramstock, Germany). Glycidamide synthesised. Both test materials reported to be reagent grade. Blood samples were taken from three male, healthy donors into heparinised tubes. Blood was incubated with acrylamide (1000-6000 µM) and glycidamide (100-3000 µM) for 1, 2 or 4 hours. Alkaline comet was undertaken according to Singh 1988. Tail intensity was quantified by computer assisted microscopy.

Major Findings:

Acrylamide was inactive at all concentrations tested. Glycidamide induced a dose-dependent at 300 µM and above. Incubation of 300 µM glycidamide and 10 mM acrylamide reported the same extent of DNA damage as 300 µM glycidamide. Reduced NDI seen at 2,500 µM acrylamide.

Additional Comments:

Bleomycin gave appropriate positive response in all trials. Authors note that glycidamide response was similar or greater than bleomycin depending on concentration and duration of incubation used.

Glycidamide Positive

JOHANSSON2005: Johansson F;Lundell T;Rydberg P;Erixon K;Jenssen D; (2005) Mutagenicity and DNA repair of glycidamide-induced adducts in mammalian cells, Mutat Res 580(1-2):81-89

Methods:

This study examined the effects of glycidamide (up to 10 mM h, >95% purity) on several CHO cell lines; these lines were wild type, base excision repair (BER) deficient, nucleotide excision repair (NER) deficient, or homologous recombination (HR) deficient. The Detection of Repairable Adducts by Growth Inhibition (DRAG) assay was used to determine the required DNA repair pathway. Cells were exposed to glycidamide for 1 h, followed by a 96 h incubation. Plates were fixed, stained (aqueous neutral red) and cell growth and number was assessed.

Single strand breaks were also measured in these CHO cell lines in presence or absence of inhibitors of BER (hydroxyurea/cytosine arabinoside) and NER (ISQ). Cells were pre-labelled with 3H TdR (24 h), exposed to glycidamide (1 h). DNA was unwound using ice cold NaOH (30 min) followed by neutralisation and addition of SDS. Single and double strands were separated by hydroxyapatite chromatography and scintillation data for single and double strand breaks was compared. The difference was used to calculate the number of single strand breaks, calibrated to a known number of single strand breaks induced by gamma radiation.

Major Findings:

The DRAG assay is based on the principle that a DNA repair deficient cell growth and survival is likely to be more affected than a repair proficient cell. Cells deficient in HR or BER were three or five times, respectively, more sensitive to GA in terms of growth inhibition than were wild-type cells.

In single strand break assays, the use of ISQ in BER deficient cells provided evidence that BER was responsible in part for glycidamide adduct DNA repair. Use of cell line UV4 (for long patch BER and NER) and inhibitors suggested that NER was not responsible for glycidamide repair. The authors also reported reduced survival in homologous recombination deficient cells, which suggests that this is important in the repair of glycidamide adducts.

Mechanistic Data:

Authors suggest that a large part of ss-breaks induced by glycidamide resulted from alkylation of the backbone phosphate. These are mis-repaired by homologous recombination during replication resulting in a clastogenic rather than mutagenic effect.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Positive (human & hamster) Acrylamide Negative (rat) Glycidamide Positive (human, hamster & rat)

PUPPEL2005: Puppel N;Tjaden Z;Fueller F;Marko D; (2005) DNA strand breaking capacity of acrylamide and glycidamide in mammalian cells, Mutat Res 580(1-2):71-80

Methods:

Acrylamide (VWR GmbH, Darmstadt, Germany, highest research grade) and glycidamide (synthesised according to Payne and Williams, J. Org. Chem, 1961, 26, 651-9; no purity data). Caco-2 (human), V79 (hamster) cells and primary rat hepatocytes (obtained from male Wistar rats using collagenase perfusion (>90% cell viability) and incubated with the test materials for 24h (acrylamide 100-6000 µM. Glycidamide 1-600 µM). Single cell gel electrophoresis, was undertaken according to Gedik (Int J Radiat Biol, 62, 313-320, 1992) under alkaline conditions (pH>13.0). %0 cells/slide, 100 cells/treatment analysed. Triplicate trials undertaken. Data reported as tail intensity (%of DNA in comet tail). Cell viability assessed with trypan blue exclusion. Western blotting using rabbit polyclonal antibody for human/rat CYP450E1 and anti rabbit IgG peroxidase conjugate. Data expressed as percentage of control. Data analysed using Students' t-test.

Major Findings:

No increase in strand breaks was reported in trials using 1, 3, or 6 h exposure to 6 mM acrylamide. A statistically significant increase in DNA damage was reported at 6 mM after 24 h exposure in caco-2 cells (P<0.05) and a slightly greater increase was reported in V79 cells at the same exposure period (P<0.01). For glycidamide a significant increase in DNA damage was reported in V79 cells at 6h at 30 µM glycidamide (substantial at =300 µM) and a dose related increase at =100 µM after 24 h exposure. For Caco-2 cells an increase was reported at =300 µM at 3h 6h and 24h exposures. In rat liver, a significant increase in DNA damage was reported at 600 µM following 3h exposure. A concentration related increase at =100 µM was reported following 24 h exposure.

Pre-treatment of V79 cells and rat hepatocytes with BSO (DL-buthionine-[S,R]-sulfoxime (1mM for 24 h) did not affect DNA damage levels. In rat hepatocytes no increase in DNA damage was reported following incubation of 6 mM acrylamide for up to 3 h. In V79 cells a significant increase in DNA strand breaks was observed at 6 mM acrylamide. After 24 h exposure to acrylamide, a slight but significant increase in DNA stand breaks was reported in V79 cells at 0.5 mM and 1mM acrylamide (in presence of BSO). In rat hepatocytes a significant increase in DNA strand breaks was reported following incubation with 1mM acrylamide after a 24 h incubation (in presence of BSO). Under BSO treatment =1.25 MM acrylamide resulted in a strong cytotoxic response in V79 cells and rat hepatocytes. In additional studies V79 cells were pre-treated with BSO and then exposed to glycidamide. At 100 µM glycidamide, significant increases in DNA strand beaks were observed in BSO treated cells. At 600 µM and 1000 µM slight but not significant DNA damage was reported. BSO did not have a significant effect on glycidamide induced DNA damage. (Viability was 95% in these trials with no indication of cytotoxicity).

Mechanistic Data:

Depletion of intracellular glutathione substantially increased the DNA damage associated with acrylamide in V79 and Caco-2 cells

Role of Metabolism:

V79 and Caco-2 cells were not expected to have any CYP2E1 activity. The positive results with acrylamide in these cell lines imply an effect of acrylamide.

Additional Comments:

CYP2E1 protein levels were investigated by Western blotting. CYP2E1 was not detected in V79 cells. CYP2E1 was detectable in the rat hepatocytes throughout the experiment, although levels had declined by at 30 and 50 hours. The authors conclude that there would have been some metabolic capacity in the hepatocytes; however, the presence of the protein does not necessarily mean that it is still active. The authors also note that acrylamide induced DNA damage in V79 cells but not in rat hepatocytes, indicating that high expression of CYP2E1 per se is not necessarily associated with increased genotoxicity of acrylamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Positive

CHICO2006: Chico G;Massart C;Jin L;Vanvooren V;Caillet-Fauquet P;Andry G;Lothaire P;Dequanter D;Friedman M;Van Sande J; (2006) Acrylamide, an in vivo thyroid carcinogenic agent, induces DNA damage in rat thyroid cell lines and primary cultures, Mol Cell Endocrinol 257-258:6-14

Methods:

Purity of acrylamide not stated. Rat thyroid cell line PC C13 (and one study with FRTL5 thyroid cells, although the authors noted a high spontaneous background aneuploidy in this cell line) and primary thyroid cultures from dog, sheep and humans used in comet assays. SCGE undertaken according to Olive 1990 and Singh 1988. A variety of exposure concentrations between 10 µM and 3mM used in experiments where exposure lasted between 1h up to 48 h depending on cell type used. Comets were categorised into 5 groups with those presenting with DNA damage scaled as 3-5, considered as positive. The percentage positive cells from examination of 200-250 cells per slide was calculated. Duplicate slides were used for each experiment. No overview of statistical approach used was given.

Major Findings:

Purity of acrylamide and glycidamide not stated. Rat thyroid cell line PC C13 (and one study with FRTL5 thyroid cells, although the authors noted a high spontaneous background aneuploidy in this cell line) and primary thyroid cultures from dog, sheep and humans used in comet assays. SCGE undertaken according to Olive 1990 and Singh 1988. A variety of exposure concentrations between 10 µM and 3mM used in experiments where exposure lasted between 1h up to 48 h depending on cell type used. Comets were categorised into 5 groups with those presenting with DNA damage scaled as 3-5, considered as positive. The percentage positive cells from examination of 200-250 cells per slide was calculated. Duplicate slides were used for each experiment. No overview of statistical approach used was given.

Major Findings: A concentration-related increase in DNA damage was reported in PC C13 cells over the range 10 µM and 3mM. In 34 trials the maximum increase in DNA damage was reported to be 1.5-4 fold. Similar effects were reported for either 3 h or 28 h exposures. In FRTL 5 cells an overnight exposure with 140 µM acrylamide lead to an increase in DNA damage from 11% to 23%. In primary cell cultures, using a 24 h exposure to 1mM, the increases in DNA damage were reported to be; 20% to 39.9% in dog, 19% to 37% in sheep and 18.4% to 29.85% in human tissue. A concentration related effect on DNA damage was reported following a 48 h exposure of human thyrocytes to 10 µM to 3mM acrylamide. Hydrogen peroxide was used as a positive control in these studies. The authors didn't quantitatively compare acrylamide with hydrogen peroxide, but qualitatively similar responses were seen in PC13 cells at 14 µM acrylamide (3h) and 33 µM hydrogen peroxide (1h) and 3mM acrylamide for 48h in human thyrocytes compared to 100 µM hydrogen peroxide for 10 minutes.

The authors note that induction of phosphorylation of serine 139 in H2AX histone in PC13 cells is a marker for double strand DNA breaks. Hydrogen peroxide (40 µM for 1h) and acrylamide (140 µM for 17h) were negative in this assay, whereas etoposide gave a positive result (10 µM for 1h). In additional studies acrylamide (14 or 140 µM) and glycidamide (115 µM) using a variety of exposure times between 15 minutes and 40 hours had no effect on H2AX phosphorylation.

Neither acrylamide (1.4-14 µM) or glycidamide (1.15-115 µM) had a proliferative effect on FRTL 5 cells (using BrdU to determine proliferation in presence or absence of added TSH (1mU/ml or 3mU/ml).

Additional Comments:

Data suggest acrylamide induced predominantly single strand breaks in mammalian thyroid cells.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Damage

Acrylamide Negative Glycidamide Positive

KOYAMA2006: Koyama N; Sakamoto H; Sakuraba M; Koizumi T; Takashima Y; Hayashi M; Matsufuji H; Yamagata K; Masuda S; Kinae N; Honma M; (2006) Genotoxicity of acrylamide and glycidamide in human lymphoblastoid TK6 cells, *Mutat Res* 603(2):151-158

Methods:

Induction of DNA single strand breaks by acrylamide and glycidamide was assessed by modified alkaline single cell gel electrophoresis (alkaline comet assay). TK6 human lymphoblastoid cells were treated with acrylamide (0, ~7, & 14 mM) or glycidamide (0, 0.6, ~1.1, & 2.2 mM) for 4 h. Cells were mounted onto slides in agarose and lysed (1 h). After unwinding, slides were subjected to electrophoresis (15 min), then stained with SYBER green. At least 50 cells were analysed (for each slide/dose?).

Major Findings:

Acrylamide did not increase tail length at the two concentrations tested. Significant dose related increases were seen with glycidamide.

Role of Metabolism:

Cells would not necessarily be able to metabolise acrylamide to glycidamide at potentially cytotoxic levels.

Glycidamide Positive

THIELEN2006: Thielen S; Baum M; Hoffmann M; Loepky RN; Eisenbrand G; (2006) Genotoxicity of glycidamide in comparison to (+/-)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide and alpha-acetoxy-N-nitroso-diethanolamine in human blood and in mammalian V79-cells, *Mol Nutr Food Res* 50(4-5):430-436

Methods:

Induction of DNA single strand breaks by glycidamide (prepared according to Payne and Williams, *J. Org. Chem*, 1961, 26, 651-9; no purity data) was assessed by modified alkaline single cell gel electrophoresis (alkaline comet assay with and without treatment of cells with formamido-pyrimidine-DNA-glycosylase (FPG)) in human lymphocytes from whole blood (3 healthy male donors). Blood samples (990 µl) from each donor were incubated for 1, 2 or 4 h; in the presence of glycidamide (0, 3, 10, 30, 100, or 300 µM), alpha-acetoxy-N-nitroso-diethanolamine (a-A-NDELA; 0, 0.3, 1, 3, 10, 30 or 100 µM), or (±)-anti-benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE; 0, 10, 30, 100 or 300 µM).

Cells were mounted onto slides in agarose, lysed (1 h, 37 °C) followed by incubation ±FPG (30 min). After unwinding (pH >13, 20 min), slides were subjected to electrophoresis (20 min, 25 V, 300 mA), then stained with ethidium bromide. 50 cells were analysed per gel.

Major Findings:

In the absence of FPG, significant increase in tail intensity was only observed at 300 µM with 4 h incubation). In the presence of FPG an incubation time and concentration dependent increase in tail length was observed, with significant increases at 10 µM with 4 h incubation time. Significant increases in mean tail intensity were seen with BPDE and a-A-NDELA at 30 and 10 µM respectively; this was not enhanced by FPG treatment.

Mechanistic Data:

The authors state that the increase in single strand breaks in the presence of FPG suggests that glycidamide forms N7-adducts of guanine. These lead to 5-N-alkyl-2,6-diamino-4-hydroxyformamidopyrimidine (alkyl-FAPy-G) lesions which is a substrate for FPG.

Role of Metabolism:

This study used the proposed active metabolite glycidamide

Additional Comments:

There were no significant differences in response between donors.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Adducts

Acrylamide Positive +S9 (N7-GA-Gua)

SEGERBACK1995: Segerback D;Calleman CJ;Schroeder JL;Costa LG;Faustman EM; (1995) Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [¹⁴C]acrylamide, *Carcinogenesis* 16(5):1161-1165

Methods:

¹⁴C Acrylamide (0.074 MBq/ml, Sigma, purity >98%, specific activity 40 MBq/mM) was incubated (2h, 37°C) with calf thymus DNA (1mg/ml) with S9 from un-induced Sprague-Dawley rats. DNA was isolated, its concentration determined, N7-GA-Gua standard was added, and aliquots were hydrolysed to release N7-alkylated bases (10 mM sodium citrate buffer, pH 6, 20 min, 100°C). DNA was precipitated and re-dissolved in water, then fractionated by HPLC. The fractions associated with the UV peak of the cold standard were collected and the radioactivity assessed by liquid scintillation counter. Adduct levels were calculated from the radioactivity co-eluting with the recovered standards and the amount of DNA analysed.

Major Findings:

The major adduct detected was N7-(2-carbamoyl-2-hydroxy-ethyl)guanine (N7-GA-Gua), with the structure confirmed by UV, MS and C13-NMR.

Additional Comments:

The authors state that the DNA hydrolysis conditions would favour the release of N7-alkylated bases, but little or no common bases or other alkylation products. The authors note that acrylamide is metabolised to glycidamide by S9 in their experiment, but suggest that the amount of glycidamide produced by S9 liver microsomes may not be sufficient to yield a significant increase in the Ames test.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Adducts

Glycidamide Positive (N7-GA-Gua > N3-GA-Ade and N1-GA-dA)

GAMBOA2003: Gamboa dC;Churchwell M;Hamilton LP;Von Tungeln LS;Beland FA;Marques MM;Doerge DR; (2003) DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice, Chem Res Toxicol 16(10):1328-1337

Methods:

Glycidamide (synthesised by epoxidation of acrylonitrile, purity ~98%) was incubated with salmon testis DNA in aqueous buffer (pH 7.1, 37°C, overnight), using 7 concentrations of glycidamide, in order of magnitude steps, between 1 ng and 1 mg per mg of DNA. DNA was precipitated and depurinating adducts were released by neutral thermal hydrolysis. Samples were spiked with a 15N labelled adduct standard (synthesised through reaction of glycidamide with 15N-dA, 83-99% purity) and passed through a size exclusion column, before quantification by LC/MS/MS. Deoxynucleoside adducts were released by enzymatic hydrolysis and analysed by LC/MS/MS.

Major Findings:

¹³C NMR spectroscopic analysis for the dG adduct, N7-(2-carbamoyl-2-hydroxy-ethyl)guanine (N7-GA-Gua), was generally consistent with that of Sergerbäck 1995. Together, ¹³C and ¹H spectra confirm attachment of the glycidamide moiety via the methylene carbon.

The ¹H NMR spectrum confirmed the structure of the N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade) adduct, showing that it is a depurinating adduct.

The structure of the N1-(2-carboxy-2-hydroxyethyl)-dA (N1-GA-dA) adduct was confirmed by ¹H NMR with support from the UV spectrum. As with N7-GA-Gua, this shows attachment of the glycidamide moiety via the methylene carbon. Mass spectrometry data indicated the glycidamide adduct contained a carboxyl rather than carbamoyl moiety. Extended treatment at pH 13 yielded a chromophore consistent with an N6 substituted adenosine derivative, suggesting a Dimroth rearrangement to N6-GA-dA. An additional cyclic N6-(2-hydroxypropanoyl)-2'-deoxyadenosine adduct was identified immediately after the reaction of glycidamide and dA; although this adduct rapidly hydrolysed to form N1-GA-dA.

The predominant adduct formed was N7-GA-Gua at levels 10 to 70-fold higher than either N3-GA-Ade or N1-GA-dA.

Additional Comments:

Although the depurination of N7 adduct may be mutagenic, the authors consider potential for disruption of base pairing and thermal stability of the N1 adduct may make it a directly mutagenic lesion. The low signal to noise ratio of the N1-GA-dA adduct meant it was necessary to convert the adduct to N6-GA-dA at pH13.

Glycidamide Positive (N3-GA-dThd and N3-GA-Cyd)

BACKMAN2004: Backman J;Sjoholm R;Kronberg L; (2004) Characterization of the adducts formed in the reactions of glycidamide with thymidine and cytidine, Chem Res Toxicol 17(12):1652-1658

Methods:

Glycidamide (Toronto Research Chemicals, ON, Canada) was reacted with thymidine and cytidine (Sigma) in aqueous-buffered solutions in vitro. The nucleoside adducts were isolated by reversed phase HPLC and characterised by UV absorbance (Liquid Chromatography - Diode Array Detector: LC-DAD), ¹H and ¹³C NMR spectroscopic, and mass spectrometric features (Liquid Chromatography – Electrospray Ionisation – Tandem Mass Spectrometry: LC-ESI-MS/MS)

Major Findings:

Reactions with thymidine yielded one major product peak by LC-DAD and LC-ESI-MS/MS. The structure of the product was determined to be N3-(2-carbamoyl-2-hydroxyethyl)thymidine (N3-GA-dThd) by NMR, UV spectroscopy and MS. Reactions with cytidine yielded three major product peaks by LC-DAD and LC-ESI-MS/MS. These were identified a diastereomeric pair of N3-(2-carboxy-2-hydroxyethyl)cytidine (N3-GA-Cyd-1 and N3-GA-Cyd-2) and N3-(2-carboxy-2-hydroxyethyl)uridine (N3-GA-Urd) by NMR, UV spectroscopy and MS.

Additional Comments:

The reactions were performed at non-physiological pH to give optimum yields.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Adducts

Glycidamide Positive (N1-GA-dG, N1-GA-dA, N6-GA-dA and N1-GA-dI)

BACKMAN2007: Backman J;Kronberg L; (2007) Reaction of glycidamide with 2'-deoxyadenosine and 2'-deoxyguanosine--mechanism for the amide hydrolysis, *Nucleosides Nucleotides Nucleic Acids* 26(2):129-148

Methods:

Glycidamide (Toronto Research Chemicals, ON, Canada) was reacted with 2'-deoxyadenosine (dA; Sigma) and 2'-deoxyguanosine (dG; Sigma) in aqueous phosphate buffered solutions in vitro. The nucleoside adducts were isolated by reversed phase HPLC and characterised by UV absorbance (Liquid Chromatography - Diode Array Detector: LC-DAD), ¹H and ¹³C NMR spectroscopic, and mass spectrometric features (Liquid Chromatography – Electro spray Ionisation – Tandem Mass Spectrometry: LC-ESI-MS/MS)

Major Findings:

Reactions with dA at pH 7 yielded three adducts, N1-(2-carboxy-2-hydroxyethyl)-dA (N1-GA-dA), N6-(2-carboxy-2-hydroxyethyl)-dA (N6-GA-dA) and N1-(2-carboxy-2-hydroxyethyl)-2'-deoxyinosine (N1-GA-dI), as determined by NMR, UV spectroscopy and MS. The N1-GA-dA adduct was reported to be identical to that identified by Gamboa da Costa et al. 2003 (*Chem Res Toxicol*, 16 1328-37). Reactions with dG at pH 7 yielded a single N7-GA-dG adduct previously identified by Sergerback 1995 (*Carcinogenesis* 16(5) 1161-5). This adduct was not found in reactions with dG at pH 9, which instead yielded two N1 adducts, N1-(2-carboxy-2-hydroxyethyl)-dG (N1-GA-dG I) and N1-(2-carbamoyl-2-hydroxyethyl)-dG (N1-GA-dG II) as determined by NMR, UV spectroscopy and MS.

Additional Comments:

The authors observe that, with the exception of the N1-GA-dG II adduct, the amide function in all the other adducts had undergone hydrolysis to a carboxy function. Consistent with this observation, they suggest a mechanism whereby N7-GA-Gua, N3-GA-Ade and N1-GA-dG II are formed through direct attack of the endocyclic N1 in the purine on the β-carbon of the oxirane ring. However, The N1-GA-dA and N1-GAdG I adducts may be formed through attack of the carbonyl carbon of glycidamide by the exocyclic amino groups of dA and dG, followed by deamination and ring closure through reaction of the oxirane ring with the nucleophilic ring nitrogen of purine. The N6-GA-dA and N1-GA-dI adducts may form through Dimroth rearrangement and subsequent deamination.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Adducts (Cultured Cells)

Acrylamide Positive

BESARATINIA2003: Besaratinia A;Pfeifer GP; (2003) Weak yet distinct mutagenicity of acrylamide in mammalian cells, *J Natl Cancer Inst* 95(12):889-896

Methods:

Cultured Big Blue mouse embryonic fibroblasts, carrying the lambda phage cII transgene in a recoverable shuttle vector, were treated with acrylamide (4 h, 3.2 and 16 mM, 99.9% purity, in distilled water). DNA Adducts were detected after a further 12 h incubation. Adducts identified using terminal transferase dependent PCR. Sites of adduct formation were identified as the locations where the adduct stopped the progress of DNA polymerase. Stained amplified DNA bands were visualised on a sequencing gel.

Major Findings:

Preferential adduct formation was observed at specific nucleotide positions along the gene. These were mostly dose dependent. Some mutation sites co-localised with DNA adduction sites, there was no direct relationship between pattern of induced cII mutations (see separate report) and mapping of DNA adducts.

Role of Metabolism:

No metabolic activation although the cell line may have had some metabolic competency

Additional Comments:

Cell viability reduced at 3.2 mM and above. Positive control benzo[a]-pyrene diol epoxide (0.01, 0.1 & 1 µM). DNA adducts identified using terminal transferase PCR.

Acrylamide Positive Glycidamide Positive

BESARATINIA2004: Besaratinia A;Pfeifer GP; (2004) Genotoxicity of acrylamide and glycidamide, *J Natl Cancer Inst* 96(13):1023-1029

Methods:

Normal human bronchial epithelial cells were treated with acrylamide (320 and 3200 µM) and glycidamide (5 and 500 µM) for 4 h in distilled water. Cultured Big Blue mouse embryonic fibroblasts, carrying the lambda phage cII transgene in a recoverable shuttle vector, were treated with acrylamide (3.2, 320 and 16,000 µM) and glycidamide (0.05, 0.5, 5 and 1000 µM), for 4 h in distilled water. Cells were either harvested immediately after treatment or at various time points up to 72 h.

DNA Adducts were identified using terminal transferase dependent PCR. Sites of adduct formation were identified as the locations where the adduct stopped the progress of DNA polymerase. Bronchial cell genomic DNA was digested with N-methylpurine-DNA glycosylase to increase the sensitivity for detecting N7-GA-dG adducts. Stained amplified DNA bands were visualised on a sequencing gel.

Major Findings:

Acrylamide and glycidamide formed DNA adducts at similar specific locations within TP53 and cII, and DNA adduct formation was more pronounced after glycidamide treatment than after acrylamide treatment at all doses tested. Acrylamide-DNA adduct formation was saturable, whereas the formation of most glycidamide-DNA adducts was dose-dependent. There was evidence that some adducts observed immediately after treatment were repaired by 24 to 48 h post treatment. There was a similar distribution of adducts in both of the genes examined, although adduction was more pronounced in the glycidamide treated cells at all concentrations tested.

Role of Metabolism:

No metabolic activation although the cell line may have had some metabolic competency. Authors concluded that adduct formation in human and mouse cells is based on the formation of acrylamide's epoxide metabolite glycidamide.

Additional Comments:

Acrylamide and Glycidamide reduced cell viability in the mM range in both bronchial and fibroblast cell lines.

The authors suggest that the reason for the difference in mutagenic spectra might be due to the ability of acrylamide to directly react with DNA by Michael addition, in addition to the reactivity its epoxide metabolite, suggesting multiple pathways of DNA adduction.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vitro DNA Adducts (Cultured Cells)

Acrylamide Positive (N7-GA-Gua)

Glycidamide Positive (N7-GA-Gua and N3-GA-Ade)

MARTINS2007: Martins C;Oliveira NG;Pingarilho M;Gamboa dC;Martins V;Marques MM;Beland FA;Churchwell MI;Doerge DR;Rueff J;Gaspar JF; (2007) Cytogenetic damage induced by acrylamide and glycidamide in mammalian cells: correlation with specific glycidamide-DNA adducts, *Toxicol Sci* 95(2):383-390

Methods:

Acrylamide (>99.5% pure, Fluka (Buchs Switzerland)) and glycidamide (>98.5%, with 1% acrylamide, Toronto Research Chemicals, Ontario, Canada). Approximately 8×10^5 V79 Mz cells were exposed to 1-2000 μM acrylamide and glycidamide for 19 and 29 h (to parallel chromosomal aberrations and SCE assays). DNA adducts (N7-GA-Gua, and N3-GA-Ade) were measured following thermal release from DNA using HPLC and tandem mass spectrometry. Cytotoxicity was reported as effects on mitotic index.

Major Findings:

Glycidamide induced a dose-related increase in N7-GA-Gua at doses as low as 1 μM using either 18 or 29 h cultures (no difference between cultures). N3-GA-Ade was detected in glycidamide cultures at concentrations of 250 μM and above at levels of approximately 2 orders of magnitude below N7-GA-Gua, with a dose-response reported. N-7-GA-Gua and N3-GA-Ade formation appeared to be independent of culture time (except for a 2-3 fold increase at the highest concentration in the 29 h culture). Acrylamide did result in formation of N7-GA-Gua but concentrations of 2000 μM were needed to result in an equivalent adduct level to 1 μM glycidamide. (Negative controls did not show any GA-induced DNA adducts). There was a strong correlation between DNA adducts and SCEs reported (see in vitro chromosomal aberrations).

Mechanistic Data:

It was noted that there was only a moderate increase in clastogenicity of glycidamide compared to acrylamide but DNA adducts were formed at 2-3 orders greater magnitude.

Role of Metabolism:

Authors cite Glatt 2005 and report that V79Mz cells have no CYP2E1 activity.

Acrylamide Negative -S9

Glycidamide Positive (N3-GA-Ade and N7-GA-Gua)

MEI2007: Mei N; Hu J; Churchwell MI; Guo L; Moore M; Doerge DR;Chen T (2007) Genotoxic effects of acrylamide and glycidamide in mouse lymphoma cells, *Food Chem Toxicol* E Pub

Methods:

L5178Y/Tk+/- cells (6×10^6 cells/plate) were treated with acrylamide (2-18 mM, Sigma, purity >99.9%) or glycidamide (0.125-4 mM; Toronto Research Chemicals, Ontario, Canada; purity >99.5%) for 4 hours in the absence of metabolic activation. Cells were washed, DNA purified and subjected to neutral thermal hydrolysis; then N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade) and N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua) glycidamide adducts were quantified by isotope dilution LC-ES-MS/MS (as used by Gamboa da Costa et al. 2003 and Doerge et al. 2005). N3-GA-Ade (LOD: 0.5 LOQ:1.5 adducts in 10^8 nucleotides), N7-GA-Gua (LOD: 0.5 LOQ:1.0 adducts in 10^8 nucleotides). 4-nitroquinoline-1-oxide (0.53 μM) was used as a positive control

Major Findings:

Within the dose range tested, glycidamide induced DNA adducts of adenine and guanine (N3-GA-Ade and N7-GA-Gua) in a linear dose-dependent manner. The levels of guanine adducts were consistently about 60-fold higher across the dose range than those of adenine. In contrast, no glycidamide derived DNA adducts were found in the cells treated with any concentrations of acrylamide, consistent with a lack of metabolic conversion.

Role of Metabolism:

No glycidamide derived DNA adducts in the absence of metabolic activation or endogenous CYP2E1

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua; Mouse & Rat)

SEGERBACK1995: Segerback D;Callemann CJ;Schroeder JL;Costa LG;Faustman EM; (1995) Formation of N-7-(2-carbamoyl-2-hydroxyethyl)guanine in DNA of the mouse and the rat following intraperitoneal administration of [¹⁴C]acrylamide, *Carcinogenesis* 16(5):1161-1165

Methods:

¹⁴C Acrylamide (Sigma, purity >98%, specific activity 40 MBq/mM) was administered to 3 male Sprague-Dawley rats by single i.p. injection (46 mg/kg bw: 26.1 MBq/kg) and 3 male Balb/c mice by single i.p. injection (53 mg/kg bw: 30.3 MBq/kg). Animals were sacrificed after 19 h (rats) and 6 h (mice), and organs collected for DNA extraction. DNA was isolated, its concentration determined, N7-GA-Gua standard was added, and aliquots were hydrolysed to release N7-alkylated bases (10 mM sodium citrate buffer, pH 6, 20 min, 100°C). DNA was precipitated and redissolved in water, then fractionated by HPLC. The fractions associated with the UV peak of the cold standard were collected and the radioactivity assessed by liquid scintillation counter. Adduct levels were calculated from the radioactivity co-eluting with the recovered standards and the amount of DNA analysed.

Major Findings:

The level of N7-GA-Gua adducts in rodent liver was found to be in the region of 22 to 32 pM/mg DNA. At the time of sacrifice (19 h), similar levels of adducts were found in the various rat tissues, indicating an even distribution. In mouse, only liver, kidney and brain were analysed (30, 220 and 62 pM/mg DNA, respectively).

Additional Comments:

The authors comment that they find much lower levels of adducts than previous publications (that were considered in the EU-ESR report). They suggest that this is because their assay specifically examines radioactivity associated with N7-GA-Gua adducts, rather than just DNA associated radioactivity. The authors suggest the interspecies difference in organ distribution may be due to the higher degree of metabolism to glycidamide, longer $t_{1/2}$, or the shorter period to repair the lesion before sacrifice at 6 h. Sacrifice times were chosen to be consistent with previous studies. The interspecies difference in rodent adduct levels is consistent with findings by Sumner et al 1992 (*Chem. Res. Toxicol.*, 5, 81-9) that mice excrete higher proportion of urinary metabolites than rats when dosed at similar levels to the present study.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua and N3-GA-Ade) Glycidamide Positive (N7-GA-Gua and N3-GA-Ade)

GAMBOA2003: Gamboa dC;Churchwell MI;Hamilton LP;Von Tungeln LS;Beland FA;Marques MM;Doerge DR; (2003) DNA adduct formation from acrylamide via conversion to glycidamide in adult and neonatal mice, *Chem Res Toxicol* 16(10):1328-1337

Methods:

Adult male (C3H/HeNMVT), female (C57B1/CN) or 3 day old (B6C3F1) mice were administered 50 mg/kg bw i.p of either acrylamide (ICN, Ohio; no purity data) or glycidamide (synthesised by epoxidation of acrylonitrile, purity ~98%). An additional dose response study was performed with acrylamide at 0, 1, 10 and 50 mg/kg bw. Animals were sacrificed 6 hours post dosing. Tissues were taken from adult mice and DNA extracted from hepatocyte nuclei, and kidney and lung homogenate. Neonatal mice were frozen (-80°C) and powdered for DNA extraction. DNA was precipitated and depurinating adducts were released by neutral thermal hydrolysis. Samples were spiked with a 15N labelled adduct standard (synthesised through reaction of glycidamide with 15N-dA, 83-99% purity) and passed through a size exclusion column, before quantification by LC/MS/MS. Deoxynucleoside adducts were released by enzymatic hydrolysis and analysed by LC/MS/MS in the MRM mode. N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) adducts were directly detected, using 15N labelled standards as internal controls.

Major Findings:

In neonatal mice, the background level of N7-GA-Gua was 8.6 ± 3.1 adducts per 10^8 nucleotides, which significantly increased (100-fold; ANOVA $P < 0.05$) upon acrylamide treatment. N3-GA-Ade was below the LOD in control animals, but detectable in acrylamide treated mice. Levels of both adducts in glycidamide treated mice were significantly (5 to 8-fold; ANOVA $P < 0.05$) higher than equivalent doses of acrylamide. No sex differences were observed in neonatal mice.

In adult mice, background levels in liver, lung and kidney were 7 to 11 adducts per 10^8 nucleotides with N3-GA-Ade adducts below the LOD. Treatment with acrylamide significantly increased adduct levels in all tissues, e.g. Liver 160 to 240-fold increase in N7-GA-Gua. Glycidamide treatment caused modest increases in both adducts, but was only significant (ANOVA $P < 0.05$) in females. Levels in male mice were significantly greater than female mice in liver and lung, whereas female kidneys had higher levels than males. The acrylamide dose response study revealed a supralinear dependence on dose, with N7-GA-Gua always >70-fold higher than N3-GA-Ade.

The N1-(2-carboxy-2-hydroxyethyl)-dA (N1-GA-dA) adduct could not be detected in any tissue, even when converted to N6-GA-dA.

Role of Metabolism:

Glycidamide adducts were identified in vivo following acrylamide administration. Treatment with equivalent concentrations of glycidamide only produced only modest (1.2 to 1.5-fold) increases in adduct levels compared to acrylamide.

Acrylamide Positive (N7-GA-Gua)

TWADDLE2004: Twaddle NC;Churchwell MI;McDaniel LP;Doerge DR; (2004) Autoclave sterilization produces acrylamide in rodent diets: implications for toxicity testing, *J Agric Food Chem* 52(13):4344-4349

Methods:

B6C3F1 mice were maintained on either an autoclaved (NIH-31) or non autoclaved diet (NIH-31IR) until ~75-120 days of age. Liver adducts were quantified by LC-ES/MS/MS as per Gamboa da Costa et al. (2003)

Major Findings:

Liver DNA from untreated male and female mice that had been maintained since weaning on the non-autoclaved NIH-31-IR diet was found to contain 1.0 ± 0.2 N7-GA-Gua adducts per 10^8 nucleotides. This was ~7-fold lower than mice consuming autoclaved diet. No N3-GA-Ade adducts were detected. Acrylamide levels were determined to be 17 and 240 µg/kg for non autoclaved irradiated (NIH-31IR) and autoclaved (NIH-31) rat diets.

Additional Comments:

This study demonstrates that a heat sterilisation procedure can lead to the formation of significant levels of acrylamide. Additional toxicokinetic analysis showed the acrylamide is bioavailable, distributed to tissues, and is metabolically activated to a genotoxic metabolite. This seems to produce quantifiable cumulative DNA damage.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua)

Glycidamide Positive (N7-GA-Gua)

DOERGE2005a: Doerge DR; Young JF; McDaniel LP; Twaddle NC; Churchwell MI; (2005) Toxicokinetics of acrylamide and glycidamide in B6C3F1 mice, *Toxicol Appl Pharmacol* 202(3):258-267

Methods:

Male and female B6C3F1 mice were administered acrylamide (0.1 mg/kg, Sigma, purity >99.9%) i.v., by oral gavage, or in the diet. Equimolar doses of glycidamide (0.12 mg/kg; Toronto Research Chemicals, Ontario; purity 99%, 1% acrylamide) were administered i.v. and by oral gavage. Animals were sacrificed for the toxicokinetics study at various time-points. Three animals were used per sex, time-point and dose route. At 8 h, livers were collected from mice dosed by gavage, and adducts were quantified by LC-ES/MS/MS as per Gamboa da Costa et al. (2003). Data was analysed using Student's two tailed t-test. Erythrocytes were also collected for Hb adduct analysis.

Major Findings:

Quantifiable levels of N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) adducts were seen in untreated animals. Acrylamide and glycidamide administration resulted in a significant increase in N7-GA-Gua adducts compared to untreated controls (4 and 6 fold, respectively; $p < 0.0001$). Glycidamide adduct levels were significantly higher than the equimolar dose of acrylamide (1.5-fold; $p < 0.01$). No significant differences were observed between male and female mice, so the data was combined.

Additional Comments:

Acrylamide and glycidamide were both rapidly absorbed from oral dosing, were widely distributed to tissues, and acrylamide was efficiently converted to glycidamide. Oral and dietary administration attenuated acrylamide bioavailability (23% and 32-52%, respectively). A marked increase in relative formation of glycidamide was reported for both oral routes of administration. The authors interpreted this as indicating first pass metabolism to glycidamide. When compared to predicted concentrations based on linear extrapolation from previous studies (Gamboa da Costa et al. 2003) at 500-fold higher concentrations; the AUC for acrylamide was 47% of expected and glycidamide was 130%. The authors suggest this indicates bioactivation is more efficient at lower doses.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua) **Glycidamide Positive (N7-GA-Gua)**

DOERGE2005b: Doerge DR; Young JF; McDaniel LP; Twaddle NC; Churchwell MI; (2005) Toxicokinetics of acrylamide and glycidamide in Fischer 344 rats, Toxicol Appl Pharmacol 208(3):199-209

Methods:

Male and female F344 rats were administered acrylamide (0.1 mg/kg, Sigma, purity >99.9%) i.v., by oral gavage, or in the diet. Equimolar doses of glycidamide (0.12 mg/kg; Toronto Research Chemicals, Ontario; purity 98.5%, 1% acrylamide) were administered i.v. and by oral gavage. Animals were sacrificed for the toxicokinetics study at various time-points. Three animals were used per sex, time-point and dose route. At 10 h, livers were collected from rats dosed by gavage, and adducts were quantified by LC-ES/MS/MS as per Gamboa da Costa et al. (2003). Data was analysed using multivariate ANOVA with Tukey's HSD test for multiple comparisons. Erythrocytes were also collected for Hb adduct analysis.

Major Findings:

Levels of the N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) adduct were below the limit of detection in untreated animals (<1 adduct per 10^8). Acrylamide and glycidamide administration yielded quantifiable levels of N7-GA-Gua adducts, which were proportional to internal exposure to glycidamide (based on the serum glycidamide AUC determined in animals dosed with either acrylamide or glycidamide). This equated to 2.0 ± 0.19 adducts per AUC unit ($\mu\text{M} \times \text{h}$) regardless of sex or chemical. This was comparable to the mouse study (Doerge et al. 2005 Toxicol Appl Pharmacol 202:258-67) which found 2.2 ± 0.5 adducts per AUC unit ($\mu\text{M} \times \text{h}$).

A significant sex difference in adduct levels was observed following acrylamide and glycidamide dosing ($p < 0.05$). In male, but not female rats, adduct levels following glycidamide administration were significantly increased compared to acrylamide administration ($p < 0.05$).

Role of Metabolism:

Levels of N7-GA-Gua adducts were proportional to the internal exposure to glycidamide.

Additional Comments:

Acrylamide was rapidly absorbed following oral dosing and was present in all tissues at 2 and 4 h. The glycidamide metabolite was detectable in all tissues except liver and testes. Similarly, glycidamide was not detectable in the liver and testes of animals dosed directly with glycidamide.

As with the mouse study, oral and dietary administration attenuated acrylamide bioavailability although there were significant ($p < 0.05$) sex differences: 60% (gavage) and 47% (diet) for males; and 98% (gavage) and 28% (diet) for females. Also consistent with the mouse study was the marked increase in relative formation of glycidamide reported for both oral routes of administration. Dietary administration consistently produced the highest ratio of glycidamide to acrylamide AUC.

The authors comment that the 500 fold-reduction in dose (50 to 0.1 mg/kg bw) eliminates significant differences in the formation of DNA adducts between rat and mouse (based on comparison of the three Doerge 2005 papers).

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua; acute & sub-chronic) Glycidamide Positive (N7-GA-Gua and N3-GA-Ade; acute & sub-chronic)

DOERGE2005c: Doerge DR;da Costa GG;McDaniel LP;Churchwell MI;Twaddle NC;Beland FA; (2005) DNA adducts derived from administration of acrylamide and glycidamide to mice and rats, Mutat Res 580(1-2):131-141

Methods:

Adult male and female B6C3F1 mice and F344 rats were administered acrylamide (Sigma, purity >99.9%) or equimolar concentrations of glycidamide (Toronto Research Chemicals, Ontario; purity 99%, 1% acrylamide). For DNA adduct analysis animals (n=3 to 4) were administered aqueous acrylamide (50 mg/kg bw) or equimolar dose of glycidamide (61 mg/kg bw). Mice dosed by oral gavage were sacrificed at 0, 0.5, 1, 2, 4 and 8 h. Rats and mice dosed i.p. were sacrificed after 6 h. For DNA adduct accumulation studies, rats (n=4) and mice (n=8) that had been fed low acrylamide (NIH-311R) diet since weaning, were administered acrylamide (0.14 mM, equivalent to ~1 mg/kg bw) in drinking water for 28 days.

DNA was prepared from whole tissue homogenates using a Qiagen Blood and Cell Culture Maxi kit with a modified protocol to reduce spontaneous thermal hydrolysis of labile adducts. Adducts were quantified by LC-ES/MS/MS as per Gamboa da Costa et al. (2003). The LOD for N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) was 1 adduct in 10⁸ nucleotides and N3-(2-carbamoyl-2-hydroxyethyl)adenine (N3-GA-Ade) was 1.5 adducts in 10⁸ nucleotides. LOQ was approximately 0.5 adducts in 10⁸ nucleotides for both. Statistical analysis by ANOVA with log transformation of data where necessary. Pair-wise comparisons by Student-Newman-Keuls.

Major Findings:

Following gavage dosing with Acrylamide (50 mg/kg bw), maximal serum levels were found at 0.5 h (450 µM) for acrylamide and 2 h (190 µM) for glycidamide. Acrylamide was eliminated (0.94 h⁻¹) at a similar rate to glycidamide formation (0.91 h⁻¹). Adducts accumulated throughout the time-course with a similar rate constant (0.36 h⁻¹) to glycidamide elimination (0.37 h⁻¹).

In mice, single i.p. administration of acrylamide (50 mg/kg bw) or equimolar glycidamide resulted in N7-GA-Gua and N3-GA-Ade adducts in all tissues (liver, lung, testes, kidney and leukocytes), N7 adduct levels being approximately 75-fold lower than N3 (consistent with in vitro result of Gamboa da Costa, 2003). Sex differences were observed in certain organs following acrylamide dosing (liver M>F, lung M>F and kidney F>M), but only in the liver (F>M) following dosing with glycidamide. Glycidamide dosing resulted in significantly (p<0.05) higher adduct levels in most tissues (up to 1.5-fold). In rats, both adducts were detected in all tissues (liver, brain, thyroid leukocytes and mammary gland or testes) following similar i.p. administration. Acrylamide dosing resulted in generally lower levels and larger differences in adduct levels between tissues, compared to mice. Compared to acrylamide, glycidamide dosing resulted in significantly (p<0.05) higher adduct levels in most tissues (up to 5.6-fold). There were no significant sex differences except for thyroid (F>M) in glycidamide dosed rats.

In mice, repeated administration (~1 mg/kg bw/day, drinking water) resulted in an accumulation of N7-GA-Gua adducts in the livers, reaching a steady state level (3-400 adducts per 10⁸ nucleotides) after 14 days. N3-GA-Ade adducts were only detectable at ~42 days. No sex differences were observed, so data sets were combined. In rats, steady state levels N7-GA-Gua levels were also reached after 14 days in female rats. Adduct levels were consistently lower in male rats and slowly declined from the maximum on day 14. N3-GA-Ade adducts were not detected in rat livers. There were no significant variations in serum levels over time for either acrylamide or glycidamide. Average acrylamide concentrations were 0.58 ± 0.07 and 0.57 ± 0.30 µM for males and females respectively. Glycidamide concentrations were 0.48 ± 0.07 and 0.65 ± 0.10 µM, respectively.

Role of Metabolism:

Liver adducts accumulated with a similar rate constant to glycidamide elimination.

Additional Comments:

The authors comment that differences in adduct level alone cannot account for tissue specificity of tumours in the rat carcinogenicity studies. N1-GA-dA adducts could not be analysed due to methodological limitations, although the authors note that N1 adducts have the potential to cause miscoding.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua)

FARMER2005: Farmer PB;Brown K;Tompkins E;Emms VL;Jones DJ;Singh R;Phillips DH; (2005) DNA adducts: Mass spectrometry methods and future prospects, Toxicol Appl Pharmacol 207(2 Suppl):293-301

Methods:

Acrylamide (purity not stated) was administered to male BALB/c by oral gavage (0, 1, 100 and 2000 µg/kg). Solid phase extraction and LC-MS/MS was used to detect the N7-(2-carbamoyl-2-hydroxyethyl)guanine (N7-GA-Gua) adduct of glycidamide in urine.

Major Findings:

A dose-dependent increase in the detected level of N7-GA-Gua adduct was observed. The adduct was also present in the urine of the control animals

Role of Metabolism:

Glycidamide adduct detected following administration of acrylamide.

Acrylamide Positive (CYP2E1+/+; N7-GA-Gua and N3-GA-Ade)

Acrylamide Positive (CYP2E1-/-; N7-GA-Gua)

GHANAYEM2005: Ghanayem BI;McDaniel LP;Churchwell MI;Twaddle NC;Snyder R;Fennell TR;Doerge DR; (2005) Role of CYP2E1 in the epoxidation of acrylamide to glycidamide and formation of DNA and hemoglobin adducts, Toxicol Sci 88(2):311-318

Methods:

Acrylamide (50 mg/kg; Sigma, purity >99.5%) was administered i.p. in saline to CYP2E1 null or wild type mice (129Sv crossed with C57BL/6N2). Mice were sacrificed 6 h post administration, and blood and tissues were collected. Liver, lung and kidney adducts were quantified by LC-ES/MS/MS as per Gamboa da Costa et al. (2003). Haemoglobin (Hb) adducts and plasma levels were also determined by LC-ES/MS/MS. Group mean comparisons were made using Student's t-test, two tailed assuming equal variances.

Major Findings:

Low levels of N7-(2-carbamoyl-2-hydroxy-ethyl)guanine (N7-GA-Gua) adducts were detected in liver, testes and lung of untreated wild type, but not null mice. Administration of acrylamide caused a significant ($p < 0.05$) increase in both N7-GA-Gua and N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade) adducts in wild type mice. N7-GA-Gua adducts were also detectable in tissues from null mice, but at significantly lower levels (52 to 66-fold; $p < 0.05$). N3-GA-Ade adducts occurred at levels ~100-fold lower than N7-GA-Gua in treated wild type mice and were below the limit of detection in treated null mice and untreated controls.

Role of Metabolism:

The authors comment that the low levels of glycidamide adducts in CYP2E1 null mice indicates other pathways may be involved in formation of glycidamide in vivo, but that these pathways contribute less than 2% of the total GA DNA adducts.

Additional Comments:

At 6 h post dosing, plasma acrylamide levels in wild type mice were significantly (137-fold; $p < 0.05$) lower than null mice. Glycidamide levels were significantly (19-fold; $p < 0.05$) higher in wild type mice than null mice. Acrylamide treatment significantly increased glycidamide and acrylamide Hb adducts in wild type mice. The level of acrylamide Hb adducts in null mice was approximately twice that of wild type mice, whilst glycidamide adducts were 33-fold lower than wild-type mice.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua and N3-GA-Ade)

MANIERE2005: Maniere I; Godard T; Doerge DR; Churchwell MI; Guffroy M; Laurentie M; Poul JM; (2005) DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide, Mutat Res 580(1-2):119-129

Methods:

Acrylamide (Sigma; purity >99%). Groups of four male Sprague-Dawley rats were dosed orally with either 18 mg/kg bw or 54 mg/kg bw in distilled water and tissue samples collected (brain, liver and testes; at 5, 24, 48, and 72 h) and blood (for plasma) at 5, 24, and 48 h. DNA was extracted purified and adducts quantified (N7-GA-Gua, and N3-GA-Ade) using an isotope dilution LC-ES/MS/MS approach (as given in detail in Gamboa da Costa Chem Res Tox, 16, 1328-1337, 2003). Statistical approach used ANOVA and depending on distribution Student-newman Keul procedure.

Major Findings:

N3-GA-Ade was formed at approximately 50-100 fold less than N7-GA-Gua. At 54 mg/kg bw similar levels of N7-GA-Gua were formed in brain and liver with lower levels in testes. Peak levels were reported in all tissues at 5 to 24 h slightly decreasing to 72 h. DNA adducts disappeared slowly from rat tissues. At 18 mg/kg bw similar DNA adduct was reported except that levels were approximately two fold lower. The $t_{1/2}$ for N7-GA-Gua was 50-70 h in liver, testes and brain at 54 mg/kg bw and 50-680 h at 18 mg/kg bw. The N3-GA-Ade DNA adduct was removed faster with a $t_{1/2}$ at 54 mg/kg bw in tissues studies of 20 h and at 18 mg/kg bw a $t_{1/2}$ of 20-30 h.

Additional Comments:

The authors suggested that glycidamide was formed at a proportionately higher level at the lower dose in this study. The in vivo decline in DNA adducts formed from acrylamide was consistent with in vitro stability data on the DNA adducts investigated.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Adducts

Acrylamide Positive (N7-GA-Gua; rapid repair)

TAREKE2006: Tareke E; Twaddle NC; McDaniel LP; Churchwell MI; Young JF; Doerge DR; (2006) Relationships between biomarkers of exposure and toxicokinetics in Fischer 344 rats and B6C3F1 mice administered single doses of acrylamide and glycidamide and multiple doses of acrylamide, *Toxicol Appl Pharmacol* 217(1):63-75

Methods:

Adult male and female B6C3F1 mice and F344 rats that had been fed low acrylamide (NIH-311R) diet since weaning, were administered acrylamide (10 µg/ml; equivalent to 2.25 ± 0.37 , 0.71 ± 0.056 , and 1.10 ± 0.15 mg/kg bw day in mice, and female and male rats respectively; Sigma, purity >99.9%) in drinking water for 21 days, then returned to control water. Erythrocytes (rats only), serum and livers were collected at several time-points up to 42 (mice) or 63 days (rats). Adducts were quantified by isotope dilution LC-ES/MS/MS as per Gamboa da Costa et al. (2003). Statistical analysis used linear regression and 2-tailed t-test.

Major Findings:

Liver N7-GA-Gua adduct removal times were faster in mice (half-time 2.6 days), than female (4.1) and male (4.5) rats. Although the adduct levels were similar in rat liver and leukocytes, DNA adducts in the latter were removed more slowly (6.7 and 7.4 days for females and males, respectively)

Role of Metabolism:

Levels of N7-GA-Gua and haemoglobin adducts were proportional to the internal exposure to glycidamide.

Additional Comments:

The authors note that in vivo adduct removal times were slower than observed in vitro (1.8 days) by Gamboa da Costa et al. (2003), but also slower than Manière et al. (2005), who reported 50 to 80 h in rats gavaged with acrylamide at 18 and 54 mg/kg bw. The authors suggest that rapid hydrolytic depurination of N7-GA-Gua occurs in vivo as well as in vitro, and that no enzymatic repair processes are apparently involved.

This study was conducted to compare DNA and haemoglobin glycidamide adduct levels with AUC for acrylamide and glycidamide. In analysing the data for both rats and mice administered acrylamide in drinking water; both N7-GA-Gua ($r^2=0.997$, $p<0.002$) and haemoglobin-glycidamide ($r^2=0.99$, $p<0.006$) adducts were significantly correlated with glycidamide AUC (but not acrylamide AUC). These adducts were also correlated with each other ($r^2=0.99$, $p<0.001$), which suggests haemoglobin adducts are a readily measured biomarker for liver N7-GA-Gua adduct levels. By taking published haemoglobin adduct levels (17 to 29 fmol/mg globin), the empirical relationship predicts corresponding steady state N7-GA-Gua levels in the range 0.2 to 0.3 adducts per 10^8 nucleotides. An alternative approach using allometric scaling predicts a level of 0.06 adducts per 10^8 nucleotides in a non-smoking human.

Acrylamide Positive (N7-GA-Gua)

FENNELL2007: Fennell, T.R., Snyder, R.W. and Friedman, M.A. (2007) Unpublished results, (Abstract Only)

Methods:

Male Fisher rats were administered triply labelled ^{13}C Acrylamide (50 mg/kg, oral gavage, no purity data). After 24 hours, DNA was isolated from liver, lung, kidney, brain, spleen, lung, white blood cells and thyroid, and the abundance of labelled N7-GA-Gua was determined by LC-MS/MS.

Major Findings:

Similar levels of the N7-GA-Gua adduct were found in all tissues examined, except for the testes, which showed significantly fewer N7-GA-Gua adducts.

Additional Comments:

Pre publication abstract only

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation

Acrylamide Negative

KREBS1997: Krebs O; Favor J; (1997) Somatic and germ cell mutagenesis in lambda lacZ transgenic mice treated with acrylamide or ethylnitrosourea, Mutat Res 388(2-3):239-248

Methods:

Acrylamide (50 or 100 mg/kg bw, Sigma-Aldrich) was administered i.p in distilled water to groups of 3-5 animals. Animals were killed at 3, 10 and 100 days post dose and liver and testes (including epididymis and vas deferens) isolated and frozen (-80oC). Genomic DNA was extracted from liver (homogenisation, proteinase k treatment, chloroform/phenol extraction). Genomic DNA was extracted from germ cells using similar procedures (involving SDS/mercaptoethanol and proteinase K treatment and chloroform/phenol extraction) except that four methods of preparing spermatozoa were undertaken including vortex, hypotonic lysis, sonication (in sucrose solution) and homogenisation (with column separation of DNA instead of solvent extraction). DNA was packaged using a commercial kit (Gigapack II Gold Stratgene) in a final volume of 500µl. Phage titre was determined in 10µl by addition to E coli in presence of maltose for 30 min and subsequent culturing on LB agar plates. For mutant selection 490µl phage was incubated with E. coli and then cultured on LB agar plates in the presence of phenyl-β-D-galactopyranoside. The mutation frequency data was analysed using Fisher's exact test.

The transgenic mouse strain 40.6 was developed at TNO, The commercial Muta™Mouse was derived from Hazelton Research Product Inc (Denver Pennsylvania), and E coli lac- galE- Kan' (gal E- AMpr) was supplied by Gossen and Vijg (Ingenvy BV Leide, The Netherlands). Ethylnitrosourea used as positive control, i.p. at 80/160 mg/kg bw.

Major Findings:

A slight increase in mutation frequency was reported for the liver at all doses and time points, although no increase achieved statistical significance. It was reported that the increase was more prominent at 50 mg/ kg bw compared to 100 mg/kg bw. It was reported that the increases tended to be greater at the earlier sampling time points. There were two animals which displayed 'Jackpot' mutations (one in acrylamide group and one in the ethylnitrosourea group).

Additional Comments:

Ethylnitrosourea gave a positive response (between 2—7 fold increase in mutation frequency) at both dose levels and at all time points in the liver with evidence for an increase in response with duration after dosing. Initial experiments using germ cell DNA showed that vortexing of spermatozoa was an inefficient method for isolating genomic DNA. The authors reported that no meaningful data could be derived for germ cell investigations as the extraction methods were either inefficient or degraded the DNA or had both effects on the isolation of germ cell DNA.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation

Acrylamide Positive Glycidamide Positive

MANJANATHA2006: Manjanatha MG;Aidoo A;Shelton SD;Bishop ME;McDaniel LP;Lyn-Cook LE;Doerge DR; (2006) Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice, *Environ Mol Mutagen* 47(1):6-17

Methods:

Acrylamide (Sigma, >99.9% purity) and Glycidamide (Toronto Research, Ontario; >98.5% purity, approx 1% acrylamide). Purity was confirmed by GC-FID and LC-ES/MS. Male and female Big Blue mice (Taconic Farms, Germantown NY) were obtained as weanlings. Diet used was NIH 31R (selected for low acrylamide content, ca 10 ppb). Acrylamide was administered via the drinking water at 100 and 500 mg/l. Equimolar amounts of glycidamide were also used (120 mg/l and 600 mg/l). Animals were treated for 7 d per week for up to 4 weeks. The test materials were stable in water for up to a week (solutions changed weekly). Animals were sacrificed at termination and liver tissue snap frozen. It was noted that high dose acrylamide animals developed hind leg paralysis and sluggish movement and treatment was halted after 3 weeks. These animals were reported to recover and were included in the mutation analyses. Spleen samples were taken for lymphocyte hprt assay.

For the hprt assay. Cloning efficiency of non selected cells was measured following incubation of 8 cells/well for 2 days. Cloning efficiency of 6-thioguanine selected cells was measured following addition of 4 x 10⁴ (4) cells/well and incubation for 10 days. The mutation frequency was determined as ratio of cloning efficiency in selection cultures compared to non selective cultures. Data were analysed by one way ANOVA followed by Holm-Sidak test to evaluate mutation frequencies. A logarithmic transformation was performed before conducting the analyses

Major Findings:

The authors noted that high dose levels of acrylamide and glycidamide were associated with lower water intakes compared to low dose and control animals. The average daily doses were between 19-25 mg/kg bw/day acrylamide or glycidamide in the low dose and 88-111 mg/kg bw/day for the high dose acrylamide and glycidamide animals. No effect on body weight was reported. Mice treated with low dose levels of acrylamide had a mean increase of three fold in hprt mutation frequency and animals treated with the low dose of glycidamide had a mean increase in mutation frequency of eight fold (increases significant at P<0.05 in sex matched groups) . At the dose levels the increases in mutation frequency were 16 and 25 fold respectively for acrylamide and glycidamide (increases significant at P<0.01 in sex matched groups).

Additional Comments:

Overall the authors report a dose-related increase in mutation frequency. They note the low spontaneous mutation frequency for hprt in lymphocytes (and size of hprt gene compared to cII) might have aided the identification of a treatment related effect at the low dose level. They note other investigators (Blasiak J et al *Chem Biol Interact* 149, 137-149, 2004) have reported CYP2E1 activity in lymphocytes.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation

Acrylamide Positive Glycidamide Positive

MANJANATHA2006: Manjanatha MG;Aidoo A;Shelton SD;Bishop ME;McDaniel LP;Lyn-Cook LE;Doerge DR; (2006) Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice, *Environ Mol Mutagen* 47(1):6-17

Methods:

Acrylamide (Sigma, >99.9% purity) and Glycidamide (Toronto Research, Ontario; >98.5% purity, approx 1% acrylamide). Purity was confirmed by GC-FID and LC-ES/MS. Male and female Big Blue mice (Taconic Farms, Germantown NY) were obtained as weanlings. Diet used was NIH 31IR (selected for low acrylamide content, ca 10 ppb). Acrylamide was administered via the drinking water at 100 and 500 mg/l. Equimolar amounts of glycidamide were also used (120 mg/l and 600 mg/l). Animals were treated for 7 d per week for up to 4 weeks. The test materials were stable in water for up to a week (solutions changed weekly). Animals were sacrificed at termination and liver tissue snap frozen. It was noted that high dose acrylamide animals developed hind leg paralysis and sluggish movement and treatment was halted after 3 weeks. These animals were reported to recover and were included in the mutation analyses. Spleen samples were taken for lymphocyte hprt assay.

For the liver cll mutation assay; Genomic DNA was extracted, packaged, plated and mutation frequency determined using lambda Select-cll Mutation Detection system for BB rodents using the Strategene protocol. The lambda shuttle vectors were rescued using Transpack packaging extract E.coli host strain G1250. Selection of mutants was achieved by plating at 37oC (non selective) and 24oC (selective) conditions. Mutation frequency was determined by number of plaques identified under selective compared to non selective conditions. DNA from mutant plaques was amplified using PCR using primers for cll gene. Purified cll DNA was sequenced for the cll gene. Alterations in sequence were verified at least once. Data were analysed by one way ANOVA followed by Holm-Sidak test to evaluate mutation frequencies. A logarithmic transformation was performed prior to conducting analyses. Chi squared and the approach used by Cariello (*Carcinogenesis*, 15, 2281-2285, 1994) was used to analyse mutation profiles.

Major Findings:

There were no statistically significant increase in the cll mutation frequency at the low dose levels for acrylamide or glycidamide. A slight but not statistically significant increase was reported in female mice (cf 33.5±6.2 x 10(6) cf 26.5±4.5 x10(6) in female mice control group). There was a statistically significant (P<0.05) increase in cll mutation frequency in the high dose acrylamide and glycidamide groups (= 2 fold compared to sex matched controls). cll sequence data from 52 independent mutations in controls, 57 acrylamide and 71 glycidamide treated mice were analysed. There was no difference between sexes and thus data were combined. Simple bp substitutions comprised most of the mutations recovered.

Substitutions at G:C accounted for 69% of control mutations, with 12% A:T. Most frequent was G:C to A:T (25/52, 48%) with 80% of these in CpG sites. In addition 19% (10/52) were deletions or insertions leading to frameshifts. For acrylamide 37/57 (65%) were base pair substitutions, and 20 (35%) were frameshift mutations. 27/37 of the base pair mutations occurred at G:C while 10/37 (27% were A:T. The predominant base pair substitution G:C to T:A transversion (25%, 14/57 followed by G:C to A:T (10%, 6/57) and A:T to G:C (7%, 4/57) transitions. The acrylamide mutation spectrum was significantly different from the cll control spectrum (P=0.0002).

Of the 71 independent glycidamide mutations 64% were single base pair mutations and 25 (35%) were frameshift mutations. Of the 46 single base pair substitutions 27 (59%) were at G:C while 19 (41%) were at A:T. The predominant base pair substitution was G:C to T:A transversion (27%, 19/71). The glycidamide spectrum also included a few A:T to T:A (10%, 7/71) and A:T to C:G (8% 6/71) transversions. The glycidamide mutation spectrum was significantly different from cll control spectrum (P+0.0001).

There was no difference between the acrylamide and glycidamide mutation spectra.

Mechanistic Data:

The authors note G to T mutations were consistent with N7-dG-GA adduct formation and that A to G transitions, A to C and A to T transversions (all in range 5-10%) might be associated with N1 and N3 dA adducts. The authors noted the frameshift mutations were not reported in the in vitro studies with acrylamide and glycidamide (citing Besaratinia 2003, 2004). The authors noted the suggestion that slippage by DNA polymerase at nucleotide repeats enhanced by bulky adducts is a possible mechanism for frameshift mutations.

Role of Metabolism:

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation

The authors suggest that the similar mutation spectrum for acrylamide and glycidamide is consistent with metabolism to glycidamide. They noted that the data were consistent with either efficient metabolism of acrylamide by CYP2E1 or the route of administration reduced the effective dose of glycidamide in the liver. The authors note this would be consistent with DNA adduct data on glycidamide in the liver of mice. The authors also suggest the finding of a similar amount of frameshift mutations with acrylamide and glycidamide also supports the metabolism of acrylamide to glycidamide and N&-dG-GA adducts.

Additional Comments:

The authors attributed the lack of a statistically significant increase in mutation frequency at the low dose to the high spontaneous mutation frequency in the *cll* gene and speculated that a longer sampling time might have resulted in a significant elevation in mutation frequency at the lower dose. The authors noted that no frameshift mutations were found in homopolymeric run of dAs in the *cll* gene at bp 241-246 which would be attributable to N3 dA-GA. It was speculated that either these adducts were not formed in sufficient quantities or polymerase slippage does not occur in the *cll* gene.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Chromosomal Aberrations

Acrylamide Positive

KLIGERMAN1991: Kligerman AD;Atwater AL;Bryant MF;Erexson GL;Kwanyuen P;Dearfield KL; (1991) Cytogenetic studies of ethyl acrylate using C57BL/6 mice, *Mutagenesis* 6(2):137-141

Methods:

12 week old male C57BL/6 mice (n=5) were injected i.p. with acrylamide (100 mg/kg, Sigma) in saline as positive control for study of ethyl acrylate. Spleen removed under anaesthesia 24 hr post exposure. Cells were concentrated and washed and 1x10⁶ cells added to 2ml culture medium containing 2µg/ml concavalin A. After 21hr washed and added to medium containing 5 µM 5-bromo-2'-deoxyuridine. Harvested at 52hr after 0.5 µg/mol 3h colcemid treatment. Slides prepared and stained by fluorescence-plus-Giemsa. 1st div cells analysed for CAs. 2nd div cells analysed for SCEs. Per animal 50 second division and 50 first division metaphases were scored

Major Findings:

No significant changes in no. of cells with high frequencies of SCEs.

No change in % first division metaphases

Acrylamide Negative

KRISHNA1995: Krishna G;Theiss JC; (1995) Concurrent analysis of cytogenetic damage in vivo: a multiple endpoint-multiple tissue approach, *Environ Mol Mutagen* 25(4):314-320

Methods:

Very limited details of methods provided. Author refers to previous publications dating from 1987-1994. The approach used was to develop a multi-tissue (bone marrow/spleen) multi-end point (chromosome aberrations/MN formation) assessment of clastogenicity. Acrylamide was one test chemical used along with cyclophosphamide. A single MTD dose of 100 mg/kg bw was administered to rats (route not given).

Major Findings:

No evidence of Chromosome aberrations in bone marrow or spleen in rats.

Additional Comments:

Data for studies in mice not reported in this publication.

Acrylamide Positive

GASSNER1996: Gassner P;Adler ID; (1996) Induction of hypoploidy and cell cycle delay by acrylamide in somatic and germinal cells of male mice, *Mutat Res* 367(4):195-202

Methods:

(10/E1 x C3H/E1)F1 mice (n=4) were administered acrylamide (80 and 120 mg/kg bw, Sigma, no purity data) i.p. in saline. Cell proliferation was measured by BrdU incorporation (administered as an s.c. tablet 2 h before acrylamide dosing). Colchicine (3 mg/kg bw) was administered i.p. 1 h prior to termination at 18 h. Bone marrow samples were taken and smears prepared. The number of cell cycles was evaluated using FPG staining and average generation time determined. One hundred second metaphase cells were scored for numerical chromosome changes. Data analysed by Student's t-test and Mann-Whitney u-test.

Major Findings:

There was evidence for a cell cycle delay at 120 mg/kg bw (Average Generation Time 14.2 h compared to 12.3 h in controls). A statistically significant (p<0.01) increase in hypoploidy was reported at the high dose level (12.4% cf 6.5 % in controls). A slight but not significant increase in hypoploidy was reported at 80 mg/kg bw/ The authors reported the NOEL for effects on cell proliferation was between 80-120 mg/kg bw.

Additional Comments:

The authors note the possibility of artefactual formation of hypoploidy chromosomes using this approach and that there was no change in the rate of hyperploidy.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Chromosomal Aberrations

Acrylamide Positive

NESTEROVA1999: Nesterova EV;Durnev AD;Seredenin SB; (1999) Verapamil contributes to the clastogenic effects of acrylamide, cyclophosphamide, and dioxidine on somatic cells of BALB/C and C57BL/6 mice, Mutat Res 440(2):171-179

Methods:

Acrylamide (Sigma) was administered by i.p. dosing in saline water to groups of BALB/c or C57Bl/6 mice (sex not reported group sizes ca 5-14) at 100 mg/kg bw or 5 daily doses of 50 mg/kg bw. Bone marrow samples were obtained 24 h post single dosing or 6h after the last of the repeated daily doses. Bone marrow smears were prepared and chromosome aberrations analysed. (Data recorded as chromosome/chromatid breaks and exchanges. Percent cells with aberrations reported). 100 metaphases/animal examined. Data were analysed using Statistica for Windows 95 software.

Major Findings:

A statistically significant increase in percent cells with aberrations was reported using both dosing schedules in both strains of mouse used.

Additional Comments:

Verapamil (calcium blocking agent) could increase response in acrylamide treated animals depending on dose of verapamil (= 2.5 mg/kg bw) and schedule used (effective using gavage or i.p dosing).

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive

KLIGERMAN1991: Kligerman AD;Atwater AL;Bryant MF;Erexson GL;Kwanyuen P;Dearfield KL; (1991) Cytogenetic studies of ethyl acrylate using C57BL/6 mice, *Mutagenesis* 6(2):137-141

Methods:

12 week old male C57BL/6 mice (n=5) were injected i.p. with acrylamide (100 mg/kg, Sigma) in saline as positive control for study of ethyl acrylate. Spleen removed after 24hr and splenocytes isolated on density gradient removing erythrocytes. Given concavalin A treatment then replaced with medium containing 3µg/ml cytochalasin B. Cultures harvested at 54 hr and slides prepared using a cytocentrifuge. 500 binucleated splenocytes were scored. Replicative index (RI) calculated from 100 consecutively scored metaphases. Data compared to concurrent control using one tailed (SCE CA MN) or two tailed (RI) Students t-tests.

Major Findings:

Acrylamide caused significant ($p < 0.05$) increase in MN frequency in binucleated splenocytes

Acrylamide Negative

KRISHNA1995: Krishna G;Theiss JC; (1995) Concurrent analysis of cytogenetic damage in vivo: a multiple endpoint-multiple tissue approach, *Environ Mol Mutagen* 25(4):314-320

Methods:

Very limited details of methods provided. Author refers to previous publications dating from 1987-1994. The approach used was to develop a multi-tissue (bone marrow/spleen) multi-end point (chromosome aberrations/MN formation) assessment of clastogenicity. Acrylamide was one test chemical used along with cyclophosphamide. A single MTD dose of 100 mg/kg bw was administered to rats (route not given). A column separation method was used to separate nucleated cells from bone marrow and spleen.

Major Findings:

No increase in micronuclei reported for either bone marrow or spleen.

Additional Comments:

Positive results with method reported for cyclophosphamide. Data for studies in mice not reported in this publication.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive (Clastogenic & Aneugenic)

SCHRIEVERSCHWEM1997: Schriever-Schwemmer G;Kliesch U;Adler ID; (1997) Extruded micronuclei induced by colchicine or acrylamide contain mostly lagging chromosomes identified in paintbrush smears by minor and major mouse DNA probes, Mutagenesis 12(4):201-207

Methods:

Acrylamide (Sigma) was administered by i.p. dosing in distilled water to a group of 3 male (IO2/E1xC3H/E1)F1 at 125 mg/kg bw. Bone marrow samples were obtained using a paintbrushing technique 24 h post dose and directly smeared onto slides. 5-6 slides/animal were prepared. The objective of the study was to investigate MN extrusion. (Colchicine 1mg/kg bw i.p was used as second experimental compound in this study.) FISH was undertaken using biotinylated probes (murine ? satellite DNA). Murine minor satellite DNA probe pMKB6 was linked with either biotin or digoxigenin. Hybridisation procedure (after fixation and denaturation) was undertaken overnight at 37°C under moist conditions. Biotin was detected using Cy3 streptavidin (in some experiments amplification using biotinylated anti streptavidin). FITC was used to visualise digoxigenin DNA probes. (Enhancement used FITC rabbit anti streptavidin). Counter staining used DAPI. MN was assessed in PCEs including position and evidence for extrusion. 1000 PCEs were scored/animal. 100 MN were used in signal detection analysis. Apoptotic cells were excluded. Chi square test was used in data analysis.

Major Findings:

In solvent controls there was 1/1000 MNPCEs and using May-Grunwald-Giemsa staining there was no evidence of MN extrusion. For acrylamide there were 4.7 MNPCEs/1000 PCEs, of which 1.3/1000 appeared to be extruded (i.e 22% were undergoing process of expulsion with MN in contact with cell membrane). Authors noted this did not affect the determination of a positive result. In single labelled FISH studies with acrylamide 17 MN/1000 PCEs contained whole chromosomes (3x control) indicating aneugenic potential. MNPCEs only contributed minimally to the total. In studies using double labels (i.e minor and major satellite DNA probes) there was a reduction in the number of double labelled MN in acrylamide compared to control (cf 28.3% cf 46.7% in controls). Thus acrylamide MN had 2.5 x more acentric fragments than controls. The authors noted the overall rate of MN formation with acrylamide was greater than 3x compared to controls indicating both aneugenic and clastogenic activity. The number of extruded MN using dual labels was 4% in control (all had major and minor signals) and 8.3% in acrylamide treated animals (where 28% had no signal and 72% had major and minor signals). Thus in controls all extruded MN contained lagging chromosomes, whereas in acrylamide a proportion (28%) contained acentric fragments.

Mechanistic Data:

Evidence for multiple genotoxic actions in vivo including clastogenicity and aneugenicity.

Additional Comments:

For colchicine, less MN were reported to be extruded compared to acrylamide using Giemsa staining and approximately the same using FISH staining. Most retained MN with colchicine (76%) contained whole chromosomes. The authors note that extrusion of Mn may be a common phenomena.

Acrylamide Negative

DOBZYNSKA2000: Dobzynska MM;Gajewski AK; (2000) Induction of micronuclei in bone marrow and sperm head abnormalities after combined exposure of mice to low doses of X-rays and acrylamide, Teratog Carcinog Mutagen 20(3):133-140

Methods:

Acrylamide (Bio-Rad, purity >99%). Groups of 5-6 Pzh:Sfis mice (8-12 weeks sex not given) were dose i.p. with 75 mg/kg bw and bone marrow samples taken at 24, 48 and 72 h post dose. Samples were processed and stained with May-Grunwald-Giemsa. 2000 PCEs/animal were examined for MN formation. The PCE/NCE ratio was determined. Data analysed using Students t-test.

Major Findings:

No evidence of MN formation in bone marrow at any time point.

Additional Comments:

Rationale for dose level not given but study was intended to examine the interaction between acrylamide and x-radiation, hence a non responsive dose level may have been chosen for the acrylamide only trial.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive (mouse) Acrylamide Negative (rat)

PAULSSON2002: Paulsson B;Grawe J;Tornqvist M; (2002) Hemoglobin adducts and micronucleus frequencies in mouse and rat after acrylamide or N-methylolacrylamide treatment, Mutat Res 516(1-2):101-111

Methods:

Acrylamide (Merck; 99% purity) was administered i.p. in saline, to groups (size not given) of male CBA mice (8 wk) or male Sprague-Dawley rats (8 wk). In a pilot study dose levels of 28, 55 or 109 mg/kg bw were used with peripheral blood sampling in mice (at 48 h post dose using retro-orbital sinus sampling under anaesthesia) and bone marrow sampling in rats (at 24 h). In the main study dose levels of 25, 50 or 100 mg/kg bw were used for both species with sampling in mice at 48 h and in rats at 24h and 48h. MN in peripheral blood (mice) was analysed using a flow cytometric procedure (erythrocytes fixed and stained using Hoechst 33343 and thiazole orange) Bone marrow samples were subject to purification to separate erythrocytes which were then analysed using flow cytometry. 80,000 PCEs/sample were analysed. Statistical analysis was undertaken using linear regression.

Major Findings:

There was no evidence for an increase in MN formation in either the pilot or the main study in rats. A linear dose response was reported in mice (1.7%o/mmol/kg bw (1.5-1.9 95% CI)). The coefficient of variation across all the MN assays was 145. There was no evidence for a decrease in PCEs in any test.

Role of Metabolism:

Haemoglobin adduct data suggest that, per unit dose, the mouse is able to generate 3 to 6 times higher levels of epoxide adducts than rat.

Additional Comments:

MMC (1 mg/kg bw with 24 h sampling of bone marrow) gave expected positive response in this study. Haemoglobin adduct data suggest that, per unit dose, the mouse is able to generate 3 to 6 times higher levels of epoxide adducts than rat.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive

ABRAMSSONZETTER2003: Abramsson-Zetterberg L; (2003) The dose-response relationship at very low doses of acrylamide is linear in the flow cytometer-based mouse micronucleus assay, *Mutat Res* 535(2):215-222

Methods:

Acrylamide (Merck, purity >99.9%). Two experiments were conducted. In experiment 1; Groups of two (three at 0 and 75 mg/kgw) CBA male mice were dosed i.p (in PBS) with 0, 2.5, 5.0, 6.5, 8.5, 10.0, 11.5, 13.5, 15.0, 17.5, 20.0, 22.5, 25.0, 30.0, 35.0, 40.0, 45.0, 50.0, 62.5, 75.0, 87.5, or 100 mg/kg bw. In Experiment 2; groups of five mice were dosed i.p (in PBS) at 0, 1,3,6,12,24, or 30 mg/kg bw. Blood was collected from the orbital plexus 42h post dose (determined from published literature as optimum for PCEs in bone marrow and peripheral blood). Four samples in experiment 1 and five samples in experiment 2 each 50 µl were processed. Erythrocytes were separated by centrifugation, fixed (few days at 4oC) and stained (1h at 37oC followed by 4oC overnight) using Hoechst 33342 and thiazole orange. MN PCEs were analysed using flow cytometry using 75,000 PCEs (20,000 for colchicine control 1 mg/kg bw). Data analysis was undertaken using simple regression and two-tailed Student's t-test for statistical significance.

From experiment 1 there were data generally for 280,000 PCEs/animal and from experiment 2 from 350,000 PCEs/animal. In some instances the occurrence of cell doublets in some of the samples limited the total number of PCEs evaluated.

Major Findings:

In experiment 1 linear regression for fMPCE over the range 0-100 mg/kg bw gave an R² factor of 0.87 (slope 1.35×10^2) which is significantly different from 0 (t value 17.4). Partitioning the dose response into regions indicated greater slopes for the lower dose regions. Thus 0-10, 0-15, 0-20 gave slopes of 1.8×10^2 , 2.0×10^2 and 1.5×10^2 respectively. (t values 2.3, 3.6, 4.9). However none of the slopes for the linear regression lines were significantly different from that of the corresponding higher dose region. The authors reported that even a range of 0-6.5 mg/kg bw suggested a significant dose response indicating a rapid increase in dose-response at low dose levels.

In experiment 2 the R² was 0.72 with a slope of 2.3×10^2 which differed significantly from 0 (t value 9.1). Partitioning into regions, ie. 0-6 mg/kg bw indicated a higher slope of 3.0×10^2 . However the slope of the lower dose region was not different from higher regions (6-30, or 12-30 mg/kg bw).

The results as a whole indicated that increase in effect was greater at lower dose regions than higher dose regions. The authors also reported that the mean DNA content of MN formed following acrylamide was comparatively low with respect to controls (contrasting with colchicine where significantly elevated DNA in MN was reported indicating an aneugenic effect).

Additional Comments:

The authors comment that the absence of a threshold in this study along with the tendency for a greater slope at lower dose levels indicated that risk estimation for human exposure to low doses will be difficult. Also, the authors suggest that the low DNA content of the micronuclei indicated an absence of whole chromosomes, i.e. no aneugenic effect of AA

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Glycidamide Positive (mouse) Glycidamide Equivocal (rat)

PAULSSON2003A: Paulsson B;Kotova N;Grawe J;Henderson A;Granath F;Golding B;Tornqvist M; (2003) Induction of micronuclei in mouse and rat by glycidamide, genotoxic metabolite of acrylamide, *Mutat Res* 535(1):15-24

Methods:

Glycidamide (synthesised from acrylamide, purity > 95% by NMR) was administered i.p. (in saline) to groups (group size not given) of male CBA mice (8wk) or male Sprague-Dawley rats (8wk). A pilot study was performed at 0.7 mmol/kg, resulting in significant decreases in PCEs (20% in rats and 40% in mice). For the main study dose levels of 0.18, 0.35 and 0.7 mmol/kg were dosed to mice. In dose levels of 0.7 mmol/kg and 1.4 mmol/kg were studied in rats (one animal received 0.9 mmol/kg due limited test material availability). Peripheral blood sampling in mice (at 48 h post dose from retro-orbital sinus under anaesthesia) and bone marrow sampling in rats (at 24 h) were used for MN evaluation in PCEs. MN in peripheral blood (mice) was analysed using a flow cytometric procedure (erythrocytes fixed and stained using Hoechst 33342 and thiazole orange) Bone marrow samples were subject to purification to separate erythrocytes which were then analysed using flow cytometry. Numbers of cells analysed not stated. Statistical analysis was undertaken using linear regression. Haemoglobin adducts were used as a measure of in vivo dose of glycidamide.

Major Findings:

For mice a linear-quadratic dose response was reported (combined pilot and main study data). In rats, the authors reported a non monotonic dose response when data for 0.7 mmol/kg and 0.9 mmol/kg were combined. (It is noted the data for 0.9 mmol/kg is from one animal). The increase was statistically significant using combined 0.7 and 0.9 mmol/kg data ($P=0.001$) and also with 0.7 mmol/kg data alone ($P=0.02$). There was no increase in MNPCE formation at 1.4 mmol/kg in rats.

The authors reported that test material induced reductions in PCE formation was variable between the pilot and main study for rats with a 40% reduction at 0.7 mmol/kg in the main study (cf 20% at this dose level in the pilot study). There was an approximate 2/3rds reduction in PCEs at 1.4 mmol/kg in the main study. The coefficient of variation across all the MN assays was 14%.

Additional Comments:

In the rat no positive dose-response relationship was obtained, probably due to toxic effects to the bone marrow. Glycidamide induced linear dose-dependent increases in Hb adduct levels in both species.

This study demonstrates that, after treatment with synthetic GA, the MN frequency per unit of the in vivo dose of glycidamide in the mouse is very similar to that obtained in a previous study, where animals were treated with acrylamide, forming glycidamide as a metabolite. The authors considered that this equality in potency of GA, whether its in vivo dose is established by injection of synthetic GA or through metabolism of AA, supports the view that GA is the predominant genotoxic factor in AA exposure.

Acrylamide Negative

ABRAMSSONZETTER2005: Abramsson-Zetterberg L;Wong J;Illback NG; (2005) Acrylamide tissue distribution and genotoxic effects in a common viral infection in mice, *Toxicology* 211(1-2):70-76

Methods:

¹⁴C-radiolabelled acrylamide (purity not given) was administered orally in distilled water to female Balb/c mice. Blood samples were taken by retro orbital sinus sampling 42 h post dose and MN evaluated via flow cytometry. Erythrocytes were purified from whole blood by centrifugation and fixed and stained using Hoechst 33342 and thiazole orange. Approximately 50,000 PCEs were analysed/animal. Group sizes were not given.

Major Findings:

There was no increase in the MN frequency in PCEs.

Additional Comments:

In corresponding studies the effect of Coxsackievirus type B3 infection was reported to alter the distribution of acrylamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive (CYP2E1+/+) Acrylamide Negative (CYP2E1-/-)

GHANAYEM2005A: Ghanayem BI;Witt KL;Kissling GE;Tice RR;Recio L; (2005) Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect, Mutat Res 578(1-2):284-297

Methods:

Groups 12 female CYP2E1 (+/+) wild type or CYP2e1 (-/-) null mice were given 5 daily i.p. doses of acrylamide (25 or 50 mg/kg bw, Fluka, purity >99.5%). Blood samples were taken 24 h after last dose. 20,000 PCEs/mouse were examined for Mn formation by flow cytometry. Kendall rank test used for dose-response. Mann Whitney used for pair-wise comparison. Kruskal-Wallis used for comparison between genotype. Positive result at $P < 0.025$ with adjustment for number of test groups.

Major Findings:

Significant ($p < 0.001$) dose related increases in MN PCEs were reported in wild type mice (the high dose was associated with an increase in %PCE), whereas there was no effect seen in null animals treated with acrylamide.

Role of Metabolism:

Authors considered genotoxic effects of acrylamide due to metabolism to glycidamide.

Additional Comments:

Urethane (100 mg/kg bw, dosed i.p. for 5 days) gave appropriate positive response in both wild type and null mice, although the magnitude of the response was greater in wild type mice. There was also a significantly higher baseline MNPCE frequency in null mice. Authors note baseline level of MN in CYP2e1 null male mice was higher than in females.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive **Glycidamide Positive**

HUSOY2005: Husoy T; Abramsson-Zetterberg L; Olstorn HB; Paulsen JE; Alexander J; (2005) Adenomatous polyposis coli influences micronuclei induction by PhIP and acrylamide in mouse erythrocytes, Mutat Res 580(1-2):111-118

Methods:

Acrylamide (Sigma-Aldrich; purity >99%) and glycidamide (Toronto Research Brisbane, CA, USA; no purity data). Colchicine used as positive control for aneugenicity. Groups of 4-6 Female mice (C57BL/6J wild type or C57BL/6JAp^cMin/+ (Min-mice)) maintained at the Norwegian Institute of Public Health, Oslo, Norway were dosed with 50 mg/kg s.c acrylamide. Blood samples (4/animal) obtained via cardiac puncture at 42 h post dose. In a second experiment groups of female were pre-treated with Ara C (s.c 200 mg/kg bw and hydroxyurea (200 mg/kg bw) 30 min prior to injections (s.c) with acrylamide or glycidamide (each at 50 mg/kg bw). Ara C and hydroxyl urea inhibit DNA ligation and increase the sensitivity of MN assay to DNA strand breaks. Erythrocytes were purified, fixed and stained (Hoechst 33342 and thiazole orange) (Method Craze Cytometry , 13, 750-758, 1992.) Two way ANOVA was used for data analysis.

Major Findings:

Colchicine (1 mg/kg bw s.c.) resulted in a 3.4 fold increase in the frequency of micronucleated polychromatic erythrocytes (fMPCEs) in wild type mice and a 3.9 fold increase in Min-mice (not statistically different). Colchicine reduced the %PCEs by 75% and increased the DNA content of MN by approximately 70%. With regard to acrylamide, dosing resulted in an approximate 2 fold increase in fMPCEs (P<0.001) with no effect on %PCEs or DNA content of MN.

In the second experiment no statistically significant increase in fMPCEs was reported following treatment of wild type or Min-mice either with or without AraC /hydroxyurea pre-treatment with acrylamide compared to the corresponding controls. (Pre-treatment by itself increase fMPCEs significantly compared to untreated controls and also reduced cell proliferation.)

Glycidamide gave more than a two-fold increase in fMPCEs in the absence of pre-treatment (P<0.001). Upon pre-treatment with AraC/hydroxyurea, glycidamide gave a significantly larger increase in fMPCE in Min-mice compared to wild type mice (P=0.008). However the authors considered that the reduction in PCEs analysed in this trial reduced the reliability of the results.

Mechanistic Data:

It is difficult to derive any conclusions on type of DNA damage induced by acrylamide since the second experiment which investigated the effect of DNA repair inhibition gave negative results with acrylamide (either in presence of absence of DNA repair inhibition). The authors considered that there was no biologically significant difference between wild type and Min-mice with regard to MN formation in animals dosed with glycidamide supporting the conclusion that small DNA adducts formed by substances such as acrylamide and glycidamide are not repaired by NER, but possibly BER.

Additional Comments:

Min-mice were significantly less susceptible to the mutagenic activity of PhIP (authors consider formation of bulky DNA adducts relevant). The authors considered that individual variations in the metabolism of acrylamide to glycidamide might have influence the different results obtained for acrylamide between experiment 1 and experiment 2. it was noted the increase in the first experiment was relatively small, although statistically significant. In studies using colchicine, a significantly increased mutagenic effect was reported in Min-mice heterozygous for Apc which is consistent with the deregulation of microtubule function Min-mice.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive

YANG2005: Yang HJ;Lee SH;Jin Y;Choi JH;Han CH;Lee MH; (2005) Genotoxicity and toxicological effects of acrylamide on reproductive system in male rats, *J Vet Sci* 6(2):103-109

Methods:

Male ICR mice were dosed with acrylamide (0, 18.13, 36.25, 72.5, 100, 145 mg/kg; Sigma) by oral gavage. Positive control: mytomycin C (1 mg/kg, i.p.). Peripheral blood was collected after 48 h, slides prepared and the frequency of micronucleated polychromatic erythrocytes (PCEs) was determined on the basis of 1000 PCEs.

Major Findings:

Acrylamide significantly increased the incidence of micronucleated PCEs at 72.5 mg/kg and above.

Acrylamide Positive Glycidamide Positive

MANJANATHA2006: Manjanatha MG;Aidoo A;Shelton SD;Bishop ME;McDaniel LP;Lyn-Cook LE;Doerge DR; (2006) Genotoxicity of acrylamide and its metabolite glycidamide administered in drinking water to male and female Big Blue mice, *Environ Mol Mutagen* 47(1):6-17

Methods:

Acrylamide (Sigma, >99.9% purity) and Glycidamide (Toronto Research, Ontario; >98.5% purity, approx 1% acrylamide). Purity was confirmed by GC-FID and LC-ES/MS. Male and female Big Blue mice (Taconic Farms, Germantown NY) were obtained as weanlings. Diet used was NIH 311R (selected for low acrylamide content, ca 10 ppb). Acrylamide was administered via the drinking water at 100 and 500 mg/l. Equimolar amounts of glycidamide were also used (120 mg/l and 600 mg/l). Animals were treated for 7 d per week for up to 4 weeks. The test materials were stable in water for up to a week (solutions changed weekly).

Reticulocytes were fixed and identified following staining with anti-CD72-FITC and RNase A. Micronuclei were stained by propidium iodide and cells analysed by flow cytometry (20,000 RETs /sample). Animals were sacrificed at termination and liver tissue snap frozen. It was noted that several samples from control females were lost and thus data for males only was evaluated. Data were analysed by one way ANOVA followed by Hom-Sidak test.

Major Findings:

A significant increase in %MN RETS was reported ($P < 0.05$). cf 0.28 ± 0.02 in control, 0.93 ± 0.13 in acrylamide treated, and 0.65 ± 0.07 in glycidamide treated animals.

Additional Comments:

The authors also noted that acrylamide and glycidamide produced an increase in proportion of reticulocytes in blood and this may have affected the increase in MN frequency reported in this study.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Micronucleus

Acrylamide Positive

DAVIS2007: Davis, J. and Recio, L. (2007) Determination of a Micronuclei Frequency Peripheral Blood of B6C3F1 Mice Exposed Acrylamide for Four Weeks, ILS Report C155-01

Methods:

Acrylamide (Sigma-Aldrich) was administered, in distilled water, by oral gavage, to duplicate groups of 5 male B6C3F1 mice (7 days / week for 28 consecutive days) at 0, 0.125, 0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, and 24.0 mg/kg bw/day. A concurrent positive control of 10 mg/kg bw/day cyclophosphamide was included in the study. Blood samples were taken 24±1 h prior to initiation (by retro orbital sinus) and 24±1h after the final dose by cardiac puncture. Liver, both testes, and sperm from vas deferens and epididymides (from both testes) were collected from each animal and snap frozen to -60oC (or -70oC for sperm samples). Flow cytometry was undertaken using MicroFlowplus kit (mouse) reagents. From each sample 200,000 ± 2,000 mature erythrocytes or CD71 positive reticulocytes were analysed for MN formation and percent PCEs. For one set of animals (group1-13 covering all dose groups, negative and positive controls) 1,000,000 total erythrocytes were sampled. For the duplicate blood sample from these animals and the duplicate dose groups (14-26) two sets of stop criteria were used; i) 20,000 CD71 positive reticulocytes and 200,000 CD71 positive reticulocytes. The MN data based on administered dose were evaluated using linear regression, quadratic regression and threshold methods.

Major Findings:

All animals survived to termination of the study. There were statistically significant increases in bodyweight in all groups except the 4.0 and 6.0 mg/kg bw group. No gross pathological changes were reported for liver and testes. A dose related increase in MNPCEs was reported in wild type mice. No mutagenic effect was reported in CYP2E1 null mice. Urethane gave a positive response in both null and wild type animals. A dose related increase in MN NCE and MN reticulocytes was reported. The data fitted linear regression, quadratic regression and threshold models equally well when administered dose was used as the metric (P,0.001). (Positive control gave expected response.)

Additional Comments:

Additional analyses of data were undertaken using DNA adducts as the metric of exposure were presented to COM at the October 2007 meeting.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Damage

Acrylamide Positive (CYP2E1+/+) Acrylamide Negative (CYP2E1-/-)

GHANAYEM2005A: Ghanayem BI;Witt KL;Kissling GE;Tice RR;Recio L; (2005) Absence of acrylamide-induced genotoxicity in CYP2E1-null mice: evidence consistent with a glycidamide-mediated effect, *Mutat Res* 578(1-2):284-297

Methods:

Groups 12 female CYP2E1+/+ (wild type) or CYP2E1-/- (null) mice were given 5 daily i.p. doses of acrylamide (25 or 50 mg/kg bw, Fluka, purity >99.5%). Blood samples were taken 24 hours after last dose. SGCE undertaken using alkaline PH>13 method (Tice, Hartman). Blood samples taken 24 hours post final dose. Tissues taken, minced/frozen. 10,000 cells mixed with low melting point agarose, lysed (cold) fixed. (Low MW DNA estimated for necrosis/apoptosis). 2 slides/sample exposed to alkali for 20 min then electrophoresed for 20 min (0.7v/cm, 300 mA). Separate slides electrophoresed for 40 min. DNA stained with SYBR gold. Tail length, percent migration of DNA, and olive tail moment determined for 100 cells (distance centre gravity of DNA between tail/head x fraction DNA in tail). Kendall rank test used for dose-response. Mann Whitney used for pair-wise comparison. Kruskal-Wallis used for comparison between genotype. Positive result at P<0.025 with adjustment for number of test groups).

Major Findings:

Blood leukocytes: Dose related increase in tail moment reported in wild type mice with no effect in null mice. Background levels of DNA damage similar in both genotypes. Liver: Dose related increase in wild type olive tail moment (significant at both dose levels). Null animals gave negative results in liver (a slight increase in tail moment at 50 mg/kg bw was seen following 20 min but not 40 min electrophoresis. Evidence of low molecular weight DNA at 50 mg/kg bw in null mice was not confirmed in histopathology for necrotic/apoptotic cells. Lung: No effect in either wild type or null mice.

Role of Metabolism:

Authors conclude that metabolism to glycidamide is responsible for genotoxic effects seen in mice.

Additional Comments:

Urethane (100 mg/kg bw, dosed i.p. for 5 days) positive control did not give response in this test. Authors noted that DNA adducts found in lung tissues in other studies and the selection of 100 cells in SGCE assay may have been insufficient to detect those cells with DNA damage.

Acrylamide Positive (rat thyroid and adrenal)

KLAUNIG2005: Klaunig JE;Kamendulis LM; (2005) Mechanisms of acrylamide induced rodent carcinogenesis, *Adv Exp Med Biol* 561:49-62

Methods:

Acrylamide (Sigma, purity 99.9%). Groups of 7wk old male F344 rats (numbers/group not given) were dosed with 15 mg/kg bw/day acrylamide for 7 days in drinking water (changed every other day). Alkaline comet assay (pH>13.0) was undertaken (Shimoi et al. 2001, *Mutat. Res.* 480:371-8) with liver, adrenal and thyroid. In concurrent experiments DNA synthesis were undertaken. Comet assay was unsuccessful in testes as the mesothelium could not be isolated. Electrophoresis was at 25 V, 300 mA for 30 min at 40C. Cells were stained with ethidium bromide. 100 nuclei/animal (50 cells/slide) were measured for comet tail moment. DNA damage data was analysed by ANOVA followed by Fisher Exact post hoc tests. Statistical significance was set at P<0.05. Comet Tail Moment assessed using Komet 4.0 software.

Major Findings:

A significant increase in comet tail moment was reported for thyroid (ca 1.5x control) and adrenal (ca 2x control). There was no increase in liver.

Mechanistic Data:

The authors suggest DNA damage and increases in DNA synthesis in male F344 rats were confined to cancer target tissues.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Damage

Acrylamide Positive

MANIERE2005: Maniere I; Godard T; Doerge DR; Churchwell MI; Guffroy M; Laurentie M; Poul JM; (2005) DNA damage and DNA adduct formation in rat tissues following oral administration of acrylamide, *Mutat Res* 580(1-2):119-129

Methods:

Acrylamide (Sigma; purity >99%). Groups of 4 to 5 male Sprague-Dawley rats were dosed orally with either 0, 18, 36 or 54 mg/kg bw in distilled water. Methyl methanesulphone (MMS; 110 mg/kg bw; single oral gavage) served as a positive control. Tissue samples were collected (blood, brain, liver bone marrow, adrenals and testes) at 2, 5 h post dosing (0 and 54 mg/kg bw) with tissues collected from all dose groups at 24 h. The comet assay was performed according to the method of Tice et al. 2000 (*Environ. Mol. Mutagen.*, 35, 206–221) using either blood, or tissue cell suspensions (50,000 to 100,000 cells/ml). Cells were mounted onto slides in agarose and lysed (pH 10, 1 h, 5 °C). After unwinding (pH 13.6, 20 min, room temp), slides were subjected to electrophoresis (24 min, 0.7 V/cm, 300 mA), then stained with ethidium bromide. 50 randomly selected cells were analysed per slide (2 slides per organ/tissue). Images processed by CometPro4 software (Aphelion, Adcis Soc., Caen, France). DNA damage reported as % DNA in comet tail and olive tail moment (OTM; product of the distance between barycentres of head and tail, by the proportion of DNA in the tail). Highly damaged cells with apoptotic or necrotic morphology were excluded from comet analysis and were reported separately. Individual treatment groups were compared to controls using the Mann-Whitney U-test with 5% significance.

Major Findings:

24h post exposure, significant increases ($p < 0.05$) in DNA damage was seen in blood leukocytes and brain at 36 and 54 mg/kg bw, and in testes at 54 mg/kg bw. No significant increase was seen in the other organs (liver, bone marrow and adrenals) or in the 18 mg/kg bw dose group. Earlier time points (2 and 5 h) were assessed to examine the possibility of transitory DNA damage, which was not seen later at 24h. All tissues except brain and testes showed increased comet parameters at 54 mg/kg bw. Increases in comet parameters were seen in positive controls at 24 h, although increases in bone marrow and liver were did not reach significance.

Additional Comments:

Tissues were selected to include some target (brain, testes and adrenals) and non target (blood, bone marrow and liver) sites from the animal carcinogenicity studies. The authors suggest that the organ specific DNA damage results seem to be in accordance with the known organ-specificity in acrylamide carcinogenesis in rat.

Acrylamide Positive

ZAMORANOPONCE2006: Zamorano-Ponce E; Morales C; Ramos D; Sepulveda C; Cares S; Rivera P; Fernandez J; Carballo MA; (2006) Anti-genotoxic effect of *Aloysia triphylla* infusion against acrylamide-induced DNA damage as shown by the comet assay technique, *Mutat Res* 603(2):145-150

Methods:

Groups of eight male BALB/c mice (6-8 weeks) were administered acrylamide (Sigma, purity not stated) i.p. at 0, 5, 20, 30 or 50 mg/kg bw. Bone marrow samples were prepared 6 h after dosing (trypan blue exclusion for 200 cells used to assess degree of cytotoxicity). The alkaline (Ph>13.0) Comet assay of blast cells was undertaken according to Singh et al (*Exp Cell Res*, 17, 184-191, 1988). Electrophoresis was undertaken at 25V/300 mA for 20 min. Cells were stained with ethidium bromide. Tail moment was determined for 100 cells/slide for a total of 300 cells/animal. Slides were kept humidified and evaluated within 24 h of preparation. In separate experiments the effect of pre-treatment by infusion (2x/day oral gavage dose of infusion for 20 days) with *Aloysia triphylla* was investigated on acrylamide genotoxicity (at 50 mg/kg bw i.p). Statistical analysis was by ANOVA using Levene test for homogeneity and Tukey's test for multiple comparisons.

Major Findings:

A clear dose related increase in tail moment was reported with statistically significant effects at all doses. Cell viability was reduced from 99% in control to 90% at 50 mg/kg bw. The dose-response was not modelled. Pre-treatment oral dosing with Infusion of *Aloysia triphylla* reduced acrylamide genotoxicity to control levels.

Additional Comments:

Authors report effects at 5 mg/kg bw i.p were consistent with Abramasson-Zetterberg (*Mut Res* 2003, 535,215-222). The mechanism of inhibition of acrylamide genotoxicity was not known although concurrent investigations of total Ferric reducing activity of plasma showed pre-treatment with *Aloysia triphylla* increased total anti oxidant capacity of plasma. Positive control (cyclophosphamide, 20 mg/kg b.w.) behaved as expected.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Synthesis

Acrylamide Positive (thyroid, adrenal medulla & testes) Acrylamide Negative (liver & adrenal cortex)

LAFFERTY2004: Lafferty, J. S., Kamendulis, L. M., Kaster, J., Jiang, J., and Klaunig, J. E. (2004) Subchronic acrylamide treatment induces a tissue-specific increase in DNA synthesis in the rat., *Toxicol Lett* 154(1-2):95-103

Methods:

Acrylamide (Sigma; purity >99.9%) was administered to Male F344 and Sprague Dawley (SD) rats in drinking water. Expt 1: Acrylamide dosed in both rat strains at 2 or 15 mg/kg bw for 14 and 28 days. Expt 2: F344 rats were dosed at 2 mg/kg bw ± 1-aminobenzotriazole (ABT; 100 mg/kg/day, i.p., 7 days, starting 1 day before acrylamide administration). ABT is an inhibitor of CYP2E1 oxidative metabolism of acrylamide. BrdU was administered for 7 days prior to sacrifice by osmotic mini pump. Formalin fixed tissues (thyroid, adrenals, testes and liver) were paraffin embedded, sectioned and stained to determine DNA synthesis (immunohistochemical detection of BrdU), mitosis (H&E) and apoptosis (TACS kit, Trevigen USA; verified with H&E). Statistical analysis using ANOVA followed by Dunnett's post hoc test ($p < 0.05$ significance level).

Major Findings:

Expt 1: Thyroid follicular cells: Significant increase in DNA synthesis at both doses in F344 rats (no dose response) and at 15 mg/kg bw in SD rats at all time points. Adrenal medulla: significant increase in DNA synthesis in both strains at both doses and was highest at 7 days. Testicular mesothelium: Significant increase in DNA synthesis in F344 rats at both doses and all time points and at 15 mg/kg bw in SD rats at all time points. Liver and adrenal cortex: no increases in DNA synthesis (even in the 3 separate zones of the adrenal cortex). No treatment group showed changes in mitotic/apoptotic indices.

Expt 2: Thyroid follicular cells: Both acrylamide and ABT alone increased DNA synthesis (acrylamide < ABT). No difference between co-treatment and ABT alone. Adrenal medulla: ABT does not increase DNA synthesis whereas acrylamide does. Co-treatment prevents acrylamide induced DNA synthesis. Testicular mesothelium: ABT does not increase DNA synthesis whereas acrylamide does. Co-treatment does not affect acrylamide induced DNA synthesis. Liver: ABT increases DNA synthesis in presence or absence of acrylamide, but acrylamide does not increase DNA synthesis. Adrenal cortex showed no increase in DNA synthesis with any treatment. No treatment group showed changes in mitotic/apoptotic indices.

Role of Metabolism:

Authors suggest that oxidative metabolism or glycidamide do not appear to exclusively account for the induction of DNA synthesis. This study does not preclude the oxidative stress or pathways involving thyroid hormone regulation being involved in acrylamide carcinogenesis.

Additional Comments:

The authors suggest a correlation between induction of DNA synthesis and target organs in carcinogenicity studies (Targets: thyroid, testes and adrenal medulla; Non-targets: liver and adrenal cortex). The authors suggest that inter-strain differences in CYP2E1 activity (SD < F344) may be responsible for the differential induction of DNA synthesis in the thyroid and testes. The lack of dose related increases in DNA synthesis in F344 rats could be due to inverse relationship in relative conversion of acrylamide to glycidamide at low doses. The authors suggest that glycidamide may be involved in inducing DNA synthesis and related tumours in the adrenal medulla; however, mechanisms independent of oxidative metabolism may be responsible for the effects in testicular mesothelium. Effects of inhibition in the thyroid are inconclusive as ABT causes increased DNA synthesis itself.

Liver weight and relative liver weight increased significantly in both groups treated with ABT. ABT inhibition of oxidative metabolism was confirmed by the method of Fennell et al 2003.

Expt 1 may be the same as reported by Klaunig 2005

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo DNA Synthesis

Acrylamide Positive (thyroid, adrenal medulla & testes) Acrylamide Negative (liver & adrenal cortex)

KLAUNIG2005: Klaunig JE; Kamendulis LM; (2005) Mechanisms of acrylamide induced rodent carcinogenesis, Adv Exp Med Biol 561:49-62

Methods:

Acrylamide (Sigma, purity 99.9%). Groups of 7wk old male F344 rats (numbers/group not given) were dosed with 15 mg/kg bw/day acrylamide in drinking water (changed every other day). DNA synthesis (after 7, 14, 28 days at 15 mg/kg bw/d) was measured using BrdU uptake (immunohistochemical detection in 2000 cells/animal) in thyroid, adrenals, testes and liver. In concurrent experiments, alkaline comet assay was undertaken with liver, adrenal and thyroid. DNA synthesis data was analysed by ANOVA followed by Dunett's post hoc test. Statistical significance was set at $P < 0.05$.

Major Findings:

An increase in DNA synthesis in the thyroid, testes and adrenal medulla (latter highest at day 7). No increase in DNA synthesis was seen in liver and adrenal cortex.

Mechanistic Data:

The authors suggest DNA damage and increases in DNA synthesis in male F344 rats were confined to cancer target tissues.

Additional Comments:

Authors report positive effects on DNA synthesis at 2 mg/kg bw/day in the abstract, but no mention is made to this dose level in the methods or results.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Human Biomonitoring

Acrylamide Negative

KJUUS2005: Kjuus H; Hansteen IL; Ryberg D; Goffeng LO; Ovrebo S; Skaug V; (2005) Chromosome aberrations in tunnel workers exposed to acrylamide and N-methylacrylamide, Scand J Work Environ Health 31(4):300-306

Methods:

73 Tunnel workers exposed to N-methylacrylamide (NMA) grouting (grout solution contained 26-31% N-methylacrylamide, 2.5-5.4% acrylamide, 0.02-0.03 methylene-bis-acrylamide, 12-17% methylic diesters and 0.9% formaldehyde) in a two year period 1995-1997 participated in an occupational health survey from October 1997-January 1998. A subgroup of 25 workers with the highest exposure to grouting solution was selected (method of exposure assessment involved measuring acrylamide concentration in grouting solutions and also leaks from drilled holes (which were noted to contain high levels of acrylamide)). For these 25 workers the average number of days grouting was 586 (243-743, median 639d). 20/25 reported skin contact with grouting fluid and all workers stated they had direct skin contact with tunnel water. A time weighted exposure index as calculated for each worker based on questionnaires. not given). Those with known neurological disease, or alcohol/drug abuse were excluded. 25 matched tunnel workers without exposure to NMA grouting solutions were age matched ($\pm 3y$) and smoking were used (data from 23 included in analyses). Information on smoking, alcohol use, X-ray exposure, common cold, allergy, current medication, previous work with solvents, and previous lead exposure were collected. Blood samples (10 ml) were taken from two exposed and unexposed workers together with one reference sample on the same day. The referent group comprised 7 staff members of NIOSH who provided blood samples as a further control group as it was not possible to sample all tunnel workers on the same day. Whole blood cultures were undertaken for 50-53h, cells were fixed and stained with Giemsa. 200 metaphases examined per person. (Information on colchicine/colcemid treatment not given in this paper, previous reference cited Skyberg Br J In Med, 46, 791-8, 1989). Number of cells with aberrations (less gaps) recorded. Blood samples from all individuals were genotyped for GSTM1 and GSTT1. Statistical significance was undertaken using Mann-Whitney and significance was set at $P < 0.05$. A crude exposure group of above 639 days of injection was used.

Major Findings:

There was no evidence for an increase in chromosome aberrations in exposed workers. In a separate publication cited in this report, the authors noted that detectable haemoglobin adducts were only found in three exposed workers. A slightly higher number of cells with higher levels of aberrations $\geq 3/\text{cell}$ was reported in 14 exposed, cf 9 control individuals. The data from referents was comparable to controls. The authors reported that GSTM1 null and GSTT1 null were associated with increase in chromatid gaps but the significance of this finding is unclear.

Additional Comments:

Limited evidence suggests that absorbed doses of acrylamide might have been comparable to background for most workers.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Mutation

Glycidamide Positive

GENEROSO1996: Generoso WM;Sega GA;Lockhart AM;Hughes LA;Cain KT;Cacheiro NL;Shelby MD; (1996) Dominant lethal mutations, heritable translocations, and unscheduled DNA synthesis induced in male mouse germ cells by glycidamide, a metabolite of acrylamide, Mutat Res 371(3-4):175-183

Methods:

Glycidamide (100 mg/kg bw/day; Polyscience, Washington, PA, USA; purity 100%) in Hanks Balanced Salt Solution was dosed i.p to C3H/RLx101RL F1 male mice (ca 12 weeks of age). 100 mg/kg bw was selected to result in approximately 50% dominant lethality. The sterility/semi-sterility of F1 progeny was examined. Heritable translocation was assumed from previous studies to occur in semi-sterile F1 males. Mating was performed with SEC C57BL F1 females (12 weeks of age). Full details of the dosing and mating regime were not given (authors refer to Generoso WM Mutat Res, 345, 167-180, 1995). Clear sterile F1 males were investigated cytogenetically for translocations. Details of statistical analyses referred to dominant lethal investigations in this publication but did not specifically refer to approaches used for heritable translocations.

Major Findings:

A heritable translocation rate of 20.18% was reported following glycidamide treatment (of 669 male progeny evaluated: 91 semi-sterile, 3 unclear sterility, 41 sterile with cytogenetically evaluated translocations). The frequency in historical control was 0.06% (11292 males evaluated: 6 semi-sterile males and 1 sterile male with cytogenetically confirmed translocations).

Role of Metabolism:

The authors considered that metabolism of acrylamide to glycidamide was responsible for results obtained in previous studies using acrylamide.

Additional Comments:

Glycidamide induced a similar proportion of heritable translocations to dominant lethality in this study compared to acrylamide in previous studies (Shelby, 1986, Mutat. Res., 250 431-7).

Acrylamide Positive

ADLER2004: Adler ID;Gonda H;Hrabe dA;Jentsch I;Otten IS;Speicher MR; (2004) Heritable translocations induced by dermal exposure of male mice to acrylamide, Cytogenet Genome Res 104(1-4):271-276

Methods:

Male C3H/EI mice were treated with five dermal exposures of 50 mg/kg AA and mated 1.5–8.5 days after the end of exposure to untreated female 102/EI mice. Pregnant females were allowed to come to term and all offspring were raised to maturity. Translocation carriers among the F1 progeny were selected by a sequential fertility testing and analysed by G-band karyotyping and MFISH. Data was analysed by one sided Fisher's exact test.

Major Findings:

In the heritable translocation test in the first experiment, 475 progeny were examined and 41 carried reciprocal translocations. An overall rate of 8.635% was reported which was significantly higher than the historical control rate of 0.05% ($p < 0.001$).

Additional Comments:

The authors suggest the ratio of 0.39 should be used to compare generic risk from dermal to i.p. dosing, although they note difference in mating after dosing between dermal and i.p. studies.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Mutation

Acrylamide Positive (CYP2E1+/+) Acrylamide Negative (CYP2E1-/-)

GHANAYEM2005B: Ghanayem BI;Witt KL;El Hadri L;Hoffler U;Kissling GE;Shelby MD;Bishop JB; (2005) Comparison of germ cell mutagenicity in male CYP2E1-null and wild-type mice treated with acrylamide: evidence supporting a glycidamide-mediated effect, *Biol Reprod* 72(1):157-163

Methods:

Acrylamide (0, 12.5, 25 or 50 mg/kg bw, Sigma-Aldrich, purity >99.5%) in saline was administered to groups of male CYP2E1+/+ (wild type) or CYP2E1-/- (null) mice derived from B6C3F1. The i.p. dosing regime which varied between studies but was based on Shelby 1986 and Generoso 1996. Male mice were mated at various times after dosing with untreated females and the uterine contents examined on approximately day 13 of gestation. Implants, numbers of live/dead embryos/fetuses, resorptions (early/late) were recorded.

In study 1 untreated wild type and null mice were mated with untreated female B6C3F1 mice for 5 days (control). 48h later 30 males of each genotype were administered saline (n=10) or 50 mg/kg bw acrylamide (n=20) for 5 days and then mated for 5 days (1 male with 3 females). Uterine contents were examined on day 13 after the end of mating.

In study 2, following a high level of sterility in study 1, a dose ranging study was performed where wild type and null mice were dosed i.p for 5 days with acrylamide (12.5 or 25 mg/kg bw; group size 12 and 13 animals respectively). Mice were mated 2 days after the end of dosing, with a second mating undertaken 7 days after the cessation of dosing (i.e two periods of mating designed to cover different periods of spermatogenesis). Females were sacrificed 13 days after the end of mating and uterine contents examined.

In study 3, A more definitive investigation using 12.5 mg/kg bw and 25 mg/kg bw was undertaken using wild type and null mice using the same dosing, mating regime as study 2, using 12 null and 13 wild type mice. Control groups consisted of 8 null and 11 wild type males. A mating ratio of 1 male to 2 females was used.

After modelling the data for extra binomial variability, a non parametric approach to data analyses was used involving Kruskal-Wallis analysis of variance followed by Mann-Whitney U tests. Group comparison for percentage of pregnant females used chi square in study 1 and dose related trends were evaluated using Jonckheere-Terpstra test in study 2 and 3. Overall group differences in percentage pregnancy were followed by Fisher Exact test. In studies 2 and 3 week 1 data were compared to week 2 data using Wilcoxon signed rank tests.

Major Findings:

In wild type CYP2E1 mice, in study 1 only 3/60 females became pregnant. There were no significant effects on number of pregnant females at 12.5 or 25 mg/kg bw. There was a significant reduction in the mean number of implants in the 3 pregnant females at 50 mg/kg bw ($P<0.01$ compared to controls and null treated groups). In study 2 there was no effect at the first mating but a significant reduction in the mean number of implants at both dose levels ($P<0.01$) in the second mating indicating an effect on spermatids. The same pattern of an effect on the second mating was also seen in study 3 at both dose levels. With a dose-related effect reported. The percent live fetuses reduced from 90% in controls to 44% at 50 mg/kg bw following mating of wild type mice. In study 2 the reduction in percent live fetuses was greater for the first mating compared to the second mating. Dose related effects on resorptions were reported with consistent effects noted between matings one and two.

In CYP2E1 null mice there were no treatment related effects on percent pregnancy, mean number of implants per pregnant female, percent live fetuses per pregnant female or percent resorptions per pregnant female.

Mechanistic Data:

The authors considered that glycidamide was the ultimate germ cell mutagen for dominant lethal effects binding to nucleophilic sites in chromatin in early spermatozoa. It was unclear whether glycidamide bound to sulphhydryl proteins or DNA. The authors note that DNA breakage in late spermatids/spermatozoa could be induced by protamine alkylation and chromatin strand distortion. However DNA damage in late spermatid/spermatozoa stage would persist. The possibility of weak DNA alkylation could not be excluded. The authors noted experiments from their laboratory (unpublished) showed negligible DNA adducts in mixed germ cells types from CYP2E1 null mice. The authors considered that even if acrylamide did alkylate DNA or protamines directly this action was ineffective in inducing detectable germ cell damage.

Role of Metabolism:

The authors concluded that CYP2E1 null mice lacked the metabolism of acrylamide to glycidamide suggesting

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Mutation

metabolism was a prerequisite for dominant lethal mutation. In the absence of CYP2E1 the authors considered there would be an increase in glutathione conjugation. The authors quoted additional studies of CYP2E1 mice to report that glycidamide was not formed in these mice (Sumner, J Chem Res Tox 12, 1110-1116, 1999 and Ghanayem, Tox pathol, 28, 839-850, 2000) Not specifically examined in this study. The authors also report preliminary (unpublished) results indicating high levels of acrylamide in CYP2E1 null mice in plasma in animals dosed with acrylamide whereas negligible levels of acrylamide were found in CYP2E1 wild type mice dosed with acrylamide.

Additional Comments:

The authors considered that data on implantations tended to be more variable than effects on resorption/live fetuses. Overall they considered the data supported the view that the critical sensitivity was condensed spermatids and early epididymal spermatozoa. Authors consider this study is first demonstration that germ cell effects of acrylamide are due to glycidamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Dominant Lethal

Acrylamide Positive

WORKING1987: Working PK;Bentley KS;Hurt ME;Mohr KL; (1987) Comparison of the dominant lethal effects of acrylonitrile and acrylamide in male Fischer 344 rats, Mutagenesis 2(3):215-220

Methods:

Three groups of 50 male Fischer 344 were gavaged daily for 5 days with acrylamide (30 mg/kg, Aldrich, >99% purity) in normal saline. Starting 1 day after exposure, each male was bred to one female per week for 10 weeks (4 weeks in the TEM group). Females were necropsied 13 days after the end of the mating week and the amount of pre- and post-implantation loss calculated. Positive control: triethylenemelamine (TEM, 0.2 mg/kg) dosed i.p. on day 5

Major Findings:

Acrylamide induced significantly elevated amounts of post-implantation loss for 3 weeks after exposure and pre-implantation loss for 4 weeks post-exposure; both measures returned to control values for the remaining 6 weeks of the study. The positive control TEM caused both indices to increase during all 4 weeks examined.

Pregnancy rates were reduced only during week 2 in the AA-exposed group and week 4 in the TEM-treated group. Mating rates were only reduced only in the TEM group (week 1).

Role of Metabolism:

This rat strain may bioactivate acrylamide to glycidamide.

Additional Comments:

The authors conclude that acrylamide is a dominant lethal mutagen in male rat germ cells in vivo, specifically in mature spermatozoa and late-stage spermatids.

Acrylamide Positive

DOBRZYNSKA1990: Dobrzynska M;Lenarczyk M;Gajewski AK; (1990) Induction of dominant lethal mutations by combined X-ray-acrylamide treatment in male mice, Mutat Res 232(2):209-215

Methods:

Fertile Pzh:SF1SS male mice (n=14 to 19) were dosed i.p. with Acrylamide (75 & 125 mg/kg, BioRad, purity > 99.9%) in Hanks' balanced salt solution. Each male was caged with 3 virgin females per week for 7 weeks. Females were sacrificed a 18 days post mating, living fetuses, resorptions and dead fetuses were counted.

Major Findings:

A significant ($p < 0.05$, t-test) increase in dominant lethality was observed in weeks 1 and 2 post dosing at 125 mg/kg. No effect was seen at 75 mg/kg.

Role of Metabolism:

This mouse strain may bioactivate acrylamide to glycidamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Dominant Lethal

Glycidamide Positive

GENEROSO1996: Generoso WM;Sega GA;Lockhart AM;Hughes LA;Cain KT;Cacheiro NL;Shelby MD; (1996) Dominant lethal mutations, heritable translocations, and unscheduled DNA synthesis induced in male mouse germ cells by glycidamide, a metabolite of acrylamide, Mutat Res 371(3-4):175-183

Methods:

Glycidamide (Polyscience, Washington, PA, USA; purity 100%) in Hanks Balanced Salt Solution was administered i.p to C3H/RLx101RL F1 male mice (about 12 weeks of age). In the dominant lethal assay a single dose level of 125 mg/kg bw was used (based on range finding lethality test using 150, 175 and 200 mg/kg bw). Mating procedures were undertaken for a 50 day period (13 x 3 day periods from 0.5 day up to 49.5 days), mating 1 male with 2 female mice. Data analysed by ANOVA (total implants, live embryos and proportion resorptions/female). Freeman-Tukey Poisson transformation for count data and arcsine transformation for proportion data.

Major Findings:

No effect on mating performance was noted at 125 mg/kg bw. There was a marked reduction in living implants and an increase in resorptions in the interval 2.5-11.5 days post treatment. It is also possible that there were increases in dominant lethality a day before and after this period. The maximum dominant lethal response was 4.5-9.5 days post treatment. This corresponds to late spermatids and early spermatozoa. Split doses of 2 x 87.5 mg/kg bw or 2 x 75 mg/kg bw reduced mating ability.

Mechanistic Data:

The authors considered the response of glycidamide showed a similar pattern to that of acrylamide (with regard to maximal response time). They considered such a response was typical of monofunctional alkylating agents (MMS, EMS and ethylene oxide) that bind to nitrogen positions of guanine and also to sperm protamines.

Role of Metabolism:

Glycidamide induced a similar proportion of heritable translocations to dominant lethality in this study compared to acrylamide in previous studies (Shelby, 1986, Mutat. Res., 250 431-7) . The authors compared the maximal sperm UDS response of acrylamide with the data on glycidamide and noted the time point of maximum for acrylamide (ca 5h) was the longest delay for mouse germ cells reported to date and it was inferred from the shorter maximum peak effect for acrylamide that the data were consistent with metabolism of acrylamide. The authors calculated that the maximal response seen with 150 mg/kg bw glycidamide equated to that seen from 125 mg/kg bw acrylamide at the respective maximum response times to glycidamide for the germ cell effects.

Additional Comments:

Maximum response of glycidamide exceeded that of acrylamide (Shelby, 1986, Mutat. Res., 250 431-7). Authors commented on a significant drop in dominant lethal response when dose of glycidamide was reduced from 125 mg/kg bw to 100 mg/kg bw, although this experiment was not reported or referenced in this paper.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Dominant Lethal

Acrylamide Positive

HOLLAND1999: Holland N; Ahlborn T; Turteltaub K; Markee C; Moore D; Wyrobek AJ; Smith MT; (1999) Acrylamide causes preimplantation abnormalities in embryos and induces chromatin-adducts in male germ cells of mice, Reprod Toxicol 13(3):167-178

Methods:

Male C57B1/6J mice (7 to 12 weeks of age) were randomised into groups (8 to 10 animals per group) and administered acrylamide (0, 40 and 50 mg/kg bw; Biorad; no purity data) i.p. in PBS, for 5 days. Treated males were mated with 3 or 4 untreated female C3H/J mice (10 to 12 weeks of age) 4 or 5 times per week, over a 5 week period. 6 Females with vaginal plugs were sacrificed 15 to 16 days post mating and uterine horns examined for total implants, early death (moles), early resorptions, late death. Preimplantation loss estimated from the number of corpora lutea on the ovaries. Fishers exact test was used to compare exposed to control groups.

Major Findings:

The highest increase in preimplantation loss was in animals mated 1 to 2 weeks post treatment (72.1%), which was significantly ($P < 0.05$) lower than 3 to 4 weeks (41.3%) and 5 weeks (18.5%). This closely correlated with the embryo abnormality study where the increase in abnormal embryos at day 4 were 72.9%, 40.1% and 10.1% respectively (see other summary). There were also increases in post-implantation loss (12.7%, 8.2% and 10.1% respectively).

Additional Comments:

The authors note that their results are in accordance with Shelby 1982 (Mutat. Res., 250 431-7).

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Dominant Lethal

Acrylamide Positive

ADLER2000: Adler ID; Baumgartner A; Gonda H; Friedman MA; Skerhut M; (2000) 1-Aminobenzotriazole inhibits acrylamide-induced dominant lethal effects in spermatids of male mice, *Mutagenesis* 15(2):133-136

Methods:

Male (102/EIXC3H/EI)F1 mice were administered acrylamide (125 mg/kg, Sigma) i.p. in distilled water ± 1-aminobenzotriazole (ABT, 50 mg/kg, Sigma) i.p. in physiological saline. In Expt 1, two groups of mice (n=20) were treated with acrylamide ± pre-treatment with ABT for three days prior to acrylamide dosing. Vehicle and ABT alone groups (n=20) were also included. Males were mated with untreated virgin females (ratio 1:2) 6h post treatment, females were replaced every week for a total of 4 weeks. Expt 2 used the same experimental design, but with group sizes of 34 male animals, selecting 30 males for mating with untreated virgin females (ratio 1:1). Females were replaced every 4 days for a total of 4 mating intervals.

Females with vaginal plugs were removed from mating cages. On day 14-16 of gestation, females were killed and uterus contents inspected for live and dead implants. Numbers were compared between control and treated groups using the Chi² test. In Expt 2, 4 males from each treatment group were killed 1 week after treatment and testes and epididymes were dissected for spermograms. Epididymes were placed in 300 µl fetal calf serum and sperm allowed to actively leave for 1h. In 100 sperm each from 2 slides per animal, sperm concentration, mobility and morphology were determined by microscopic evaluation (WHO method, 1992). Parameters were compared between treatment groups and control using t-tests.

Major Findings:

Expt 1: Significant increases in percentage of dead implants in the first and second mating week of males dosed with acrylamide. This effect was not seen in acrylamide exposed animals pre-treated with ABT. Expt 2: Significant increase in the percentage of dead implants and rates of dead implants per female in 2nd, 3rd and 4th mating intervals after treatment with acrylamide. No dominant lethal effect in 2nd mating interval when ABT and acrylamide were combined. In 3rd mating interval combined treatment significantly increased rate of dead implants per female and percentage of dead implants above ABT and control groups but also significantly lower than acrylamide group. In 4th mating interval combined treatment resulted in dominant lethal effects at similar levels to the acrylamide group. Reduced pregnancy rates and reduced numbers of total implants were observed in first mating interval in both experiments.

Acrylamide treatment dramatically reduced fast moving sperm and this was not affected by ABT pre-treatment. ABT alone also significantly decreased the percentage of fast moving sperm compared to control. Percentage of immobile sperm, and sperm counts and morphology were all unaffected by treatment.

Role of Metabolism:

ABT inhibits/significantly reduces acrylamide induced effects supporting the hypothesis that p450 mediated oxidative metabolism to glycidamide is the ultimate clastogen in mouse spermatids.

Additional Comments:

Authors suggest in Expt 2 that either CYP inhibition is not complete or there is additional mechanism involved in AA-induced clastogenicity. Reduced fertility might be explained by mobility impairment in sperm in acrylamide, ABT and combination groups; which the authors consider may involve inhibition of motor proteins by acrylamide.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Dominant Lethal

Acrylamide Positive

TYL2003: Tyl RW;Friedman MA; (2003) *Effects of acrylamide on rodent reproductive performance, Reprod Toxicol* 17(1):1-13

Methods:

Groups of 30 male and 30 female F344 rats were given acrylamide (purity not stated) via the drinking water at concentrations to achieve dose levels of 0, 0.5, 2.0 or 5.0 mg/kg bw/day for 10 weeks (F0) and 11 weeks F1 prior to mating as part of a multigenerational study. After the first mating (Fo), treated males were removed from acrylamide exposure for 2 days and mated for a second time with untreated females and a dominant lethal analysis undertaken on day 14 of gestation. Reproduction indices reported included gestational length, implantations/female, number of live births/litter. The animals were also observed for signs of neurotoxicity.

Major Findings:

There was a significant reduction in reproduction indices at 5 mg/kg bw/day in the multigenerational study. Examination for dominant lethal effect revealed a significant reduction in the number of implants/litter, and number of live implantations/litter. There was a statistically significant increase in both pre-implantation and post implantation loss. The authors calculated the Frequency of dominant lethal mutations as $1 - (\text{ratio of live implants in treated/control}) \times 100$. There was a significant increase in the dominant lethal frequency (20.21% of 0 in controls and negative estimates at 0.5 and 2.0 mg/kg bw/d (-1.06 and -2.13 respectively).

Additional Comments:

The authors reported that dominant lethal effects did not account for all the fetal loss in the reproduction studies. Additional data are provided in Tyl, *Reproduct Toxicol*, 14, 147-151, 2000).

Acrylamide Positive

ADLER2004: Adler ID;Gonda H;Hrabe dA;Jentsch I;Otten IS;Speicher MR; (2004) *Heritable translocations induced by dermal exposure of male mice to acrylamide, Cytogenet Genome Res* 104(1-4):271-276

Methods:

Male (102/EI x C3H/EI)F1 mice were administered acrylamide (50 mg/kg bw, Sigma) in saline, i.p. for 5 days. One day after the end of exposure, the males were mated to untreated females of the same hybrid stock for four days and females were changed every four days for a total of five matings. This study compared i.p. dosing to dermal exposure (50 mg/kg bw in corn oil, applied to shaved backs for 5 days). Data was analysed by Mann-Whitney U test.

Major Findings:

Increased dominant lethality was observed during the first 3 matings. (81.7, 85.7 and 45.4%, corresponding to 1.5-4.5, 5.4-8.5 and 9.5-12.5 days after treatment). The response reported for dermal treatment was 2-4 times lower than following i.p. treatment.

Additional Comments:

Authors note their data is consistent with Shelby 1986 (*Mutat. Res.*, 250 431-7), and Holland 1999 (*Repro Tox*, 13, 263-8)

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Embryo Abnormalities

Acrylamide Positive

HOLLAND1999: Holland N; Ahlborn T; Turteltaub K; Markee C; Moore D; Wyrobek AJ; Smith MT; (1999) Acrylamide causes preimplantation abnormalities in embryos and induces chromatin-adducts in male germ cells of mice, *Reprod Toxicol* 13(3):167-178

Methods:

Male C57B1/6J mice (7 to 12 weeks of age) were randomised into groups (8 to 10 animals per group) and administered acrylamide (Biorad; no purity data) i.p. in PBS, for 5 days. Treated males were mated with 3 or 4 untreated female C3H/J mice (10 to 12 weeks of age) 4 or 5 times per week. Three studies were performed: Study 1 tested 0, 40 and 50 mg/kg bw; Study 2 tested 50 mg/kg bw using 42 males; and Study 3 tested 10, 25 and 50 mg/kg bw. In Study 1, mice were mated over a 5 week period, mice were only mated during weeks 2 and 3 post treatment in Studies 2 and 3. Females with vaginal plugs were sacrificed 86 to 88 hours post mating. Uterine horns were flushed with warm RPMI tissue culture medium to collect embryos. The number of ovulated eggs was estimated by counting corpora lutea on the ovaries. Blastocyst / morula morphology was examined under a dissecting microscope. Normal and abnormal embryos were assessed; the latter being differentiated into embryos with retarded cleavage and a small number of blastomeres (<10), embryos with lysis or abnormal cell structure, and unfertilised eggs. Fishers exact test was used to compare exposed to control groups.

Major Findings:

Study 1 showed a significant ($p < 0.01$) dose related increase in embryo abnormalities, during week 1 of mating, at 40 and 50 mg/kg bw/day over 5 days (61.1% and 86.2% respectively). At 50 mg/kg bw, there was a gradual decline in frequency of abnormal embryos over the 5 weeks of mating, whereas there was a plateau at 60% abnormalities for the first 4 weeks in the 40 mg/kg bw group. Of the various types of abnormal embryo, unfertilised eggs and single cell zygotes were predominant during weeks 1-3 of mating compared to weeks 4 and 5 ($p < 0.001$), whereas frequencies of the other abnormalities for the two other abnormalities remained constant.

Study 2 examined abnormal embryo frequencies in a larger sample size, daily, for weeks 2 and 3 of mating, at 50 mg/kg bw/day administered over 5 days. The average frequency in week 2 was 93.5% compared to 81.8% in the third week ($p = 0.003$). Logistic regression modelling suggested a significant trend for reduction in abnormal embryos over time ($p < 0.001$).

Study 3 examined the dose response. 10 mg/kg bw did not significantly increase the frequency of abnormal embryos above control levels. There was a strong dose related increase in abnormal embryo frequency at both 25 and 50 mg/kg bw, with a greater increase during the 2nd week compared to the 3rd

Additional Comments:

The authors performed logistic regression modelling to analyse the effects of various parameters on preimplantation abnormalities. This identified dose and time after treatment to have the main effect on frequency of abnormal embryos. This modelling exercise identified 10 mg/kg bw/day over 5 days as being a 'no effect dose' and 50 mg/kg bw/day over 5 days as resulting in ~90% abnormal embryos.

Slides from this study were analysed for a number of cytogenetic and cytologic criteria, reported separately (see Titenko-Holland 1998)

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Chromosomal Aberrations

Acrylamide Positive

GASSNER1996: Gassner P;Adler ID; (1996) Induction of hypoploidy and cell cycle delay by acrylamide in somatic and germinal cells of male mice, *Mutat Res* 367(4):195-202

Methods:

(10/E1 x C3H/E1)F1 mice (n=3) were administered acrylamide (120 mg/kg bw, Sigma, no purity data) i.p. in saline. Animals were sacrificed at 0, 2, 6, 10, 14, 18 and 22 h post treatment. Germ cell preparations were obtained by the method of Evans et al. 1964 (Cytogenetics, 3:289-94). Slides were stained with lacto-aceto-orcein (2%) with additional slides (6 to 22 h) by the Giemsa C-banding technique. Cell proliferation was estimated by counting spermatogonial mitoses, and meiotic metaphase (MM) I and II cells per 1000 mid pachytene cells. The 'meiotic index' (MM II / MM I) was calculated to indicate meiotic delay (index <2). 100 well-spread secondary spermatocyte metaphases per animal were scored for aneuploidy. Data analysed by Student's t-test and Mann-Whitney u-test.

Major Findings:

Acrylamide significantly slowed meiotic progression ($P < 0.01$). There was no significant effect on the rate of hyperploid cells. Initially, the incidence of hypoploid cells was reduced 0.7% at 10 h compared to 2.4% in controls ($P < 0.05$); however, levels increased at later time points (6.8% at 18 h and 4.8% at 22h).

Acrylamide Positive

MARCHETTI1996: Marchetti F;Lowe X;Moore DH;Bishop J;Wyrobek AJ; (1996) Paternally inherited chromosomal structural aberrations detected in mouse first-cleavage zygote metaphases by multicolour fluorescence in situ hybridization painting, *Chromosome Res* 4(8):604-613

Methods:

Male B6C3F1 mice (8-12 weeks) were administered five consecutive doses of acrylamide (5x50 mg/kg bw, Fisher Scientific) i.p. in distilled water. Untreated females were administered pregnant horse serum i.p. to induce superovulation, followed by human chorionic gonadotrophin. Mice were mated (1:1) for 8 h, whereupon females with vaginal plugs were administered colchicine (2 mM, i.p.) to prevent union of the parental pronuclei and arrest zygote development at metaphase of the first cleavage division. Zygotes from 10-15 females were harvested, fixed and processed for FISH analysis. Biotin labelled probes for chromosome 1,2,3 and X were detected with avidin FITC. Digoxigenin labelled probe was used to visualise chromosome Y. DAPI was used as to counter-stain. Microscopic examination included translocations acentrics, dicentrics and other chromosome aberrations. The PAINT nomenclature system was used. This combination of probes covers 25.6% (Y-) or 22.9% (Y+) of the genome, meaning 38.1% and 36.5% of potential chromosomal exchanges could be detected; therefore, assuming a random distribution of breaks, the PAINT analysis was expressed in terms of cell equivalents to correct for the fraction of the genome painted. Chi squared analysis was used for effects on fertilisation and zygote development.

Major Findings:

Structural chromosomal aberrations were observed in the sperm-derived, but not in the egg-derived, pronuclei. Significant increases in structural aberrations by DAPI analysis and independently by PAINT analysis ($P < 0.001$). While 59.4% of the zygotes had structural aberrations by DAPI analysis, 94.1% of the same zygotes had structural aberrations by PAINT analysis ($P < 0.001$). The two methods were in close agreement for most types of aberrant chromosomes, with the exception of translocations and insertions, where PAINT analysis was more sensitive than DAPI

Additional Comments:

The authors noted a pre-fertilisation effect (reduced fertilisation rate) and post-fertilisation (reduced zygotic development)

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Chromosomal Aberrations

Acrylamide Positive

MARCHETTI1997: Marchetti F;Lowe X;Bishop J;Wyrobek AJ; (1997) Induction of chromosomal aberrations in mouse zygotes by acrylamide treatment of male germ cells and their correlation with dominant lethality and heritable translocations, *Environ Mol Mutagen* 30(4):410-417

Methods:

Male B6C3F1 mice (8-12 weeks) were administered five consecutive doses of acrylamide (5x50 mg/kg bw, Fisher Scientific) i.p in distilled water and mated (for 8h) with untreated females at 8 time points between 2.5-48.5d post final dose (interval 4-8days between mating periods). Females were pre-treated with pregnant mare serum to increase the number of maturing ovarian follicles and human chorionic gonadotrophin to induce ovulation. Females were caged with males for 8h and then 24h after HCG treatment dosed with colchicine to prevent the union of parental pronuclei and arrest zygote development at metaphase of the first cleavage division. Zygotes from 10-15 females were pooled and processed for FISH analysis. Biotin labelled probes for chromosome 1,2,3 or X were detected with avidin FITC (and amplification using biotinylated antiavidin antibodies). Digoxigenin labelled probe was used to visualise chromosome Y. Hybridisation used DAPI counterstain. Microscopic examination included translocations acentrics, dicentrics and other chromosome aberrations. The PAINT nomenclature system was used. Overall 36.55 (and 38.1% including Y) of the total chromosomes were analysed (ca factors for G banding 2.63 and 2.71 respectively). Chi squared analysis was used for effects on fertilisation and zygote development. Fisher exact was used to analyse frequencies of aberrations.

Major Findings:

A reduction in the fertilisation rate was noted at all time points excluding 48.45d (i.e 2.5-41.5d). A post fertilisation reduction in the number of zygotes occurred on days 2.5-12.5 post treatment which equated to repair deficient (late spermatids and early spermatozoa). There was an increase in the percentage of fertilised eggs still at the pronuclear stage which was consistent with cell cycle delay at 2.5 and 6.5 d post treatment (epididymal and early spermatozoa). The authors also note a significant reduction in mated females at 2.5d.

Chromosome aberrations were detected at up to 27.5d post treatment. At 6.5d 76% of zygotes had chromosome aberrations (<50% at 2.5 d and 9.5d) The small but significant increase in chromosome aberrations at 27.5 d equated to an effect on pachytene spermatocytes. The type of aberrations seen at 27.5d consisted of acentric fragments (not in the painted chromosomes). There were no chromosome aberrations in zygotes from animals mated 41.4 and 48.5 d post treatment. The authors reported that all types of chromosome aberration (acentric, dicentrics, translocations, RFL, insertions) peaked at 6.5d. The sum of acentrics or translocations (number per cell equivalent) was 1.5 and 1.3 respectively at 6.5 d and 0.4 at 2.5d and 9.5d. The number of zygotes with 2 breaks/cell was 60% at day 6.5 and 20% at days 2.5, 9.5 and 12.5d the ratio of balanced/unbalanced translocations was 1 at all time points except for a significant increase in unbalanced translocations was recorded at 9.5d ($P<0.02$). With two exceptions out of 310 zygotes chromosome aberrations were found in zygotes from treated males. The authors analysed breaks across painted chromosomes and considered the pattern was random between chromosomes. The cytogenetic response reported in this study (unbalanced translocations) was consistent with the dose-response for dominant lethality previously reported by Shleby in 1986. The response for balanced translocations in this study (28%) was also consistent with the response for heritable translocations reported by Shelby (39%) and by Adler (22%).

Additional Comments:

These data suggest the time interval for inheritable/dominant lethal mutagenicity from later spermatids and early spermatozoa to include pachytene spermatocytes.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Chromosomal Aberrations

Acrylamide Negative

SCHMID1999: Schmid TE;Xu W;Adler ID; (1999) Detection of aneuploidy by multicolor FISH in mouse sperm after in vivo treatment with acrylamide, colchicine, diazepam or thiabendazole, *Mutagenesis* 14(2):173-179

Methods:

Acrylamide (purity not stated) was administered i.p. to male (102/E1xC3H/E1)F1 mice at 60 and 120 mg/kg bw in physiological saline. Caudal epididymides were obtained 22 days post administration, sperm collected, smears prepared and stored at -20°C for FISH analysis. In situ hybridisation was undertaken using probes for chromosome 8 (biotin), chromosome X (biotin and digoxigenin) and chromosome Y (digoxigenin). Labelled probes and mix were denatured in formamide and dehydrated in an alcohol series. Hybridisation was undertaken for 24-48h at 37°C under moist conditions. Detection used streptavidin Cy3 (8), digoxigenin-FITC (y) and a combination for chromosome X; nuclei were counterstained with DAPI. 10,000 cells/animal were scored (total ca 870,000). Scoring included X8 and Y8 as normal, five hyperhaploid classes were scored for first meiotic division (XX8, YY8, XY8, X88, Y88) and two for second meiotic division as autodiploids (XX88, YY88). Hypohaploidy was scored but not included in statistical analyses. Statistical analysis was by chi square test.

Major Findings:

There was no increase in the diploidy or hyperhaploidy frequency reported at either dose level. Concurrent studies using 1.5 and 3.0 mg/kg bw colchicine revealed positive results for hyperhaploidy using summed data for the three FISH probes used in this study.

Mechanistic Data:

Authors suggest acrylamide did not have an aneugenic effect in mouse sperm under the test conditions.

Additional Comments:

Positive with colchicine (1.5 and 3 mg/kg single dose, i.p.), diazepam (300 mg/kg single dose by oral intubation) or thiabendazole (300 mg/kg daily for 11 days by oral intubation).

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Embryo Micronucleus

Acrylamide Positive

TITENKAHOLLAND1998: Titenka-Holland, N., Ahlborn, Thomas, Lowe, Xiu, Shang, Nang, Smith, Marilyn T., and Wyrobek, Andrew J. (1998) Micronuclei and Developmental Abnormalities in 4-Day Mouse Embryos After Paternal treatment with Acrylamide, Environmental and Molecular Mutagenesis 31:206-217

Methods:

Acrylamide (50 mg/kg, BioRad) was administered to male C57BL/6J mice i.p. in sterile PBS for 5 consecutive days. These were mated with untreated female C3H/J mice (1:3-4) for 5-17 days after the end of treatment to sample various postmeiotic cells. Pregnancy was determined by vaginal plug and females were sacrificed at 86-88h after mating. Females were injected with colcemid (0.2 ml, 0.01%) 2 hr prior to embryo isolation. 6-10 embryos were recovered per female. Each embryo was processed for cytological evaluation using DAPI staining for interphase, metaphase, fragmented and pyknotic nuclei, micronucleated cells and the number of micronuclei per micronucleated cell. FISH staining was performed to analyse centromere DNA content of MN. Nuclear and micronuclear areas were determined. 10 nuclei were measured in normal embryos and nuclear areas of all cells with MN were measured. Generalised linear models were used to analyse the data. Pair-wise comparisons were performed by Fishers exact test.

Major Findings:

10% loss of treated males within 24 hours of last treatment but no detectable effect on mating. Ovulated eggs and embryo recovery per female were similar.

Large increase $p < 0.001$ in frequency of abnormal embryos after acrylamide treatment (e.g. single-cell embryos, embryos with one lysed blastomere and embryos with < 10 cells). Average cell number/embryo significantly ($p < 0.01$) higher in control than treated group. More embryos in treated group lacked metaphases ($p < 0.001$) and more than 65% of embryos in the treated group had < 10 cells.

Embryos with one or more pyknotic nucleus were more common in control than treated groups ($p < 0.001$) and more prevalent in normal rather than abnormal embryos of both groups ($p < 0.001$). Fragmented nuclei were more frequent in abnormal than normal embryos and more frequent in normal embryos of treated group than control group ($p < 0.05$).

More preimplantation embryos had at least one micronucleus in the treated group compared to vehicle control ($p < 0.05$), particularly in normal embryos ($p < 0.01$). Average micronuclei per embryo doubled compared to control ($p < 0.05$). When adjusted for cell number, micronucleus frequencies were 4 / 1000, control; 41 / 1000, treated normal embryos ($p < 0.001$), and 93 / 1000, treated abnormal embryos ($p < 0.001$). Difference between normal and abnormal cells was also significant ($p < 0.001$). No association was found between cell number per embryo and presence of MN.

Both centromere positive and negative micronuclei were increased ($p < 0.001$) with acrylamide treatment, which was also significant in abnormal vs normal treated embryos ($p = 0.002$). There was no change in ratio of centromere positive and negative micronuclei between control and treated and normal and abnormal embryos.

Contingency table analysis was used to examine the data, which suggested that micronuclei, fragmented and pyknotic nuclei appeared to be induced by independent processes in preimplantation embryos

Additional Comments:

Authors suggest that lacking metastases and lower number of cells is due to reduced proliferative activity and that the results suggest that both clastogenic and aneugenic mechanisms are involved. (see Holland 1999)

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell DNA Adducts

Acrylamide Positive

HOLLAND1999: Holland N;Ahlborn T;Turteltaub K;Markee C;Moore D;Wyrobek AJ;Smith MT; (1999) Acrylamide causes preimplantation abnormalities in embryos and induces chromatin-adducts in male germ cells of mice, *Reprod Toxicol* 13(3):167-178

Methods:

Performed as per Turteltaub 1993 (*J Cell Biochem, Suppl*:17F, 138-48). 10 Male C57B1/6J mice (7 to 12 weeks of age) were administered ¹⁴C-acrylamide (50 mg/kg bw, 3 mCi/mol, no source or purity data). 2 mice were sacrificed every three days. Sperm was extracted from cauda epidymes, and then the nuclei were isolated and converted to graphite for ¹⁴C analysis by AMS

Major Findings:

Chromatin associated radioactivity was significantly ($p < 0.001$; t-test) increased 3 and 9 days post treatment (16.5 ± 2.7 and 19.3 ± 2.6 μ g acrylamide/g sperm, respectively)

Additional Comments:

The authors consider that this indicates that sperm and late stage spermatids have the highest level of adduct formation, which coincides with the period when the maximum increase is seen in abnormal embryo frequency (see summary). They also note that this technique is unable to differentiate between DNA adducts and protamine adducts.

Acrylamide Positive

XIE2006: Xie Q;Sun H;Liu Y;Ding X;Fu D;Liu K; (2006) Adduction of biomacromolecules with acrylamide (AA) in mice at environmental dose levels studied by accelerator mass spectrometry, *Toxicol Lett* 163(2):101-108

Methods:

[2,3-¹⁴C]-acrylamide (0, 0.075, 0.75, 7.5, 93, 240 and 1000 μ g/kg bw; 5 Ci/mol; American Radiolabeled Chemical Inc., St Louis, MO, USA) was administered i.p to adult ICR mice (n=20 per dose and 5 per sample). Animals were sacrificed 24 h post administration and blood and testes were epididymes were collected. Haemoglobin was isolated according to Li et al. 2003 (*Toxicol Lett* 139:25-32), protamine was isolated according to Balhorn et al. 1997 (*Biochemistry* 16:4074-80). Sperm DNA precipitated during protamine isolation was purified according to Sega et al. 1974 (*Mutat Res* 24:317-33). Sperm heads and tails were isolated by density gradient centrifugation

Isolated biomacromolecules were converted to graphite according to Vogel 1992 (*Radiocarbon* 34:344-50). ¹⁴C, ¹³C and ¹²C were measured simultaneously using a 2 x 0.6 MV Tandem AMS facility (National Electrostatic Co., USA) at Peking University, and normalised to the ¹⁴C/¹²C ratio (with a machine background of $< 4 \times 10^{-16}$ and precision $< 1\%$). The isotope ratio was converted to acrylamide equivalents (ng) per gram of tissue. This method is unable to differentiate between the parent compound and metabolites.

Major Findings:

A plot of log acrylamide adducts (ng/g) against log acrylamide dose showed a linear relationship for haemoglobin, serum albumin, protamine, and sperm head and tail adducts. Sperm DNA adducts showed a similar linear response within the dose range 7.5 to 1000 μ g/kg bw, but lower doses (0.075 and 0.75 μ g/kg bw) were not significantly different from background. Comparison of the plotted DNA and protein acrylamide adducts appears to show that, to achieve an equivalent level of adducted acrylamide, DNA adducts require a dose that is two orders of magnitude greater than that for protein adducts.

Additional Comments:

This study used relevant doses 50 to 50,000 fold lower than that used by Holland et al. (1999). The authors point out that the lowest doses in this study (0.075 and 0.75 μ g/kg bw) are below the WHO estimated average daily intake for humans (1 μ g/kg bw)

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell UDS

Glycidamide Positive

GENEROSO1996: Generoso WM;Sega GA;Lockhart AM;Hughes LA;Cain KT;Cacheiro NL;Shelby MD; (1996) Dominant lethal mutations, heritable translocations, and unscheduled DNA synthesis induced in male mouse germ cells by glycidamide, a metabolite of acrylamide, Mutat Res 371(3-4):175-183

Methods:

Glycidamide (150 mg/kg bw; Polyscience, Washington, PA, USA; purity 100%) in Hanks Balanced Salt Solution was administered i.p to male C3H/RLx101RL F1 mice (12-20 weeks n= 3-4). Subsequently, 3H thymidine was administered (intra-testicular) at 0, 2, 4 & 6 hours post glycidamide exposure. Sperm were recovered from caudal epididymides 16 days post treatment (and analysed animal by animal) using 3H-dpm/million sperm as the index of incorporation. Methyl methanesulphonate (MMS) served as a positive control.

Major Findings:

There was a significant incorporation of 3H-thymidine at all time points including 0 (ca 6 x control). The maximum UDS response was seen after 2 h (ca 18 x control). The UDS response was significant at 4 and 6 h but was reduced compared to 2 h.

Role of Metabolism:

The authors compared the maximal sperm UDS response of acrylamide with the data on glycidamide and noted the time point of maximum for acrylamide (ca 5h) was the longest delay for mouse germ cells reported to date and it was inferred from the shorter maximum peak effect for acrylamide that the data were consistent with metabolism of acrylamide. The authors calculated that the maximal response seen with 150 mg/kg bw glycidamide equated to that seen from 125 mg/kg bw acrylamide at the respective maximum response times to glycidamide for the germ cell effects.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Germ Cell Other

Acrylamide Positive

GASSNER1995: Gassner P;Adler ID; (1995) Analysis of chemically induced spindle aberrations in male mouse germ cells: comparison of differential and immunofluorescent staining procedures, *Mutagenesis* 10(3):243-252

Methods:

Acrylamide (80 & 120 mg/kg bw, Sigma, purity not stated) was administered to male (102/E1 x C3H/E1)F1 mice i.p. in PBS. Animals were killed 6 h (120 mg/kg bw) and 24 h (80 & 120 mg/kg bw) after treatment, the testes were removed and germ cells extracted from coarsely cut tubulus mass. Cells were allowed to settle onto slides in an isotonic solution (calcium & magnesium chloride to preserve spindle), then fixed for staining. Differential staining was performed with Brilliant Blue R (0.3%) and Safranin O (0.5%); method slightly modified from Wissinger et al. 1981 (*Stain Technol.*, 56:221-6). 100 cells analysed per slide. Immunofluorescent spindle staining was performed using anti-alpha-tubulin with a FITC conjugated secondary antibody and counterstained with propidium iodide; method modified from Wichenlaub-Ritter et al. 1986 (*Chromosoma*, 94:337-45). 50 cells analysed per slide. 2 slides were used from each animal for each staining technique. Vinblastine used as a positive control. Data was probed for binomial dispersion (Snedecor-Cochran test) then significant differences determined by Pearson's chi-square test.

Major Findings:

Acrylamide caused significant increases in spindle disturbances, identified by immunofluorescence at (17.0% at 120 mg/kg bw) and differential staining (4.75% at 120 mg/kg 24 h post treatment). Increases in misplaced chromatin identified by differential staining (4.75% at 80 mg/kg (24 h), and 3.75% (6 h) and 3.00% (24 h) with 120 mg/kg). Acrylamide predominantly caused the formation of multipolar spindles.

Mechanistic Data:

The authors considered that the effects of acrylamide cannot be assigned to interactions with specific elements of the spindle; but possibly acting by binding to various spindle proteins.

Additional Comments:

This experiment aimed to compare the two staining techniques comparing slides from the same animals. Acrylamide mainly caused multipolar spindles which the authors suggest possibly develop from a separation of mother and daughter centrioles during an unspecific meiotic block. Multipolar spindles indicate malfunction of centrioles or centrosomes, which may be an effect of acrylamide on microtubule associated protein, delaying meiosis and causing premature centrosome separation. Authors also suggest as acrylamide binds SH-rich proteins, interactions with kinetochore proteins may be reason for loss of chromosome-spindle contacts.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation (Drosophila)

Acrylamide Positive

VOGEL 1993: Vogel EW; Nivard MJ; (1993) Performance of 181 chemicals in a Drosophila assay predominantly monitoring interchromosomal mitotic recombination, *Mutagenesis* 8(1):57-81

Methods:

Acrylamide ('commercially available') was applied to the surface of the food (5, 15 & 30 mM) or by direct acute larval exposure (40 & 80 mM, 15 min) Drosophila strain used C1- y (yellow) females with w (white) males. Per compound 2 separate expts either at the same exposure dose or in the case of a strong response at a lower exposure dose and to score 250 eyes per dose group. With chemicals not showing an effect in the w/w+ test at least 1500 eyes were scored. Estimation of genotoxic effectiveness in eye disc cells by calculating frequency of clones per 104 cells $f=2nm/NC$ (n=number of mosaic spots, m=mean clone size, N=number of eyes analysed, C= \sim 800 ommatidia).

Major Findings:

Acrylamide clearly positive according to statistical criteria but unable to establish a dose response relationship. Surface treatment: 'weak' positive at 5 mM, negative at 15 mM, & weak positive at 30 mM. Acute treatment: 'weak' positive at 40 and 80 mM.

Additional Comments:

Test system has limited relevance for human health hazard assessment.

Acrylamide Positive

BATISTEALENTORN 1994: Batiste-Alentorn M; Xamena N; Creus A; Marcos R; (1994) Further studies with the somatic white-ivory system of *Drosophila melanogaster*: genotoxicity testing of ten carcinogens, *Environ Mol Mutagen* 24(2):143-147

Methods:

Acrylamide (1.5 & 2 mM; Sigma) was tested using the drosophila white ivory system (males: y2 DP (1:1:1:1)wi; females: attached X-chromosomes (C(1)DX, y f)). 200 larvae/vial were treated 72 hr after egg laying. Emerging males were examined for eye-colour mosaicism and the no. of pigmented ommatidia determined; expressed as frequency of sectors with altered pigmentation. Where multiple sectors occurred in different eyes or as widely separated patches (separated by at least 4 non-mutated ommatidia) in the same eye these were attributed to independent events. Treatment was considered positive if the mutation frequency in the treated series is 2 x that of the control series.

Major Findings:

Positive result at 1.5 mM but inconclusive at 2 mM.

Role of Metabolism:

Unable to activate B[a]P

Additional Comments:

Positive result for cyclophosphamide, 4-nitroquinoline N-oxide, and propyleneimine. 'Weak Positive' results for Diethylstilbestrol and acrylamide. induced significant positive results but without a dose-response relationship, and safrole was weakly positive. On the other hand, acetamide, benzo(alpha)pyrene, thiourea, and o-toluidine were unable to increase the frequency of mutant clones. Test system has limited relevance for human health hazard assessment.

Acrylamide Mutagenicity Review - Annex 1 Table 1

In Vivo Gene Mutation (Drosophila)

Acrylamide Positive

BATISTEALENTORN1995: *Batiste-Alentorn M; Xamena N; Creus A; Marcos R; (1995) Genotoxic evaluation of ten carcinogens in the Drosophila melanogaster wing spot test, Experientia 51(1):73-76*

Methods:

Acrylamide (1, 1.5 & 2 mM; Sigma) was administered in water to 2 Drosophila strains: multiple wing hairs strain (y: mwh jv) and the flare strain (flrA/ln(3LR)TM3, Ser. Eggs from mwh virgin females crossed with flr3 males were collected. 3 days after emergence, larvae were placed in vials (50/vial) and treated with acrylamide. Larvae fed until pupation. Trans-heterozygous (mwh + / + flr3) were selected and wings were scored for presence of clones showing malformed wing-hairs. Single spots showed either mwh or flr3 phenotype, twin spots show adjacent mwh and flr3 areas. Small single spots (1-2 cells), large single spots (>2) and twin spots were scored. Negative control was Drosophila Instant Medium and distilled water.

Major Findings:

Acrylamide induced a small but statistically significant increases in the appearance of visible mutant hair clones on the adult wing blade. Small single spots frequency (m=2) positive at highest dose tested (2.0 mM). Large single spots frequency (m=5) positive at 1.0 mM but inconclusive at higher doses. Twin spots (m=5) Negative at all doses. Total spots (m=2) positive at 2.0 mM.

Role of Metabolism:

Unable to activate B[a]P

Additional Comments:

Positive result with acetamide, cyclophosphamide, 4-nitroquinoline N-oxide, propyleneimine, and o-toluidine. Negative result with benzo(a)pyrene, diethylstilbestrol, safrole and thiourea. Test system has limited relevance for human health hazard assessment.

Acrylamide Negative

PONTECORVO2006: *Pontecorvo G; Fantaccione S (2006) Recombinogenic activity of 10 chemical compounds in male germ cells of Drosophila melanogaster, Ecotox. Environ. Safe. 65(1):93-101*

Methods:

(1 and 2 mM, source not stated, >99.5% pure). Wild type (Oregon-R) Drosophila melanogaster and a stock marked with four recessive mutations on the second chromosome (dp (dumpy) (13.0), b (black) (48.5), cn (cinnabar) (57.5 and bw (brown)104.5, centromere located between b and cn). Three day larvae heterozygous for these markers were orally exposed to test materials for up to 3 days and the phenotype of F1 males was examined microscopically. For analysis clusters of identical and complimentary recombinant phenotypes were considered as a single independent event. Three regions were evaluated dp-b (1) b-cn (2), cn-bw (3), and total regions (1+2+3). Significance testing was based on Kastenbaum and Bowman and standard tables of cumulative binomial distribution prepared by these authors.

Major Findings:

Acrylamide did not induce any recombinogenic effect. Positive controls Nitroquinoline N-oxide (10 mM) and Hydroxylamine (60 mM) induced positive results.

Role of Metabolism:

Not specifically examined in this study. The authors considered lack of metabolism to glycidamide might have been important with regard to the negative results from this study.

Additional Comments:

Test system has limited relevance for human health hazard assessment.