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Acrylamide effects on kinesin-related proteins of the mitotic/meiotic spindle

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Abstract

The microtubule (MT) motor protein kinesin is a vital component of cells and organs expressing acrylamide (ACR) toxicity. As a mechanism of its potential carcinogenicity, we determined whether kinesins involved in cell division are inhibited by ACR similar to neuronal kinesin [Sickles, D.W., Brady, S.T., Testino, A.R., Friedman, M.A., and Wrenn, R.A. (1996). Direct effect of the neurotoxicant acrylamide on kinesin-based microtubule motility. *Journal of Neuroscience Research* 46, 7–17.] Kinesin-related genes were isolated from rat testes [Navolanic, P.M., and Sperry, A.O. (2000). Identification of isoforms of a mitotic motor in mammalian spermatogenesis. *Biology of Reproduction* 62, 1360–1369.], their kinesin-like proteins expressed in bacteria using recombinant DNA techniques and the effects of ACR, glycidamide (GLY) and propionamide (a non-neurotoxic metabolite) on the function of two of the identified kinesin motors were tested. KIFC5A MT bundling activity, required for mitotic spindle formation, was measured in an MT-binding assay. Both ACR and GLY caused a similar concentration-dependent reduction in the binding of MT; concentrations of 100 μM ACR or GLY reduced its activity by 60%. KRP2 MT disassembling activity was assayed using the quantity of tubulin disassembled from taxol-stabilized MT. Both ACR and GLY inhibited KRP2-induced MT disassembly. GLY was substantially more potent; significant reductions of 60% were achieved by 500 μM, a comparable inhibition by ACR required a 5 mM concentration. Propionamide had no significant effect on either kinesin, except KRP2 at 10 mM. This is the first report of ACR inhibition of a mitotic/meiotic motor protein. ACR (or GLY) inhibition of kinesin may be an alternative mechanism to DNA adduction in the production of cell division defects and potential carcinogenicity. We conclude that ACR may act on multiple kinesin family members and produce toxicities in organs highly dependent on microtubule-based functions.

Keywords: Acrylamide; Glycidamide; Kinesin; Microtubule; Cell division; Mitosis; Meiosis

Introduction

Acrylamide (ACR) is a monomer used in the manufacture of water-soluble polymers with a wide variety of industrial and scientific applications including water purification, paper and fabric manufacture, as well as the mining industry and gel electrophoresis (Dearfield et al., 1995). Interest in acrylamide has been stimulated recently by the finding of acrylamide in carbohydrate-rich foods, such as potato chips, French fries, breads, and cereals, that have been cooked at high temperatures (Tareke et al., 2000). Therefore, ACR mechanistic studies are clearly relevant to both human occupational and environmental risk assessments. The majority of mechanistic research has been directed towards understanding the well-documented neurotoxicity of ACR. As a result, ACR studies have been both useful in defining the functionality of neuronal proteins as well as defining the consequences of loss-of-function or intracellular proteinaceous accumulations. Collectively, these studies have been critical in revealing potentially viable mechanisms of neurodegenerative disease pathogenesis (Sickles et al., 2003). Other toxicants that are dissimilar in chemical structure but with similar systemic toxicities (e.g., organic diketone solvents) may produce similar toxicities by action on the same target protein(s) (Sickles, 1989b). Therefore, identifying the target proteins of ACR action has even farther reaching significance.

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Examination of homologous target proteins in non-neuronal cells may prove productive in determining mechanisms of ACR reproductive toxicity and/or carcinogenicity (see below).

ACR serves as a prototypic chemical for neuropathic mechanisms. It produces a distal, dying-back, sensory-motor polyneuropathy of the central and peripheral nervous systems (Spencer and Schaumburg, 1988). Axons accumulate both neurofilaments and tubulovesicular organelles, particularly on the proximal sides of nodes of Ranvier in distal axons. The mechanism of action has been investigated for five decades. Accumulating data support the emerging pathogenic mechanism of inhibition of the microtubule-based motor protein kinesin (Sickles et al., 1996) with subsequent compromise in fast anterograde axonal transport (Sickles, 1989a) and distal axonal protein deficiencies. Specific protein deficiencies cause dysfunction and/or degeneration of distal axons as well as the neurotoxicological symptoms. Kinesin is the most sensitive identified target of acrylamide to date with significant inhibition of bovine brain kinesin by as little as 50 μM (Sickles et al., 1996), more than one order of magnitude below the typical daily neurotoxic dose of 0.7 mM (50 mg/kg) in rats.

In addition to neurotoxicity, acrylamide is classified as a probable human carcinogen as well as a male reproductive toxicant (Dearfield et al., 1988). Studies have focused on DNA adduction as a possible mechanism for the production of mutations and/or subsequent carcinogenicity (Segerback et al., 1995; Gamboa da Costa et al., 2003; Doerge et al., 2005). However, acrylamide is a weak mutagen (Allen et al., 2005). When the time course of induction of dominant lethal mutations is compared with sperm DNA and protein alkylation, protein (and not DNA) alkylation correlates with the mutations. These results suggest that interaction with proteins is more critical than reaction with DNA (Sega et al., 1989; Sega et al., 1990). A major confusing factor in understanding the relevance of acrylamide DNA adduction is that acrylamide is not mutagenic in cells containing cyp2e1 which metabolizes acrylamide to glycidamide. Glycidamide is the DNA reactive metabolite of acrylamide and alone is mutagenic (Hashimoto and Tanii, 1985; Cliffer et al., 2005; Ghanayem et al., 2005). However, when glycidamide is produced as a result of acrylamide metabolism in vitro, it appears to have a very different mutagenicity profile. Therefore, while the metabolism of acrylamide to glycidamide is a significant toxicological event (Adler et al., 2000; Ghanayem et al., 2005), explanations of the clastogenicity appear to require an alternative mechanism other than DNA adduction (Wozniak and Blasiak, 2003).

The potential for ACR action on proteins of the mitotic/meiotic spindle has been given little consideration. A number of adverse effects of acrylamide, including aneuploidy (Cao et al., 1993; Parry et al., 1996; Schmid et al., 1999; Pihan and Doxsey, 2003), generation of micronuclei with kinetochores (Schriever-Schwemmer et al., 1997; Jie and Jia, 2001), dominant lethal effects (Marchetti et al., 1997; Adler et al., 2000), delays in the cell cycle (Gassner and Adler, 1996), blocks in mitosis or meiosis (Adler et al., 1993; Sickles and Welter, 1995; Sickles et al., 1995; Schmid et al., 2001), heritable translocations (Adler et al., 1994, 2004; Dearfield et al., 1995) and chromosomal aberrations (Marchetti et al., 1997) suggest the action of acrylamide on proteins of the mitotic spindle (Preston, 1996). Studies on the effects of acrylamide on microtubules have either been negative (Shiver et al., 1992; Harris et al., 1994) or demonstrated a very limited effect (Gupta and Abou-Donia, 1997). Microtubule-associated motor proteins are also essential components of the spindle. Mutations, deletions or antibody blocking (Nislow et al., 1990; Barton and Goldstein, 1996; Heald, 2003; Marcus et al., 2005) of these motors produces similar phenotypic outcomes as those identified above for ACR. Therefore, kinesin motors are logical targets due to their critical involvement in cell division (Heald, 2003) and our previous observations of acrylamide-induced neuronal kinesin inhibition (Sickles et al., 1996). This potential site of action of acrylamide and glycidamide has not been given any previous consideration.

The kinesin superfamily of proteins in mammals is composed of approximately 45 members. The common feature of this superfamily is a 340-amino-acid globular motor domain possessing both microtubule binding and ATPase activity. A combination of reversible MT binding, conformational changes and ATP hydrolysis allows most kinesins to walk along microtubules in their delivery of cargos to various cellular domains. Most kinesins possess the motor domain in the N-terminal domain and are predominantly plus end directed motors. Due to the radial disposition of most cytoplasmic microtubules with plus ends towards the plasmalemma, kinesins typically transport their cargo towards the cell periphery. A few members possess C-terminal motor domains and are minus end directed, while a third category possess a centrally (middle) placed motor domain and have been identified as MT disassemblers rather than locomotors. Genes coding for C-terminal motors have been associated with formation of the spindle and its maintenance (Gaglio et al., 1997; Walczak et al., 1997; Mountain et al., 1999), while M(or I)-type kinesins are correlated with the regulation of spindle dynamics and chromosomal movement during anaphase A (Walczak et al., 1996; Maney et al., 1998, 2001; Desai et al., 1999; Mountain et al., 1999). Variation in the non-motor domain contributes to kinesin diversity in the cargo that it transports, including organelles, membrane-bound vesicles, protein complexes, ribonuclear proteins and even chromosomes (Brady and Sperry, 1995; Mitchell, 2003).

To test the hypothesis that kinesin is a common site of action of acrylamide in producing a variety of toxicant effects, in particular cell division effects, we have isolated genes of kinesin-related proteins from rat testes (Navolanic and Sperry, 2000), expressed their proteins in bacteria using recombinant DNA techniques and tested the effects of acrylamide, glycidamide (its P450 oxidative product and potential toxic metabolite) and propionamide (a non-neurotoxic metabolite) on the function of two of the identified kinesin motors. The current results indicate that acrylamide and glycidamide specifically inhibit C-type and M-type kinesin motors in a similar concentration range as the neuronal N-type kinesins (Sickles et al., 1996). This is the first observation of acrylamide-induced inhibition of motor proteins associated with the mitotic/meiotic spindle. The inhibition of these kinesin motors has relevance to mutagenicity, cell cycle, and reproductive effects as well as potential carcinogenicity of acrylamide.

Materials and methods

Unless otherwise indicated, all chemicals were obtained from Sigma-Aldrich and were of the highest available purity. Acrylamide was electrophoresis grade (Bio-Rad 161–0100), and propionamide was from Aldrich #14,393-6. Glycidamide was a kind gift from Dr. Tim Fennel, Research Triangle Institute. Based on NMR analysis, the glycidamide was 84% pure. Two major impurities, glyceramide and 3-hydroxypropionamide, made up 12 and 4% of the sample, respectively. Neither of the contaminants are known toxicants. The same batch of glycidamide was injected into mice for another study and valine adducts on hemoglobin were almost purely due to glycidamide; the glycidamide to acrylamide adduct ratio was 720:1.

Details of the identification of kinesin-related protein (KRP) genes, their cloning and incorporation into expression vectors may be obtained from the well-established protocols described in detail elsewhere (Sperry and Zhao, 1996; Navolanic and Sperry, 2000). Specific details relevant to the two kinesin motors tested, KRP1 (KIFC5A) and KRP2, are provided below. Gene expression of motor proteins was conducted using previously published methods (Saxton, 1994), kinesin motor activity assays and Western blots were conducted as previously (Sickles et al., 1996).

Cloning and sequencing of KRP1 and KRP2, bacteria transformation, and optimization of KRP expression. Messenger RNA was isolated from rat testes and subjected to RT-PCR using degenerate primers to conserved sequences in the kinesin motor domain. Six different sequence classes were identified in rat testis mRNA by this method and designated KRP1-6. The specific sequences are provided elsewhere (Sperry and Zhao, 1996). The motor head domain fragments for KRP1 and KRP2 were then used to isolate full-length clones from either a mouse 15-day embryonic cDNA library (Navolanic and Sperry, 2000) or a rat testis cDNA library (Sperry and Zhao, 1996), respectively.

For KRP1, twelve potential positive clones were identified in the initial screen using the rat KRP1 motor domain sequence as a probe (Sperry and Zhao, 1996). The insert size for each clone was determined by PCR using oligonucleotide primers to either the vector sequences flanking the cloning site or the vector and head domain sequences. One clone was identified with an insert size of about 2 kb, sufficient to encode KIFC1, a related motor from mouse (Saito et al., 1997) and was further purified by two rounds of infection, plating, and hybridization to the KRP1 head probe. This positive clone was sequenced, in both directions, with overlapping oligonucleotide primers either directly from the phage DNA or after subcloning into Bluescript plasmid, using the fmol sequencing kit from Promega. Sequence analysis revealed that this clone is an isoform of the KIFC1 motor. Alignments were also generated against sequences in the Genbank databases using the National Center for Biotechnology Information (NCBI) Blast server. This sequence is reported elsewhere (Navolanic and Sperry, 2000) and deposited in the database as accession number AF221102. Sequence analysis of this clone showed that it is related to motors involved in stabilizing the spindle pole, including *Xenopus* XCTK2, *Drosophila* ncd, and hamster CHO2. All are C-terminal motors that both bind MT and move towards MT minus ends. Comparison to KIFC1 (at first thought to be identical) showed that KRP1 was a closely related isoform. KRP1 is identical to KIFC1 except for the insertion of two sequence blocks (120 nt and 60 nt) in its tail domain. This is interesting because the tail domain attaches to cargo, in this case other MTs. According to the nomenclature of mouse kinesin-related proteins, this protein was named KIFC5A (Navolanic and Sperry, 2000).

The KRP2 head domain isolated from rat testis mRNA by the PCR method described above was used to screen a lambda-gt10 rat testis cDNA library (Sperry and Zhao, 1996). Sixteen positive plaques were identified and purified twice by infection and replating. One clone was identified with an approximately 3 kb insert containing a 2015-nt open reading frame with high sequence homology to the depolymerizing kinesin MCAK. This clone was named KRP2 and was sequenced, in both directions, with overlapping oligonucleotide primers either directly from the phage DNA or after subcloning into Bluescript plasmid, using the fmol sequencing kit from Promega. The KRP2 sequence was previously reported (Navolanic and Sperry, 2000) and is available as accession number U44979. Both the full-length KRP2 ORF and a smaller fragment of 1.3 kb were cloned into a QE vector for protein expression. The 1.3-kb fragment encodes an approximately 50-kDa protein containing the motor core of this protein. This smaller form of KRP2 expressed more efficiently than the full-length clone and is used for all experiments described here.

Both KIFC5A and KRP2 were cloned into the bacterial expression vector QE31 and expressed as fusion proteins to the 6HIS tag. The 6HIS tag allowed for subsequent identification of the expressed proteins on Western blots, using antibodies against the 6 histidine residues. M15 cells were transformed according to the well-established $CaCl_2$ method (Morrison, 1977). DH5α cells (Invitrogen) were transformed using the manufacturer's protocol. Briefly, the DNA was mixed with an aliquot of cells and incubated on ice for 30 min. The cells were then heat shocked at 42 °C for 45 s followed by incubation on ice for 2 min. One milliliter of LB (Luria Broth; Sigma T-9410, Y-1625, S-7653) was added and the cells were grown, with shaking, at 250 rpm for 1 h to allow expression of the selectable marker (ampicillin; Sigma A-9518). The cells were plated at the appropriate dilution on selectable plates. Colonies were selected, DNA prepared by miniprep, and the presence of the correct plasmid confirmed by restriction enzyme digestion.

KIFC5A or KRP2 was expressed in both M15 and DH5α cells. Transformed bacteria were cultured numerous times to identify the optimal conditions for protein expression. Modifications in time and temperature of bacterial growth as well as the concentration of the inducer isopropylthiogalactopyranoside (IPTG; Sigma I-6758), temperature and time of induction were all tested for optimal expression. The optimal conditions for M15 bacterial growth was 1.5 to 2 h in a large culture flask at 37 °C. DH5α bacteria were optimally grown for 4.5 to 5.5 h at 37 °C. The final optical density at 600 nm for each batch of bacteria was 0.7 to 0.9. KIFC5A was optimally induced by 0.2 mM IPTG at room temperature for 16–17 h. KRP2 expression was optimal at 0.4 mM IPTG at 37 °C for 4 h.

Following expression of KIFC5A or KRP2, cells were collected by centrifugation at 4000×*g* for 10 min at 4 °C, washed with PME buffer (0.1 M Pipes (Sigma P6757), pH 6.9, 5 mM $MgSO_4$ (Sigma M7506)1 mM EGTA(Sigma E4378)) plus protease inhibitors (0.1 mM PMSF (Sigma P7626), 10 μg/ml leupeptin (Sigma L8511), 10 μg/ml pepstatin (Sigma P5318), 1 μg/ml aprotinin (Sigma A6103)) and spun again. The resultant pellet was frozen overnight at −80 °C. The pellet was resuspended in 20 ml of PME buffer plus 100 μg/ml lysozyme (Sigma L7651) and incubated on ice for 15 min. Protease inhibitors were added and the preparation sonicated on ice four times for 20 s each. After sonication, DNAse I (Roche #104159) was added to 50 μg/ml and incubated on ice for 30 min or overnight at 4 °C. KIFC5A was purified from the lysate using a single cycle of AMP-PNP (Sigma A2647) induced binding to taxol (Sigma T7402) stabilized MT, ultracentrifugation and ATP induced release of KIFC5A from the microtubules during a second ultracentrifugation (Boleti et al., 1996). We repeatedly expressed and purified KIFC5A with the proper molecular weight of 66 kDa (see results below), which reacted with the His6 antibodies (gift from Dr. Sperry). We could identify KRP2 in bacterial lysates (MW=48K) using the His6 antibody. However, KRP2 could not be purified with the MT affinity binding method. This is likely due to MT disassembly activity of this kinesin (clarified below). Both KRPs were also reactive with the sea urchin kinesin (SUK4) antibody (Developmental Studies Hybridoma Bank), which recognizes the common motor domain of kinesin proteins (data not shown).

KIFC5A expression and purification. Bacterial lysates or purified kinesin (from AMP-PNP induced MT affinity purification) was analyzed by SDS-PAGE gels and/or Western blots. Fig. 1 demonstrates that purification eliminates almost all of the bacterial derived proteins and greatly enhances the concentration of KIFC5A. The purified KIFC5A lane demonstrates a protein of approximately 66 kDa that was confirmed as KIFC5A in the Western blot using His6 antibodies directed against the multiple histidine amino acids engineered into the protein. This protein band was also identified using SUK4 antibody that recognizes kinesin motor domains (data not shown). The band at 55 kDa in the SDS-PAGE gel of the purified KIFC5A is residual tubulin used in the purification (confirmed on other blots with α-tubulin antibody; data not shown). The Coomasie-stained SDS-PAGE gel did not show concentration of any other protein. No other proteins were identified on the Westerns following immunostaining with SUK4 antibody which recognizes the common motor domain of kinesins. Therefore, it appears that no endogenously expressed kinesin could be responsible for the results.

KIFC5A functional assay. Initially the supernatant from the MT affinity purifications was tested for KIFC5A-based MT motility activity as previously conducted (Sickles et al., 1996). Motility was not observed despite varying the concentration of KIFC5A or enhancing KIFC5A adsorption using either multiple adsorptions or precoating coverslips with His6 antibodies. Kinesin expression was conducted at either 37 °C or room temperature overnight. Room-temperature

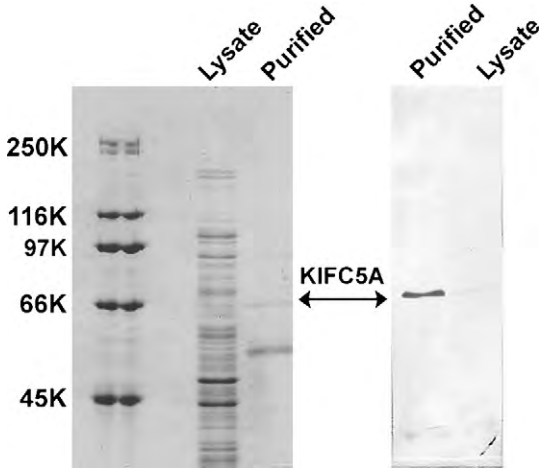

Fig. 1. SDS-PAGE gel (left) and Western blot (right) of the lysates of transfected bacteria expressing KIFC5A as well as the purification supernatant following a single cycle of MT affinity purification. The band at 66 kDa was confirmed as KIFC5A using an antibody to 6 histidine residues (His6) engineered into KIFC5A. Identical results were obtained using sea urchin kinesin (SUK) antibody directed against the common motor domain of kinesins (data not shown). The band at 55 kDa in the SDS-PAGE gel is residual unpolymerized tubulin.

expression has been effective in enhancing the quality of protein processing, folding and development of the proper quaternary structure to produce kinesins with motility functionality (Pesavento et al., 1994). None of these modifications produced KIFC5A with the ability to locomote MT. However, during all these attempts we consistently observed KIFC5A-dependent binding of MT to the coverslip at some concentrations. At higher KIFC5A concentrations large MT bundles in solution above the coverslip were observed. Since the KIFC5A sequence was similar to other kinesins possessing MT binding and bundling activities, we evaluated the feasibility of using the KIFC5A-induced binding of MT to the coverslips as our functional assay.

The KIFC5A-induced binding of MT to coverslips was observed to be concentration-dependent (Fig. 2). With full strength (0.15 mg/ml protein) KIFC5A preparations the predominant feature was the presence of bundled MT in solution above the coverslip [note that this protein concentration includes the residual tubulin in the preparation and is not to be considered the KIFC5A concentration and therefore referred to as protein concentration of the KIF5CA fraction]. With the dilution of the KIFC5A fraction to less than 0.75 mg/ml, the number of MT attached to the coverslip declined in parallel with protein concentrations. More attached MT at the higher protein concentrations was not observed presumably due to bundling of MT in solution eliminating MT from availability to bind to the coverslip. Loss of MT adherence was observed at <0.01 mg/ml. Fig. 2 demonstrates concentration-dependent MT binding between 0.02 and 0.075 mg/ml protein of the KIFC5A fraction. The MT binding and bundling observations are consistent with the function of homologous kinesin family members (Gaglio et al., 1997; Karabay and Walker, 1999; Matuliene et al., 1999; Ems-McClung et al., 2004; Zhang and Sperry, 2004).

We first tested the ability of acrylamide to compromise the MT attachment properties of KIFC5A across the effective range of KIFC5A supernatant concentrations. Initially, freshly purified KIFC5A was preincubated with either zero (control) or 0.7 mM ACR (the concentration used to produce neurotoxicity following multiple exposures) at 37 °C for 20 min prior to adsorption of the KIFC5A onto the glass coverslips. Pictures were taken and the number of MT attached to the coverslip at each dilution of purified KIFC5A from five randomly selected microscopic fields was determined by an investigator without any knowledge of experimental conditions. The assay was performed in triplicate. The results are demonstrated on Fig. 2 and described in the Results section (see below).

KRP2 purification and development of a functional assay. Following bacterial lysis, we attempted to purify KRP2 using AMP-PNP induced MT binding and ATP release. The original bacterial lysate and the supernatant resulting from the purification were examined with Coomasie stained SDS-PAGE gels and Western blots (Fig 3). KRP2 was recognized by a His6 antibody (as well as SUK4 antibody — data not shown). KRP2 was more concentrated in the original lysate than in the purification supernatant and there was an increase in soluble tubulin in the supernatant. This observation is consistent with those of others who had attempted to purify kinesins possessing MT disassembling activity. We concluded, as did others (Walczak et al., 1996), that KRP2 was disassembling MT during the purification and therefore would not precipitate during the MT centrifugation.

Development of KRP2 functional assay. Since we could not purify the protein with the microtubule affinity assay but there was good expression of KRP2, we attempted motility assays with the bacterial lysates. All of the various permutations identified above in the KIFC5A section were attempted. No MT motility was observed. However, those samples possessing KRP2 appeared to possess MT of shorter length. We used VE-DIC microscopy of MT length changes over time to monitor KRP2 activity (data not shown). However, this approach was technically problematic due to the number of sample changes and microscope adjustments required in a small window of time. Furthermore, data points for parallel samples would not be in register temporally, making statistical analyses more complicated.

We subsequently attempted to measure the effects of acrylamide on KRP2 using gel and Western blot analysis of the polymerized versus soluble tubulin. This approach was based on the principal that tubulin assembled into MT may be pelleted by forces of 100,000×*g* for 20 min whereas unassembled tubulin remains in the supernatant. KRP2 disassembly of MT would shift tubulin content from the pellet to the supernatant. Inhibition of KRP2-induced MT disassembly would decrease this shift. To test the feasibility of this technique, full-strength KRP2 lysate was preincubated with and without 0.7 mM ACR for 20 min at 37 °C, diluted to 25% with PME buffer and mixed 1:1 with tubulin-ATP in PME (4 mM MgATP (Sigma A9187) 1 mg/ml taxol-stabilized MT). Final ratios were 12.5% KRP2 to 0.05 mg/ml tubulin. This ratio was determined in preliminary experiments using serial dilutions of KRP2 from full-strength to 0% KRP2 (data not shown). The optical density of the resultant supernatant tubulin band was linear from 0 to 12.5% KRP2. The mixture was incubated 20 min at 37 °C and centrifuged at 100,000×*g* (31K rpm, Beckman SW60Ti rotor) for 20 min.

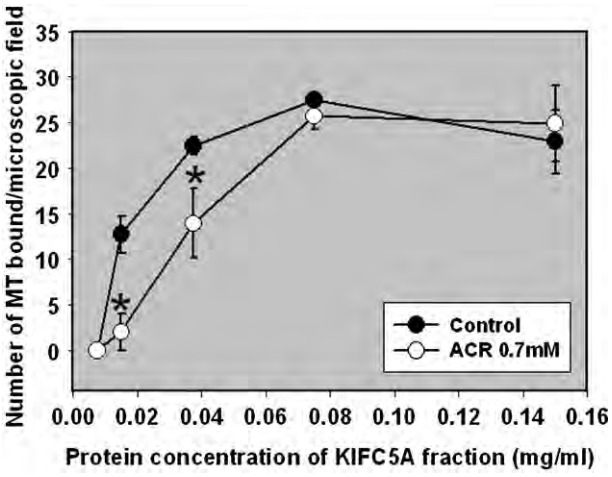

Fig. 2. A comparison of the effects of KIFC5A concentration and the number of MT observed bound to the coverslip with and without preincubation of KIFC5A with 0.7 mM ACR for 20 min at 37 °C. The maximum number of MT bound was observed using >0.75 mg/ml total KIFC5A supernatant protein. Higher concentrations also produced MT bundles in solution above the coverslip. Serial dilutions demonstrated a concentration-dependent decrease in the number of bound MT from 0.075 and 0.0075 mg/ml. Within this range of sensitivity of the assay, preincubation of KIFC5A with ACR produced a significant reduction in bound MT. All subsequent analyses were conducted using 0.04 mg/ml KIFC5A supernatant protein concentration. Each data point represents the average of 3 assays, each assay consisting of 5 randomly selected microscopic fields from different experiments. *$p<0.05$

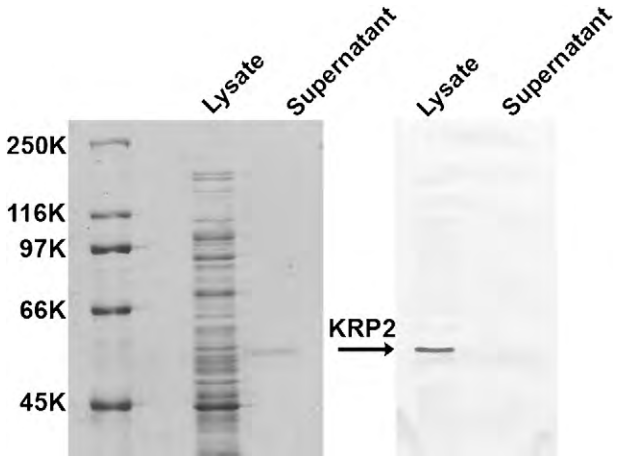

Fig. 3. Coomasie-stained SDS-PAGE gel (left) and Western blot (right) of bacterial lysate and KRP2 purification supernatant. Western blots were stained with His6 antibodies directed against a string of 6 histidine residues incorporated into KRP2. The Coomasie blue positive band in the KRP2 supernatant lane is unpolymerized tubulin. KRP2 would not purify with the MT affinity method due to its disassembling activity. The estimated molecular weight of KRP2 from these analyses is similar to the anticipated molecular weight based on gene sequence.

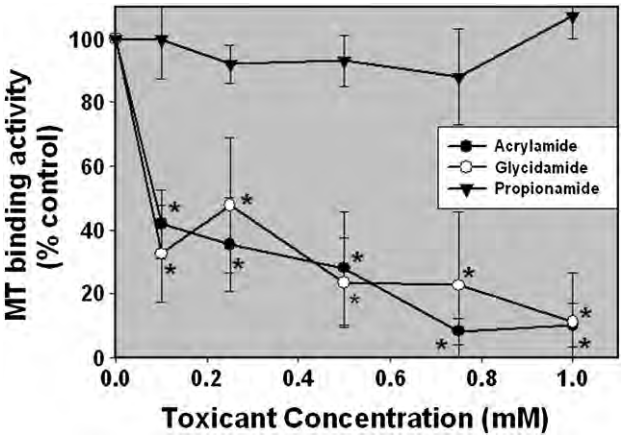

Fig. 5. The effects of acrylamide, glycidamide and propionamide on KIFC5A-dependent MT binding. KIFC5A was preincubated with 0.1–1.0 mM of each toxicant at 37 °C for 20 min prior to adsorption of 0.04 mg/ml of the KIFC5A containing sample onto coverslips and subsequent addition of taxol-stabilized MT. The numbers of MT bound to the coverslip were quantitated in 5 randomly selected fields for 3 different preparations for each dose of each toxicant. Acrylamide and glycidamide produced a similar concentration dependent reduction of KIFC5A dependent MT binding. Propionamide had no effect at any of the tested concentrations.

The pellets and supernatants were run on a 7.5% SDS-PAGE gel and stained with Coomasie blue or transferred for Western blot analysis of tubulin. The pellets were resuspended into the original volume; the supernatants were concentrated to 1/3 original volume using a Centricon 10 for 2 h at room temperature. The nitrocellulose was incubated with monoclonal anti-α tubulin (Sigma #T5168, clone B-5-1-2) mouse ascites fluid at 1:2000 dilution for 2 h at 37 °C. The secondary antibody was a goat anti-mouse IgG HRP conjugate (Bio-Rad 170-6516) at 1:2000 dilutions for 1 h at RT. The blots were developed with diaminobenzidine (DAB) (50 mg/100 ml; half this concentration for the supernatants) using 10 μl of 30% H_2O_2. Fig. 4 (left lane) shows that exposure of KRP2 to taxol stabilized MT resulted in the majority of tubulin in the supernatant fraction. In contrast, preincubation of KRP2 with 0.7 mM ACR prior to use in the assay resulted in more tubulin in the pellet (right lane), caused by ACR inhibition of KRP2 disassembly activity. Due to concerns of reliable resuspension of the extremely small high-speed pellets, all future studies measured the quantity of tubulin in the supernatants only.

Statistical analyses

The MT binding assay was used for KIF5CA activity and the disassembly of MT into soluble tubulin by KRP2 was used to assay the effects of toxicants on these two proteins. The determination of statistical significance between controls and various concentrations of acrylamide, glycidamide and propionamide on kinesin functions was determined with ANOVA, followed by Tukey's HSD post hoc test (Sigmastat 3.0 software) at a predetermined significance of $p < 0.05$.

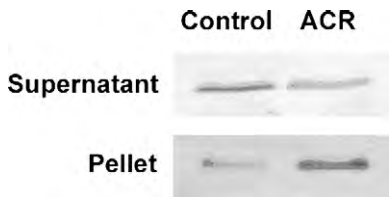

Fig. 4. Western blots of the high speed supernatant and MT pellet following exposure of microtubules to KRP2 with and without 0.7 mM ACR. Without ACR (control), KRP2-induced disassembly of MT resulting in a greater quantity of tubulin in the supernatant rather than the pellet. However, preincubations of KRP2 with ACR resulted in more tubulin in the pellet than in the supernatant, an indication that ACR inhibited the MT disassembly activity of KRP2.

Results

Effects of ACR (0.7 mM) on KIFC5A-induced MT binding

Preincubation of KIFC5A with ACR reduced the number of MT attached to the coverslips (Fig. 2). At KIFC5A adsorption concentrations of 0.0375 mg/ml, 0.7 mM acrylamide inhibited the binding function of KIFC5A by 38%; almost comparable to cutting the KIFC5A content in half. At 0.0188 mg/ml KIFC5A the number of bound MT was reduced by 84%. The lack of effect

Fig. 6. The effects of toxicants on the quantity of tubulin in the supernatant fraction following KRP2-induced MT depolymerization. Without toxicant (0 mM), the quantity of tubulin in the supernatant is maximal due to KRP2 disassembly of MT. Preincubation of KRP2 with ACR or GLY caused a concentration dependent decrease in the quantity of tubulin in the supernatant caused by a toxicant-induced reduction in KRP2 function. GLY was more potent than ACR. The non-neurotoxicant propionamide (Prop) had no effect on KRP2 activity except at the 10 mM concentration.

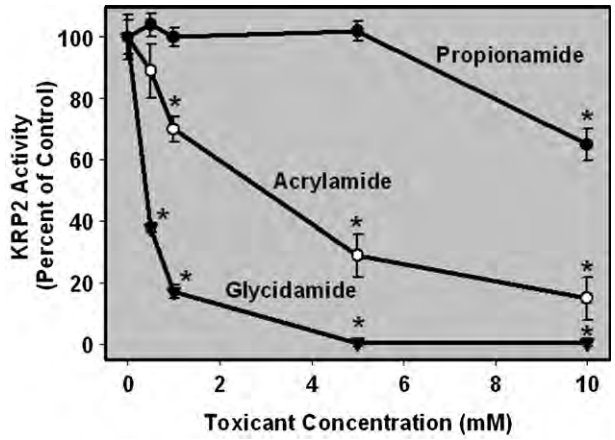

Fig. 7. The effects of acrylamide, glycidamide and propionamide on the MT disassembly activity of KRP2 expressed as percent of control (zero toxicant) from densitometric analysis of Western blots similar to the sample blot in Fig. 6. Acrylamide and glycidamide significantly reduced KRP2 activity; glycidamide was more potent than acrylamide. In contrast, the non-neurotoxicant propionamide had an effect only at the highest concentration tested (10 mM). Each data point is the average±SD from 3 assays. *$p \leq 0.05$.

at high concentrations of KIFC5A and the increasing magnitude of effect at low concentrations are the result of differential sensitivity of the assay at varying KIFC5A concentrations. We have observed similar results with acrylamide inhibition of MT motility by bovine brain kinesin (Sickles et al., 1996). In the latter case it was essential to use low concentrations of kinesins since single molecules are capable of locomoting MT. In the case of KIFC5A preparations, high concentrations of KIFC5A would be insensitive to toxicant inhibitions other than almost complete loss of activity as shown by the lack of difference in MT binding at the higher KIFC5A concentration. All subsequent assays were conducted at 0.04 mg/ml protein concentration of KIFC5A. These data also emphasize that all experimental comparisons must be made at the same protein concentration and from the same batch of KIFC5A.

Concentration-dependent effects of acrylamide, glycidamide and propionamide on KIFC5A

Based on the preliminary study above we utilized the 0.04 mg/ml concentration of KIFC5A to determine the concentration response for acrylamide, glycidamide and propionamide. KIFC5A was preincubated with 0.1 to 1.0 mM of each compound for 20 min at 37 °C before dilution of the KIFC5A supernatant to 0.04 mg/ml prior to adsorption. The control consisted of preincubations of KIFC5A with buffer only at 37 °C for 20 min. Both acrylamide and glycidamide produced a concentration-dependent inhibition of KIFC5A MT binding from 0.1 to 1.0 mM (Fig. 5). The inhibition at the lowest concentration used (0.1 mM) was sufficient to reduce the KIFC5A-induced binding of MT by approximately 60% for both ACR and GLY. The magnitude of inhibition at each concentration of ACR and GLY was almost identical to each other for all concentrations tested. Therefore, the ACR and GLY effects on KIFC5A were considered equipotent. In contrast, preincubation of KIFC5A with the non-neurotoxicant propionamide had no effect on KIFC5A MT binding at any concentration tested (up to 10 times the concentration of ACR and GLY necessary to significantly inhibit KIFC5A).

Concentration-dependent effects of acrylamide, glycidamide and propionamide on KRP2

After initial studies to identify the most effective ratio of tubulin to KRP2 to identify a toxicant effect, full strength KRP2 was preincubated with 0.5 to 10 mM acrylamide, glycidamide or propionamide or with buffer only (control) for 20 min at 37 °C before use in the assay. The quantity of tubulin in Western blots of the supernatants following a high speed spin to pull down intact MT was used to measure the quantity of KRP2-induced MT depolymerization. Fig. 6 (sample Western blots) and Fig. 7 (densitometric quantitation of 3 Western blots for each toxicant) demonstrates the effects of toxicants on KRP2-induced disassembly of microtubules. Acrylamide produced a concentration-dependent reduction in the quantity of tubulin in the supernatant fraction. In other words, ACR inhibited the KRP2-induced disassembly. This was statistically significant from 1.0 to 10 mM ACR. Glycidamide action on KRP2 was also concentration-dependent; however, it was substantially more potent than ACR. For example 0.5 mM glycidamide produced a 60% reduction in KRP2 activity, an amount greater than 1.0 mM ACR. The non-neurotoxicant propionamide had no effect on KRP2 activity, except at the highest dose (10 mM) tested.

Discussion

We have used molecular biological methods to clone motor proteins from the rat testis mRNA using conserved sequences from the kinesin motor domain. Out of six different kinesin-related proteins we selected two that had the motor domain located at different ends of the molecule and with different functionalities. We subsequently developed functional assays in order to allow determination of the effects of acrylamide and related analogues on kinesin functions. KIFC5A is homologous to kinesins that bind MT on both ends and are therefore capable of binding and/or bundling MT and, in some instances, sliding MT past each other. The function of this group of motors appears to be generation of the spindle and/or stabilization of the poles of the mitotic spindle (Gaglio et al., 1997). Some relatives of the protein have been shown to move towards the minus ends of MT; however, investigators have had difficulty showing motility with this kinesin subfamily (Kuriyama et al., 1994; Walczak et al., 1997). Similarly, we did not observe KIFC5A-based MT motility, but rather we observed bundling of MT in the solutions or attachment of single MT to the coverslip dependent on KIFC5A concentration. After identifying the proper concentrations of KIFC5A for a sensitive and linear assay, we were able to identify toxicant-specific changes in MT binding. In contrast, KRP2 possesses an internal motor domain and is homologous to kinesins with MT depolymerizing capabilities (Desai et al., 1999; Walczak, 2003). This function of

KRP2 was assayed by measuring the quantity of soluble tubulin produced by exposure of KRP2 to assembled microtubules. Dilution of KRP2 resulted in a concentration-dependent reduction in MT disassembly as measured by reduced band density of supernatants (soluble tubulin dimer) in Western blots. The assay was able to detect toxicant specific changes in KRP2 depolymerization function.

Previous studies have identified ACR effects on purified bovine brain kinesin induced MT motility (Sickles et al., 1996). These inhibitions were significant at 50–100 μM. Recent studies have demonstrated that glycidamide was slightly more potent, and propionamide had no effect (manuscript submitted). This neurotoxicant-specific action is correlated with reductions in the quantity of fast anterograde axonal transport at similar concentrations in vitro and in vivo using a variety of model systems and techniques of measurement (Sickles, 1989a; Stone et al., 1999, 2000). Studies are currently underway evaluating the impact of reduced transport of vital macromolecules to various axonal compartments in order to determine the significance of reduced transport to the functionality and survival of axons. The results of the current study indicate that other kinesin family members are also inhibited by acrylamide.

This is the first report of an acrylamide inhibition of a motor protein specifically involved in mitosis or meiosis. Furthermore, the concentration of acrylamide required to significantly inhibit KIFC5A is similar to those of bovine brain kinesin (Sickles et al., 1996). Both of these assays utilized VE-DIC microscopy to view either motility or binding of single MT. This type of assay is very sensitive as single kinesin molecule adduction by ACR can produce an observable effect. In contrast KRP2 inhibition required a concentration of ACR higher than observed for the other two kinesins. This may represent differential sensitivity of different kinesin family members to acrylamide. Alternatively, the KRP2 assay we developed measures the total quantity of disassembled tubulin in vitro on Western blots and may possess a lower sensitivity thereby resulting in a false shift in the concentration dependency. Despite the relative differences in assays, KRP2 was significantly inhibited by glycidamide concentrations of 5- to 10-fold less than acrylamide clearly indicating a strong differential sensitivity of the parent ACR versus its epoxide metabolite. These results might explain the comparative activities of ACR and GLY across genotoxic assays. Despite the quantitative differences, it is obvious that different kinesin family members are inhibited by both acrylamide and glycidamide. It is anticipated that other kinesin subfamily members involved in mitosis/meiosis and other cellular processes are also affected by these toxicants. Furthermore, the similar concentration of effects suggests that neurotoxic and cell division effects would have a similar dose response. Propionamide has been observed to be non-neurotoxic but application of this compound to reproductive, developmental studies and carcinogenicity has not been previously undertaken. Since propionamide is similar to acrylamide except for saturation of the vinyl group, the current data demonstrate that this vinyl group (or epoxide) is critical to the inhibition of several kinesin family members.

The inhibition of these two different mitotic/meiotic-related kinesins, as well as the anticipated inhibition of other kinesins and possibly dyneins, would predict a variety of cell division defects, some of which have been previously observed by acrylamide (see Introduction). The mitotic spindle is a crystalline-like structure composed of microtubule arrays emanating from the spindle poles undergoing rapid and dynamic changes in length. Superimposed on the MT arrays are a variety of kinesin and dynein motor proteins involved in generation and/or stabilization of the spindle as well as attachment and alignment of chromosomes and their eventual separation and movement to the poles. Motor proteins are associated with the properties of MT dynamics, MT sliding past each other and chromosomes (and other organelles) moving along MT. A concept emerging from multiple knockout or mutations studies is that a massive array of kinesins and dyneins are involved in a single spindle and the forces generated by these motors counteract each other resulting in a balance of forces. Therefore, differential effects on KRPs can produce a disturbance in the normal balance of forces within the spindle (Saunders et al., 1997).

KIFC5A and KRP2 are representatives of two structurally divergent subfamilies of kinesin-related proteins. KIFC5A is a member of the kinesin-14 subfamily which is characterized by location of the motor domain at the carboxyl-terminus of the polypeptide rather than at the more typical amino terminus (Lawrence et al., 2004). Members of this subfamily, including *Drosophila* ncd, human HSET, *Xenopus* XCTK2, hamster CHO2, and mouse KIFC5A, have a second microtubule binding site in their tail domain and are proposed to stabilize the spindle pole by crosslinking adjacent microtubules via microtubule binding sites in their motor and tail domains (Gaglio et al., 1997; Karabay and Walker, 1999; Matuliene et al., 1999; Mountain et al., 1999; Ems-McClung et al., 2004; Zhang and Sperry, 2004). Antibodies to XCTK2 and HSET inhibit the formation of acentrosomal spindles in vitro and in vivo (Walczak et al., 1997; Mountain et al., 1999) by preventing the formation of a focused spindle pole. KIFC5A associates with the spindle apparatus during cell division and targeting signals for both microtubule binding and nuclear import have been localized to a short, 43-amino-acid sequence in the tail domain of this motor (Zhang and Sperry, 2004). Previous tubulin fluorescence studies observed distorted spindle morphology in dividing HT1080 cells, but only after concentrations of 10 mM ACR (Sickles et al., 1995). Based on the dose response observed here and the presence of many copies of several different kinesins counteracting each other in the spindle (Saunders et al., 1997), that is consistent with the blockage of mitosis by millimolar concentrations of ACR, similar to the neurotoxic threshold.

KRP2 is a member of kinesin-12 subfamily whose motor domain is located more towards the central region of the polypeptide (Lawrence et al., 2004). This is a group of motors with unusual functional characteristics of ATP-dependent microtubule depolymerases rather than transporters of intracellular cargo (Wordeman and Mitchison, 1995; Kline-Smith and Walczak, 2002; Walczak, 2003) Several members of this class are localized to the kinetochore (*Xenopus* XKCM1 and human MCAK) along with other motor proteins and are important for

microtubule dynamics and chromosome segregation during anaphase A. Immunodepletion of XKCM1 results in disruption of bipolar spindle formation along with the formation of enormous monopolar asters with extremely long microtubules (Walczak et al., 1996). Depletion or overexpression of MCAK disrupts the separation of chromosomes in early anaphase A (Maney et al., 2001). XKCM1 has been demonstrated by electron microscopy to bind MT ends and effectively strip individual protofilaments from the tubule. The combination of location (Brown et al., 1996) and function suggests that this kinesin is associated with MT disassembly, which occurs during anaphase A when the chromosomes segregate into the daughter cells. The results from our study indicate that KRP2 is inhibited by ACR in the dose range of 1–10 mM. This is very similar to the dose range of ACR that blocked mitosis in HT1080 cells in vitro (Sickles et al., 1995). The chromosomes of ACR exposed dividing cells were retained at the metaphase plate. This is consistent with the concept that microtubule-depolymerizing kinesins may be inhibited by acrylamide, which results in failure of the migration of chromosomes from the metaphase plate and reduced mitotic activity. While millimolar doses were required to block mitosis in short experimental time periods, lower doses may affect the failure of single chromosomes to migrate properly. This would result in either cell cycle arrest or the improper segregation of chromosomes. Indeed, ACR has been demonstrated to produce aneuploidy with many of the micronuclei in possession of a kinetochore (Schriever-Schwemmer et al., 1997; Jie and Jia, 2001). This outcome is consistent with dissociation of the chromosome from the kinetochore MTs during anaphase. Therefore, the inhibition of this KRP may represent a mechanism by which action on dividing cells can produce abnormal genetic content, carcinogenicity and/or altered cell cycle events.

The concentration range of effect of ACR and GLY strongly indicate a cause–effect biological significance to ACR-induced defects in the fidelity of chromosomal separation and other mitotic spindle defects. The current study showed significant in vitro effects on KIFC5A at the lowest dose tested (100 μM). With an inhibition of 60% by both ACR and GLY, additional studies would likely find inhibition slightly lower concentrations. KRP2 activity was significantly inhibited by 1 mM ACR or 500 μM GLY (potentially lower for GLY). The neuronal kinesins were inhibited by 50 μM ACR (Sickles et al., 1996), GLY is just slightly more potent (unpublished data). While kinesin family members are likely inhibited to varying degrees, at least the three studied are strongly inhibited by 50–500 μM ACR or GLY. Significance abnormalities in cell division occur at higher concentrations. For example, in vitro chromosomal aberrations were observed at 0.7–14 mM, mitotic cell division was slowed at 0.14–2.8 mM, micronuclei were observed at 70 μM–0.7 mM and polyploidy at 1 mM. In vivo assays demonstrated dominant lethal effects at 130 μM and heritable translocations at 0.7 mM as well as loss of sperm production by multiple doses of 0.7 mM ACR (Dearfield et al., 1995). Therefore, significant mitotic spindle effects are observed in cells in vitro and in vivo at concentrations higher than required to inhibit kinesins. [Note that ACR and GLY distribute according to a whole body compartment due to lipid and water solubility.] We therefore conclude that inhibition of kinesins (and possibly dyneins) is a critical action resulting in aberrant cell division. A detailed study testing the aneugenic potential of ACR observed doubling of micronuclei by 1.4 mM and a 4-fold increase over control by 5.6 mM (Jie and Jia, 2001). Further analysis showed that these micronuclei possessed both centromeres and telomeres, consistent with the loss of chromosomes from the mitotic spindle. Chromosomes of mitotic cells exposed to ACR were either aggregated at the metaphase plate or floating throughout the cell in a colchicine-like (c-mitotic) effect (Sickles et al., 1995). The former could be the result of inhibition of KRP2 activity or the cell frozen at the metaphase checkpoint due to malalignment or attachment of chromosomes. Therefore, it is highly probable that inhibition of kinesin motors is directly related to issues of aneuploidy and blocks in cell division by ACR and/or GLY. A relationship between kinesin inhibition and chromosomal loss has during cell division has been shown in a model of mutant kinesin-2 (Miller et al., 2005).

Directly related the inhibition of kinesin motors by ACR or GLY to carcinogenicity is less conclusive. Indeed, it is arguable whether ACR or GLY is carcinogenic (see discussion below). The aneugenic potential of chemicals has been strongly linked to cancer due to the resultant instability of the genome. ACR is a well-known, albeit weak, aneugen. The current study strongly supports inhibition of kinesins as a cause of ACR-induced aneuploidy since the doses of ACR required to inhibit kinesins are substantially less than those used to demonstrate ACR aneuploidy (see paragraph above). However, lifelong doses of ACR given animals, or those human exposures are 1–2 mg/kg/day, which, assuming whole body distribution results in a tissue concentration of 14–28 μM. This is not far from the 100 μM threshold of effect on kinesin observed here. Therefore, it is reasonable to speculate that covalent binding and inhibitions of a minor number kinesins moving the same chromosome could produce aneuploidy. Therefore, even minute covalent modifications could have devastating consequences to genomic stability. Susceptibility of the endocrine organs and male gonads may be a result of their higher than average blood perfusion rate, as far as we know these data are not available. Future research relating ACR carcinogenic potential should include experimental testing of the relationship of kinesin inhibition with initiation of cancer.

The effects observed here demonstrate that acrylamide acts on vital proteins of the spindle, not just DNA. This represents a new potential mechanism by which acrylamide can have effects on the cell cycle, mitotic activity and also produce genomic instability leading to neoplasms. Studies with heavy metals have similarly concluded that direct effects on mitotic spindle proteins may account for carcinogenicity of these elements (Thier et al., 2003; Bonacker et al., 2005). An important consideration for future studies is a comparison of the relative contribution of direct DNA adduction and/or clastogenic actions by acrylamide or glycidamide versus the motor protein effects. The DNA adducts would result in the risk assessment decision of no theoretical risk-free dose (Kirsch-Volders et al., 2003; Bolt et al., 2004; Allen et al., 2005; Bonacker et al.,

2005). However, effects on the motors would predict carcinogenicity to possess a threshold and therefore result in the determination of a permissible exposure level. The importance of determining the relative contribution of DNA adducts versus motor proteins effects on cell division is therefore of paramount importance. Previous observations do provide some insight into answering this question.

The ability of glycidamide in particular to produce DNA adducts is well known (Doerge et al., 2005). Furthermore, ACR is clastogenic and aneugenic. This raises the question as to why these known genotoxic events may not result in any known human carcinogenicity (Collins et al., 1989; Cordier et al., 1995; Band et al., 1997; Granath et al., 2001; Mucci et al., 2003; Erdreich and Friedman, 2004; Granath and Tornqvist, 2005). It may be that efficient DNA repair mechanisms are sufficiently robust to overcome the DNA adduction and/or cell death mechanisms are effective in the induction of apoptosis. It may be that ACR inhibition of cell division through its action on mitotic motor proteins is anticipated to lock cells into the metaphase checkpoint and, without dispersion of chromosomes, causes apoptosis. Inhibition of cell division and repression of bowel cancers by acrylamide has been reported (Dearfield et al., 1988; Collins et al., 1989; Mucci et al., 2003). Therefore, on one hand ACR may induce genetic malformations with an increased potential for producing cancer and on the other act to retard cancer growth through blocking mitosis and induction of cell death. The feasibility of kinesin motor inhibitors acting as cancer suppressors is supported by the recent identification of antitumor agent inhibition of mitotic centromeric associated kinesin (MCAK) (Aoki et al., 2005). Furthermore, this antitumor agent blocked cells in mitosis, similar to the action of ACR (Sickles et al., 1995). ACR and this antitumor agent may share a common action on homologous kinesins. Therefore, the mutagenic effects of acrylamide with simultaneous inhibition of cell division may oppose each; the ultimate outcome may be based on chance, the strength of the repair mechanisms, the effectiveness of apoptosis and/or the ACR concentration.

The inhibition of kinesin motors involved in mitosis and meiosis is also anticipated to impact male reproductive function by reducing the ability of the seminiferous epithelium to produce sperm. This reduction may occur through two mechanisms associated with kinesins. First, the inhibition of mitosis and meiosis associated with the inability to separate chromosomes with high fidelity (discussed above). Secondly, motor proteins are involved in a variety of other cellular processes. Sertoli cells have elaborate MT networks (Boekelheide et al., 1989) and their functions may be compromised by motor inhibition. Also the dramatic alterations in morphology of the developing spermatocytes and spermatozoa and the requirement for intracellular redistribution of organelles would be compromised by kinesin inhibition. FISH analysis found KRP2 expressed in the developing spermatocytes as well. This may indicate that KRP2 has a role in destabilizing microtubule complexes in spermiogenesis as well as mitosis/meiosis. Therefore, kinesin inhibition may be the cause of testicular dysfunction and atrophy caused by ACR. This possibility is consistent with the similar dose range of neurotoxicity and male reproductive toxicity.

The discovery that several kinesin motors involved in mitosis and/or meiosis are inhibited by ACR at concentrations similar to those inhibiting neuronal kinesins is mechanistically revealing. The inhibition of kinesins offers a common site of action of ACR that may contribute to a multiple toxicities of ACR including neurotoxicity, reproductive toxicity and potential carcinogenicity. Slight differences in doses required to produce the various toxicities may be explained by slight differences in sensitivity of the different kinesin family members to ACR or the relative dependence of the organ on kinesin based MT functions. The organ selectivity of ACR is consistent with kinesin as a site of action. The most sensitive organs or tissues are those critically dependent upon motor proteins. The dependence of neuronal long axons upon intracellular transport places them particularly vulnerable to the inhibition of the anterograde motor protein kinesin. The testes susceptibility would be due to both the dependence of the spermatogenic cell lines upon microtubule based generation of the mitotic spindle and the separation of chromosomes as well as the involvement of microtubule based remodeling of spermatogenic cells during spermiogenesis. Defects or mutations in kinesin family members should be considered not only in neurological diseases (Zhao et al., 2001; Hirokawa and Takemura, 2005), but in cases of male sterility and carcinogenicity.

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