Development and Validation of an Assay for the Characterization of the DNA Damage Response to Damage Caused by Superfund Chemicals

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ABSTRACT

John Robert Ridpath: Development and Validation of an Assay for the Characterization of the DNA Damage Response to Damage Caused by Superfund Chemicals
(Under the direction of Jun Nakamura)

Determination of intracellular mode of action regarding how chemicals induce toxicity plays an important role for establishing risk assessment. Although several methods exist to ascertain how toxic substances act intracellularly, the amount of information obtained from these assays usually fails to fully clarify the route to toxicity. This projects goal was to establish and validate a new assay to provide more information with regard to the DNA damage response of cells to toxic agents. This assay uses a reverse-genetic approach similar to that used with yeast mutants. We used a cell line known as DT40 from a vertebrate source whose mutants have been derived from a single parent making the entire cell line isogenic. The DT40 cell line includes more than 50 mutants for proteins involved in DNA repair, DNA damage bypass and cell cycle checkpoints. This line is used to study several cellular metabolic processes, but we believe we are the first to exploit this exceptional tool to aid in the determination of toxicological end-points.

First we developed the assay into a relatively rapid and cost-efficient method that may be used to screen chemicals, but with accuracy, reliability and sensitivity. For this, we measured the sensitivity of DT40 mutants to chemicals using XTT, a dye reduced only in respirating cells, providing a direct measurement of cell proliferation after

treatment. We found this method both reliable and sensitive as evidenced by validations with cisplatin, methyl methanesulfonate and acrolein.

We next applied our method to study the DNA damage response to formaldehyde, an agent shown to induce DNA-protein crosslinks. Little was known as to how cells dealt with these crosslinks. Our assay was able to demonstrate the FANC/BRCA damage response pathway is important for dealing with formaldehyde-induced DNA damage.

Finally, we turned our attention to help clarify the type of DNA damage induced by hexavalent chromium whose inorganic salts are known cancer inducers but for which the mode of action is controversial. We were able to provide valuable information concerning the cellular response to chromate by showing that chromate probably induces mutagenicity by interfering with replication but not through oxidative stress.

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List of Abbreviations

3-AB	3-aminobenzamide
8-oxo-dG	8-oxo-2'-deoxyguanosine
Acr	acrolein
α-HOPdG	α-hydroxypropano-deoxyguanosine
ANOVA	analysis of variance
APE	apyrimidinic/apurinic endonuclease
ATM	ataxia telangiectasia mutated
ATR/ATRIP	ATM and Rad3 related/ATR-interacting protein
BER	base excision repair
BLM	Bloom's helicase
BRCA1	breast cancer 1
BRCA2	breast cancer 2
BrdU	bromo-deoxyuridine
CldU	chloro-deoxyuridine
Cr(III)	trivalent chromium
Cr(VI)	hexavalent chromium
DAC	DNA-amino acid crosslink
dG	deoxyguanosine
DMSO	dimethyl sulfoxide
DPC	DNA-protein crosslink
DSB	DNA double-strand break
dsDNA	double-strand DNA

FANCD2	Fanconi anemia complementation group protein D2
ES	embryonic stem
FA	Fanconi anemia
FBS	fetal bovine serum
FEN1	flap endonuclease 1
γ-HOPdG	γ-hydroxypropano-deoxyguanosine
GGE	glyoxal gel electrophoresis
GSH	glutathione
HR	homologous recombination
IARC	International Agency for Research on Cancer
ICL	DNA interstrand crosslink
IdU	iodo-deoxyuridine
LC_{50}	50% lethal concentration
MED	minimum effective dose
MMR	mismatch repair
MMS	methyl methanesulfonate
MOA	Mode of Action
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
OTX-AGE	*O*-(tetrahydro-*2H*-pyran-2-yl)hydroxylamine agarose gel electrophoresis
PAR	polymeric adenosine diphosphate-ribose
PARP1	poly [ADP-ribose] polymerase 1
PBS	phosphate buffered saline

PCNA	proliferating cell nuclear antigen
PMS	phenazine methosulfate
POLB	DNA polymerase β
POLH	DNA polymerase η
POLK	DNA polymerase κ
ROS	reactive oxygen species
Sp	spiroiminodihydantion
SSB	DNA single-strand break
TEMPO	2,2,6,6-tetramethylpiperidine-*N*-oxyl
TLS	translesion synthesis
XPA	xeroderma pigmentosum complementation group A protein
XRCC2	X-ray repair cross complement protein 2
XTT	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

Chapter 1. Introduction

Every year, a large number of new chemicals (~2000) are being added to the already vast list (~80,000) that exists. Therefore, there is a growing need to be able to quickly establish the potential dangers of these compounds in terms of genotoxicity, mutagenicity and carcinogenicity. To this end a number of procedures have been developed, such as the Ames test, the yeast reverse-genetic assay, various in vitro cell-based assays and a variety of animal studies. Each has its value but, unfortunately, they all have shortcomings, such as false negatives, false positives, lack of sensitivity or lack of an adequate correlation to humans that can muddle the decision making process for producers and regulators. Also, some of these methods are quite time consuming and/or expensive. Therefore, it is clear that any new assay providing additional information regarding how cells respond to exogenous agents and that could improve upon any or most of these weaknesses, whether it is with greater accuracy, higher sensitivity, better applicability to humans, higher throughput or lower cost, should be greatly welcomed.

In the field of toxicology, the use of biomarkers has been rapidly gaining in importance. Once one better understands the mode of action of the target compound and which DNA repair pathway is needed to alleviate the any DNA damage induced, the type of biomarker required tends to be more easily determined. Without such information, biomarkers are sometimes being established without knowledge of whether the target is

biologically important. Genotoxicity and the mechanism thereof may be indirectly inferred from knowledge of which DNA metabolic (repair, cell cycle checkpoint and/or damage sensing) pathway(s) is involved in the alleviation of the damage. In an attempt to clarify the metabolic mechanisms used to repair DNA damage induced by exogenous and endogenous agents this project has made extensive use of the chicken DT40 cell system and the great number of DNA repair, cell-cycle checkpoint and DNA damage sensing mutants that have been created.

DT40 Cell System

The reverse genetic approach provides a powerful method for the study of gene function and regulation. One such approach which has been gaining in popularity makes use of the isogenic DT40 cell line with its dozens of mutants for many cellular metabolic pathways including all known DNA repair pathways, as well as the cell cycle checkpoint and DNA damage sensing mechanisms (Table 1.).

DT40 cells originated from a chicken B-lymphocyte line derived from an avian leucosis virus induced bursal lymphoma originally isolated in 1985 (Baba et al., 1985). The DT40 cell line is rather unique in that it exhibits a high ratio (targeting efficiency 10 – 90%) of targeted to random integration of transfected DNA, whereas most transfected DNA integrates at random chromosomal positions in mammalian cells (targeting efficiency in mouse ES cells: 0.1 – 5%) and those of other higher eukaryotes (Sonoda et al., 2001; Dhar et al., 2001). This high targeting efficiency allows for the production of double and even triple mutants with relative ease where multiple mutations are virtually impossible at the lower efficiencies of the other commonly used cell lines. DT40 cells

also exhibit an exceptionally stable karyotype with very low levels of spontaneous chromosomal aberrations which allows for culturing of the cells for extended periods free from concern regarding variance in their genetic character (Hochegger and Takeda 2006).

Another unique feature of the DT40 cells is their reported lack of functional p53 (Takao et al., 1999). When testing a DNA repair mutant with a functioning p53 protein, general genomic instability may cause the cell to die due to the activation of apoptosis. The absence of functional p53 in DT40 cells presents the advantage of a compromised apoptosis pathway allowing for the observance of cell death due to defect-driven repair failures rather than by the activation of apoptosis (Hochegger and Takeda, 2006).

Due to the facility with which the DT40 line can be manipulated genetically, and the fact that DT40 mutants are observed to show a strong phenotypic resemblance to murine mutants with respect to genes involved in DNA recombination and repair (Winding and Berchtold 2001), the line has seen a steady growth of its use in genetic studies including immunoglobulin diversification, DNA repair, chromosome segregation, RNA metabolism and cell signaling (Winding and Berchtold, 2001; Yamazoe et al., 2004; Dhar et al., 2001).

Characteristics of DT40 Cells and Cells Utilized for Other Genotoxicity Analyses

Ames test

The Ames test is a bacterial reverse-mutation assay designed as a screen to detect the genotoxicity of chemicals. *Salmonella* strains deficient in their ability to produce histidine are exposed to chemicals and mutagenicity is determined by the cells' reversion to a *wild type* phenotype which is histidine-independent due to mutations caused by the chemical under test (Maron and Ames, 1983). However, although the Ames test achieves

a good association with animal carcinogenicity results, it is known that a great many of the enzymes that are used by humans (nearly all of which have homologs in DT40 cells) in DNA repair pathways simply are not known to exist in bacterial cells. Prime examples would be the DT40/human BER proteins, polymerase β (POLB) and poly [ADP-ribose] polymerase 1 (PARP1), for which no equivalents have been found in bacteria. Added to this can be HR, for which there are numerous proteins involved in vertebrate cells which do not exist in bacterial cells and the pathways are thought to operate in a very different manner (Friedberg et al., 2005). Finally, it has been recognized that *Salmonella* most likely lack the non-homologous end-joining pathway used by eukaryotes (and some other prokaryotes) as an alternative to HR in the of repair DNA double-strand breaks (Bowater et al., 2006).

Yeast assay

The use of mutant yeast strains has for decades been one of the mainstays of reverse genetic studies. However, yeasts have been found to be more tolerant to repair and cell-cycle checkpoint defects than higher eukaryotic cells (Dhar et al., 2001) leading to the possibility of underestimation of sensitivity in cell death experiments. This is perhaps due, at least partly, to the difficulty certain chemicals have in crossing the less permeable cell membrane of yeast cells. It has been recognized that there is a high level of homology between yeast genes and those of mammalian cells; whereas, function of some of the DNA damage response genes appears to be different. For example, Rainey, et al., have reported that Chk1 and Chk2 knockouts in DT40 showed that these checkpoint effector kinases control a very different range of checkpoint responses in

vertebrates compared with yeast (Rainey et al., 2006). Also, homology between yeast genes and those of mammalian cells and DT40 cells do not necessarily indicate the same mechanism in their associated pathways (Table 2.) (Sonoda et al., 2001). Furthermore, the relative role of each DNA repair pathway observed in yeast and vertebrate cells appears to differ (Yamazoe et al., 2004). For example, homologous recombination (HR) repair in yeast functions at any time during the cell cycle, whereas HR in vertebrates is only active during late S-phase to G2 (Takata et al., 1998). Thus, the overall relative contributions of HR and non-homologous end-joining (NHEJ) to DNA double-strand break (DSB) repair show a considerable variance between *S. cerevisiae* and mammalian cells with DSBs being repaired mainly through HR in the yeast while NHEJ plays only a minor role (Aylon and Kupiec, 2004; Lee et al., 1999). Critchlow and Jackson have reported that, in mammalian cells, NHEJ appears to be of greater importance (Crithchlow and Jackson, 1998), while another report indicated it is responsible for repairing more than 60% of exogenously induced DSBs in mouse embryonic stem cells (Liang et al., 2006). In fact, genes required for HR, such as *Rad51* or *Rad54*, are not expressed in quiescent vertebrate cells after insult by genotoxic agents (Tan et al., 1999) so that non-homologous end joining (NHEJ) would be the pathway of choice, whereas yeast could be using HR. Also, another important category of proteins, the Fanconi anemia complementation group (FA), which are involved in HR and DNA replication control in mammalian cells, are, with a few exceptions absent in yeast (Errico and Costanzo, 2010). Furthermore, vertebrate proteins directly involved in DSB repair also stimulate a cell-cycle checkpoint, and conversely, proteins involved in checkpoints promote DNA repair, whereas a checkpoint defect does not affect repair in yeast (Sonoda et al., 2001).

The base excision repair (BER) pathways in mammalian and yeast cells exhibit many disparities (Kelley et al., 2003). For instance, the apyrimidinic/apurinic nucleases (APE) are different proteins and function differently in mammals and *S. cerevisiae*. In fact, murine blastocysts knocked out in APE, as well as human antisense-expressing cells, are hypersensitive to γ rays and oxidizing agents such as H_2O_2, whereas yeast cells deficient in Apn1 (the protein which functions similarly to APE in yeast) showed only mild sensitivity at best (Evans et al., 2000). Yeast cells also lack some of the enzymes for dealing with 8-oxo-2'-deoxyguanosine (8-oxo-dG; i.e., MYH, MTH), enzymes that exist in mammalian cells (Kolodner and Marsischky, 1999). Therefore, in yeast, adenine which has been mispaired with 8-oxo-dG is most likely removed by the mismatch repair machinery rather than BER. Also, yeast cells contain no equivalent proteins to POLB or PARP1, both of which are used in vertebrate cells in BER (Friedberg et al., 2005). As a result, yeast perform primarily long patch BER, whereas vertebrates use mainly short patch BER (Kelley et al., 2003). In summary, it seems evident that vertebrate cells such as the DT40 line, which exhibit a much more comparable homology to those of mammals in many DNA metabolic proteins, provide an advantage over yeast in the examination of DNA repair pathways as well as cell cycle checkpoints.

Murine ES Cells

Although many gene-targeting experiments have been performed using mammalian cells, the approach has been hampered by low efficiencies (10^{-2} to 10^{-5}) in the integration of exogenous DNA through HR, as most of the DNA integrates at random positions on the chromosomes (Sonoda et al., 2001). In 1991, Buerstedde and Takeda

found that targeted integration frequencies in DT40 cells far exceeded those of random integration (more than 1:2) and were orders of magnitude higher than the frequencies in mammalian and other higher eukaryotic cells (Buerstedde and Takeda, 1991). For example, DT40 cells have 15 to 100 times greater targeting efficiency than murine embryonic stem (ES) cells (Dhar et al., 2001). As such, to detect one targeting event only 10-50 DT40 colonies need be screened in comparison to 100-1000 colonies for the mouse cells. The low gene-targeting efficiencies of ES cells also make it nearly impossible to produce double or triple mutants in those cells where it is relatively easy in DT40 cells. Also, many murine knockouts in recombination and DNA repair processes cause genomic instability with damage checkpoint stimulation. This genomic instability leads to a loss of viability of both the fetus and cultured cells making the cells of little use in the analysis of chromosomal processes because of their limited growth (Yamazoe et al., 2004). Furthermore, Sale reports that knockouts for HR proteins are embryonic lethal in mice (Sale, 2004), and, as this is not the case for most DT40 recombination knockouts, the DT40 cells possess a distinct advantage when investigating HR repair.

Many different knock-out mouse embryonic fibroblasts are available for genotoxicity testing. However, the different knock-outs are from various strains of mice which can make comparisons of responses to potential mutagens somewhat more difficult. Alternatively, all DT40 mutant cell lines have originated from a single parent and are therefore isogenic. This allows for very precise comparisons between responses to DNA damaging agents.

Another advantage of DT40 cells is their inherent ability to maintain a stable karyotype which makes them particularly valuable in assessing the effect of mutagenic

compounds. In contrast, murine ES cells tend to lose multipotency upon exposure to such mutagens (Dhar et al., 2001).

Finally, extensive work has been performed comparing the phenotypes of murine and DT40 cells with little difference found between the two. This has allowed for a favorable and widespread reception for the use of DT40 cells in reverse genetic investigations.

Applicability of DT40 Cell System to Toxicology and Genotoxicity Studies

In the field of toxicology, the use of biomarkers has been rapidly gaining in importance. Once one knows the DNA damage response to the target compound and which DNA repair pathway is needed to alleviate the damage, the type of biomarker required can be more easily determined. For example, if hypersensitivity is detected in cells that are deficient in BER, then one could focus on N^7-alkylation adducts as biomarkers of exposure to ethylene oxide or propyrene oxide.

In an attempt to illuminate the mechanisms used to repair DNA damage induced by exogenous and endogenous agents, this project has made extensive use of the DT40 system and the great number of repair and cell-cycle checkpoint mutants that have been created. This project has also endeavored to improve upon the methodology currently being used with DT40 cell assays. Presently most, if not all, researchers making use of the DT40 cell line are performing their tests using the clonogenic method and growing the cells in a methylcellulose gel (Simpson and Sale, 2006). Although the clonogenic method is certainly highly regarded for its accuracy and sensitivity, for the purpose of screening scores of chemicals it has the drawback of being overly time consuming and

costly. Also, there is a very real possibility that the methylcellulose may react with some chemicals one may wish to test, thus limiting the value of the assay. In fact, it appears that the subject of Specific Aim 2 of this project, formaldehyde, may be such a case. Here, the intent was to develop a novel (in the case of DT40 cell assays) micro-plate based assay for use with these cells that will be as accurate and sensitive as the clonogenic method but have the advantages of much higher throughput, reduced manipulation and greatly reduced cost. This new procedure has made use of the compound 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT) by which one may measure cell viability based on the activity of mitochondrial enzymes in live cells that reduce XTT but are inactivated shortly after cell death. The XTT is reduced to a formazan dye with specific absorbance at 450 nm. The percent survival can then be easily and accurately determined by comparison of absorbance measurements of test samples to those of controls. This method produces results in days instead of weeks with a dramatic reduction in materials used and much less manipulation by laboratory personnel. The method development has used cisplatin as one model for verification of the assay. Cisplatin was chosen because there is existing data obtained from DT40 mutants using the clonogenic assay (Takata et al., 1998; Nojima et al., 2005; Matsushita et al., 2005). Our cisplatin results coincide quite well with those of studies using the clonogenic assay. To further verify the assay, attention was then turned to acrolein, a highly reactive α,β-unsaturated aldehyde, as it has been the object of numerous studies over a relatively long period and although it is known to be involved in DNA adduction, there is still controversy concerning its mutagenicity and carcinogenicity in humans (Minko et al., 2009; Stevens and Maier,

2008). Our work here has indicated a virtually complete agreement with the studies of others that have shown sensitivity to acrolein in translesion DNA synthesis deficient cells but generally very little sensitivity in cells deficient in other DNA metabolic pathways, thus providing another verification of the accuracy of our DT40 assay (Kanuri et al., 2002; Minko et al., 2003).

In specific aim two, the focus was turned to formaldehyde – another highly reactive aldehyde that has recently been listed as a known human carcinogen by the International Agency for Research on Cancer (IARC) (Cogliano et al., 2005). Formaldehyde is a ubiquitous environmental pollutant found in automobile exhaust and tobacco smoke, among many other indoor and outdoor sources for which humans are known to be exposed (Cogliano et al., 2005). There also exists an endogenous exposure to formaldehyde as it is present in significant concentrations in all tissues, cells and bodily fluids (Szarvas et al., 1986; Heck and Casanova, 2004). Formaldehyde has been shown to induce DPC but very little has been learned as to how the cell deals with these lesions in terms of tolerance and repair. The DT40 system, with cell lines deficient in every known DNA repair pathway, has provided an excellent approach for the determination of the mechanism(s) used by cells for dealing with DNA-protein crosslinks (DPC) caused by formaldehyde (Ridpath et al., 2007).

In order to demonstrate the versatility of this high-throughput genotoxicity assay using the DT40 cell system we chose to use the system for the study of an inorganic toxicant. Thus, the highly toxic and carcinogenic metal chromium (hexavalent) was the focus of specific aim three of this project. Chromium is a naturally occurring element found in rocks, animals, plants and soil and can exist in all three physical forms – liquid,

solid or gas. The most important valence states of chromium to which humans are exposed are the tri- and hexavalent forms (Cr[III] and Cr[VI]) (P.H.S. US Department of HHS, Agency for Toxic Substances and Disease Registry Toxicological Profile for Chromium, Sept. 2000). While extracellular Cr(III) is somewhat toxic, it is absorbed very poorly by cells and is not considered to possess great significance as an exogenous human toxicant. However, quite the opposite is the case with Cr(VI) which is readily taken up by cells after which it is rapidly metabolized to Cr(III) which, intracellularly, is the toxic and carcinogenic state of the element (O'Brien et al., 2003; Zhitkovich, 2005; Salnikov and Zhitkovich, 2008; McCarroll et al., 2009). Therefore, exogenous exposure to Cr(VI) has been focused on here. As with acrolein and formaldehyde, the new DT40 method developed in this project will be used in this genotoxicity study of chromium.

Significance

When regulatory bodies are determining the potential carcinogenicity of a chemical one of the most integral aspects in evaluating the Weight of Evidence is to ascertain if the chemical is mutagenic and, if so, what is the mechanism of that mutagenicity. In the U.S. EPAs document, Framework for Determining a Mutagenic Mode of Action for Carcinogenicity: Using EPA's 2005 *Cancer Guidelines and Supplemental Guidance for Assessing Susceptibility from Early-Life Exposure to Carcinogen*, it states that "No single mutagenicity test is able to detect the entire spectrum of induced mutagenic events." While the DT40 test system developed in this project likewise would not be able to detect the 'entire spectrum of mutagenic (or genotoxic) events', it is believed that the ability, with this system, to determine the

distinct DNA repair pathway involved will provide additional information when used along with genotoxicity assays. Also, the applicability of this assay to be further developed into a truly high-throughput method may one day enhance any regulatory body's ability to react faster to the appearance of new chemicals to be evaluated or to a perceived necessity to re-evaluate an existing agent.

Specific Aims

Humans are exposed to a prodigious number of exogenous carcinogens in daily life. While an extraordinary amount of research has been done to identify which chemical agents induce cancer, very little, in most cases, is known of the modes of action of these compounds. However, determination of the mode of action can be very advantageous in determining biomarkers of effect which may lead to increased ability to prevent the deleterious actions of these chemicals. In addition, since a very great number of these compounds induce cancer by causing DNA damage with resulting heritable mutations, the aspect of the mode of action involving DNA repair mechanisms has gained increasing importance. The mechanisms used to cause DNA damage are known for only a very limited number of these chemicals. At present, the methods available for the determination of these DNA damage modes of action are time consuming, expensive and are either not sensitive or specific enough or involve the use of animal subjects that is becoming more and more unpopular. Therefore, the goal of this project has been to develop a simple, convenient and cost efficient method to measure the DNA damage response to exogenous agents and to provide strong evidence that the system is sensitive and accurate enough to more precisely understand mechanisms of the DNA damage

response at concentrations that are relevant to those measured in humans and to current regulations.

Specific Aim 1: Establishment of a convenient DNA damage response assay using the DT40 cell system.

While there are certainly existing methods for the determination of which DNA repair mechanisms are concerned with the repair of damage induced by exogenous agents, these methods each seem to have shortcomings such as lack of specificity with regard to the relevance to mammalian systems. It was the intent of this aim to develop an improved method using the vertebrate DT40 cell line so that DNA repair modes of action can be determined with much greater rapidity and less expense than the current methods. For this assay, cells were grown in 24-well plates and exposed to serial dilutions of the compound of interest. After a sufficient growth period, the cells were exposed to XTT dye, a tetrazolium derivative that is reduced by mitochondrial enzymes only in living cells. Thus, the amount of product generated from XTT reduction is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at a wavelength of 450 nm. Survival of mutant cell lines was then compared with that of the wild type to determine the sensitivity of the mutant to the compound under test. By collaboration, a statistical test for significance has also been developed. It was expected that cells mutated for proteins involved in the repair pathway that deals with the chemical will show greater sensitivity than either the wild type or mutants from other repair pathways and that the type of damage induced by the agent will be indicated.

Specific Aim 2: To determine, using the DT40 cell system, the mechanism by which DNA-protein crosslinks induced by formaldehyde are repaired.

Formaldehyde has recently (2006) been re-evaluated by IARC as a known human carcinogen as a result of studies that found formaldehyde induces nasopharyngeal cancer in exposed workers. While past research has determined that formaldehyde causes mutations in DNA by forming DPCs (Hubal et al., 1997), very little is known of the process the cell uses to repair these lesions. This aim also made use of the DT40 cell system along with human DNA repair deficient cells to determine which DNA repair mechanism(s) are involved in the repair of damage caused by formaldehyde. **Therefore, I hypothesized that the reverse genetic approach using the DT40 cell system will provide the answer as to which DNA repair mechanism is used to repair DNA-protein crosslinks induced by formaldehyde.**

Specific Aim 3: Analyze, using the DT40 cell system, the DNA damage response to chromium (VI).

Many metal compounds are included among the top twenty most dangerous (to humans) environmental chemicals. One of these metals, chromium – a known human carcinogen, seems to have very complex and varied modes of action. It has been suggested that Cr(VI) induces several types of DNA damage including small Cr-DNA binary adducts, DNA-Cr-protein crosslinks, DNA-Cr-small molecule ternary adducts, DNA damage due to Cr(VI) induced oxidative stress, DNA base damage, DNA single-strand breaks (SSB) and inter- and intra-strand crosslinks (O'Brien, 2003). For this aim, **I hypothesized that by making use of the DT40 cell system to determine damage repair responses which can lead to the integration of other assays, valuable information can be obtained regarding the mode(s) of action of environmental metal**

pollutants. For example, if the repair pathway, base excision repair is found to be involved in the response to Cr(VI) exposure, it could be implied that base damage (i.e., 8-oxo-dG) due to Cr induced reactive oxygen species (ROS) or the oxidation of deoxyribose is indicated. This type of damage is repaired by BER, which, through its normal process, creates DNA single-strand breaks. Therefore, to further investigate, DNA single-strand break assays were performed.

Chapter 1 Tables

Table 1-1. Example of Available DT40 Mutant Cell Lines

BER	**NER**	**NHEJ**	**HR**
- POL β	- XPA	- KU70	- RAD52
- FEN1	- XPG	- LIG IV	- RAD54
- PARP1		- DNAPKcs	- RAD51c
DNA damage	**MMR**	**TLS**	- RAD51d
sensors	- MSH2	- POL K	- XRCC2
- RAD9	- MSH3	- POL H	- XRCC3
- RAD17	- MSH6	- POL Q	- BRCA1
Helicase		- REV1	- BRCA2
- BLM		- REV3	- FANCD2
- WRN		- RAD18	

DNA damage repair/tolerance pathways represented: BER - base excision repair; NER – nucleotide excision repair; NHEJ – non-homologous end-joining; HR – homologous recombination; MMR – mis-match repair; TLS – trans-lesion synthesis

Table 1-2. Species Comparison of DNA Repair Pathways[a,b]

Pathway	*E.coli*	*S. cerevisiae*	*S. pombe*	DT40/Human	Functional Activity
BER	*DNA PolI*			*Polβ/PolB*	BER polymerase
	—	—	—	*PARP1*	
	PolA$^+$	*RAD27*	*rad2*$^+$	*FEN1*	5’ nuclease
NER	*DNA PolI*				Resynthesizes DNA
		POLδ(POL3)	*cdc6*$^+$	*Polδ/PolD*	Resynthesizes DNA
		RAD14	*rhp14*$^+$	*XPA*	Damage verification
		RAD2	*rad13*$^+$	*XPG*	3’ incision nuclease
	uvrA$^+$,*uvrB*$^+$,*uvrC*$^+$, *uvrD*$^+$				A: Binds damage; B: Helicase activity C: 3’ and 5’ incision D: Helicase activity
HR	*recF*$^+$,*recO*$^+$,*recR*$^+$	*RAD52*	*rad22*$^+$, *rti1*$^+$	*RAD52*	Recombination accessory factor
	recA $^+$	*RAD51*	*rhp51*$^+$	*RAD51c*	Mediates homologous pairing
				RAD51d	Mediates homologous pairing
				XRCC2	HR accessory factor
				XRCC3	HR accessory factor
		RAD54	*rhp54*$^+$	*RAD54*	Induction of supercoiling
				BRCA1	Recombination accessory factor
				BRCA2	Cooperation with Rad51 - essential
	—	—	—	*FANCD2*	Chromosomal stability, response to crosslinks
NHEJ		*YKU70*	*pku70*$^+$	*KU70*	DNA end binding
		LIG4		*LIG4*	Ligation of nick
TLS		*REV3*	*rev3*$^+$	*REV3*	DNA polymerase ζ sub-unit
		RAD18	*rhp18*$^+$	*RAD18*	E3 ubiquitin ligase
	dinB$^+$			*Polκ/PolK*	Lesion bypass polymerase
		RAD30	*eso1*$^+$	*Polη/PolH*	Lesion bypass polymerase
				PolQ	Lesion bypass polymerase
MMR	*mutS*$^+$	*MSH2*	*swi8*$^+$	*MSH2*	Mismatch recognition
		MSH3	*swi4*	*MSH3*	Mismatch recognition
		MSH6		*MSH6*	Mismatch recognition
RECQ helicase	*recQ*$^+$	*SGS1*	*rqh1*$^+$	*BLM*	Resolution of stalled replication/helicase
				WRN	Resolution of stalled replication/helicase/ 3’-exonuclease
CCCP		*TEL1*	*tefu tel1*$^+$	*ATM*	Strand break sensor/kinase

[a] Entries in this table are organized according to DNA repair pathway, indicating functional orthologs. In many, but not all, cases these are also sequence or structural homologs.
[b] Dashes indicate that no gene exists. Blank spaces indicate that the status is unknown.

Chapter 2. Development and Validation of a Multi-well based DT40 Cell DNA Damage Response Assay

Abstract

Chemists continually synthesize myriad new chemicals (~2000 each year), some of which make their way into the environment or otherwise pose possible threats to humans who potentially become exposed to the compounds. Regulators must determine whether these, along with the glut (~80,000) of existing, chemicals are toxic and at what exposure levels. An important component of this determination is to ascertain the mode of action (MOA) of each compound as it relates to the pathway the compound uses to induce genotoxicity. Several assays have traditionally been used to reveal these effects to the genome: the Ames test, tests with yeast and mammalian cell lines, and animal studies. Each has its adequacies and weaknesses. Here, we describe a new multi-well plate-based method which makes use of the DT40 isogenic cell line and its dozens of available mutants knocked out in DNA repair and cell cycle pathways. Although the DT40 line has existed for some time and has been used in numerous studies of DNA repair pathways, little use has been made of this valuable resource for toxicological investigations. Our method dispenses with the clonogenic assay others use with DT40 cells and introduces the use of the XTT dye scheme for the determination of cell survival in a manner that greatly increases throughput and reduces cost while maintaining

accuracy and sensitivity. We believe that this method will be very advantageous if added to the repertoire of those investigating MOAs of potentially genotoxic substances.

Introduction

The specific aim of this section of the project was to develop a new assay for which to determine how both endogenous and exogenous agents are damaging DNA. To this end we have melded two systems, the DT40 cell system and the XTT cell proliferation assay. This chapter first describes the approach to the development of the method as to the cell maintenance and test procedure processes as well as the development, by collaboration, of a statistical test for significance of the difference between the results for the *wild type* versus a mutant cell line. We then describe experiments we performed to demonstrate the reproducibility and sensitivity of the assay. In these experiments we exposed DT40 cells to methyl methanesulfonate (MMS) and were able to determine excellent reproducibility as well as sensitivity similar to the DT40 clonogenic assay.

Next, we undertook to validate the accuracy of our assay. For this purpose, we chose to compare results obtained with our assay to those obtained with the clonogenic assay after exposure to cisplatin. The cell lines chosen were ones which are known to be hypersensitive to cross-linking agents, namely Bloom's helicase (BLM), the X-ray repair cross complement protein 2 (XRCC2) and the Fanconi anemia complementation group protein D2 (FANCD2). Another protein, the base excision repair protein, flap endonuclease 1 (FEN1), was also chosen as it would be expected to indicate little, if any,

sensitivity to cisplatin. Our results compare very favorably with those of the clonogenic assay.

Finally, as a further verification of the assay, we exposed a representative group of the DT40 cell line at our disposal to the highly reactive aldehyde, acrolein. From the results we obtained, we have concluded that acrolein induced DNA damage is primarily alleviated by translesion synthesis (TLS).

The DT40 cell system

As the DT40 cell system has been described in detail in Chapter 1, only a brief recapitulation is provided here. By making use of the DT40 cell line we are using the very powerful reverse genetic approach for the determination of the DNA repair pathway(s) the cell prefers for the toleration of genotoxicity induced by exogenous and endogenous agents. DT40 cells provide advantages, especially over mammalian cells, not found in many other cells lines: 1) they exhibit a high ratio of targeted to random integration of transfected DNA which allows for relatively easy creation of double and triple mutants; 2) they exhibit an exceptionally stable phenotype and karyotype allowing for extended periods of continuous culturing without concern for genetic drift; 3) they have been reported to be deficient in p53 activity leading to reduced apoptosis due to genotoxic stress; 4) the cells are maintained in suspension culture making their handling much less tedious than that of attached cells; 5) the cell cycle time is much shorter than mammalian cells making the cell line more convenient and applicable for high-throughput screening of potential genotoxic agents; and, 6) approximately 70% of the cycle time of DT40 cells is spent in S-phase as the cells are unable to arrest at the G1/2

boundary (probably due to the p53 deficiency) (Ji et al., 2009). This last feature is especially interesting because more of the exposed DT40 cells can accumulate is S-phase whereas in mammalian cells, even the *wild types*, may arrest at the G1/S check point for up to a few days allowing for complete repair of DNA damage (Bao et al., 2001; Takao et al., 1999). In contrast, DT40 repair mutants can carry even low amounts of DNA damage into S-phase where proliferation can be significantly reduced due to interference with replication. The foregoing indicates that a cell proliferation (or survival) assay using DT40 cells can be much more sensitive than one using mammalian cells.

As a result of the advantages of the DT40 cell system, it has been used in numerous investigations into various cellular pathways. In virtually all instances the very established clonogenic assay has been employed. One downside to the method, however, is that the cells are usually grown in methylcellulose gel suspension with clonogenic assays used to rate cell survival (Simpson and Sale, 2006). Clonogenic assays are considered to be very accurate but are rather tedious. Indeed, to test just one agent at several dilutions along with controls against numerous cell lines could require hundreds of dishes with an incredible amount of manual manipulation. We felt that if the system were to be used for screening, a simpler, more rapid and less expensive method would be advantageous. Here, we describe a detailed protocol of a DT40-based response analysis that is convenient but still sensitive and precise enough for determining the genotoxicity of chemicals by making use of the compound XTT.

XTT cell proliferation assay

The ability to determine cell proliferation and viability is one of the mainstays of many cell biology approaches. Thus, methods have been developed which are sensitive, quantitative and reliable. Examples of such methods utilize the ability of cells to incorporate radioactively labeled compounds such as [^{3}H]-thymidine or to release a radioactive isotope ^{51}Cr following cell lysis. Another example takes advantage of the ability of cells to incorporate 5'-bromo-2'-deoxyuridine in place of thymidine which may be monitored by immunochemical methods such as the enzyme-linked immunosorbent assay (ELISA) or slot-blot assays. As previously mentioned there is also the clonogenic assay.

First described by Scudiero, et al., and improved upon by others, the XTT cell proliferation assay is a colorimetric method which has no requirement for radioactive compounds or immunohistochemical techniques (Scudiero et al., 1988; Weislow et al., 1989; Roehm et al., 1991). The method was originally intended for the measurement of drug sensitivity in cancer cells (Scudiero et al., 1988). The assay is based on the reduction of a yellow tetrazolium salt (XTT) by mitochondrial dehydrogenase to an orange colored formazan dye only by metabolically active cells (Fig. 2-1). Roehm, et al., found that the reduction of XTT when used alone in murine cells was not especially efficient but was potentiated with the addition of an electron coupling agent such as phenazine methosulfate (PMS) (Roehm et al., 1991). The formazan dye is water soluble and can be directly measured with a high degree of accuracy in a spectrophotometric multi-well plate reader. The amount of color developed is directly proportional to the number of living

cells in the sample. This method allows for a much higher throughput with easy on-line data collection and processing.

Materials and Methods

DT40 cell culturing and maintenance

Materials

Fetal bovine serum (FBS), 1-methoxy-5-methylphenazinium methyl sulfate, and penicillin/streptomycin were obtained from Sigma (St. Louis, MO). RPMI 1640 culture medium (+glutamine, -phenol red) and chicken serum were acquired from Invitrogen (Grand Island, NY).

Method

DT40 cells are cultured in a manner similar to most vertebrate or mammalian cell lines that are grown in suspension. Optimal propagation is provided by growing in RPMI 1640 with 10% FBS, 1% chicken serum and 1% penicillin/ streptomycin. RPMI sans phenol red should be used, as the color in the indicator may interfere during spectroscopy. FBS and chicken serum should be heat inactivated at 56 °C for 30 min. The cells are incubated at 39.5 °C and 5% CO_2 with 95% humidity. Since DT40 cells have a relatively short generation time (7-10 h), for the sake of convenience we have found that cell growth can be slowed by incubation at 37 °C or even as low as 31 °C without undue stress to adjust the growth rate during the final 16 h of cultivation. This process allows for the avoidance of overgrowth of the cells. Also, due to the rapid growth rate, the

cultures must be observed regularly to prevent overgrowth with subsequent starvation resulting in stress to or death of the cells.

Sub-culturing should be performed as needed to provide cells in the log phase of growth as an optimum test parameter and to maintain cell lines for future use. For sub-culture, cells are counted by hemacytometer. The target cell concentration for both sub-culturing and the preparation of the cell suspension for the assay that produces the most reproducible assay result lies between $0.7x10^6$ and $1.5x10^6$ cells/mL. Also, cell condition should be observed during counting. Under a light microscope, normal DT40 cells appear nearly circular. Stressed cells may appear elongated or have an uneven margin. If cells appear stressed, it is advisable to newly sub-culture before using in the assay.

For best results, a certain amount of care should be observed when handling DT40 cells. All transfers of cells should be into pre-warmed medium and mixing of the cells prior to pipetting should be very gentle but absolutely thorough – especially for cell counts or seeding for an assay. It is also advisable to use pipette tips with large bore openings such as those used in genomic studies. Our results and reproducibility improved notably with the use of these procedures. Indeed, our success rates using the assay had been very wide-ranging (from 1 to 70%) using our previous method (Ridpath et al., 2007); whereas, the current improved method provides a success rate of nearly 100%.

Although DT40 cells are considered immortal, each cell line should be restarted from frozen stock about once each month. Considering that most of our cell lines are deficient in DNA repair, this seems an appropriate choice. The cells can readily be stored under liquid nitrogen for extended periods.

Test procedure

Materials

Cisplatin, MMS, XTT, PMS and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO).

General Method

The overall scheme of this assay is shown in Figure 2-2. Cells are prepared in sterile centrifuge tubes by adding enough volume of cell-containing medium from the sub-culture dish to 6 mL warmed fresh medium to provide a cell concentration adequate to seed approximately 2500 cells in a volume of 250 μL to each of 22 wells. Before seeding, the cells should be adequately but gently mixed in the tubes. The cells are then seeded by pipetting 250 μL of the cell suspension to each of 22 wells of the 24-well plate (Table 2-1). To the two blank wells add 250 μL plain medium. These plates should be kept in an incubator at 39.5 ^{o}C until ready for treatment with the chemical compound(s). This method provides for treatment with six dilutions of the chemical, each into three wells, with four untreated wells as controls and two as blanks (Table 2-1). Treatment is performed by adding 27.8 μL (total well volume, 277.8 μL) of the chemical dilution to each appropriate well and 27.8 μL of the chemical solvent - usually either phosphate buffered saline (PBS) or DMSO - to the control and blank wells. The plates are returned to the incubator.

Once treated, the incubating cells should not be disturbed for 48 hr after which they should be observed microscopically to determine the growth of the cells. Once the cells in the control wells are nearly contiguous, each well (including the blanks) should

be treated with XTT dye (XTT cocktail is prepared with 500 mg of the XTT salt, 10.2 mg PMS and 3.3 mL DMSO). This is a critical step as the cells should not be allowed to grow until starvation begins causing cell death. Conversely, if the number of cells is too low, an inordinate amount of time may be required for the cells to metabolize the dye and adequate contrast may be difficult to obtain. The cells are exposed to the XTT cocktail by first preparing a stock solution of XTT in complete medium (9.1 μL XTT mixture/2.5 mL medium) then treating with 100 μL of the dye preparation. The plates are returned to the incubator until the dye has developed sufficient color for absorbance to be read on the plate reader (typically 2 – 4 h). Once the plates have developed sufficient color, they may be read immediately or stored at 4 ºC in relative darkness until reading is convenient (this represents another improvement over our previously reported version of the assay). Stored plates should be kept in sealable plastic bags or something similar to prevent drying of the medium in the wells. The ability to store the plates is yet another reason for using medium without phenol red as the indicator can change color due to temperature modification of the pH in the medium, which would interfere with accurate spectrometric readings later.

For measuring absorbance, our lab uses a Tecan Safire (Tecan Systems, San Jose, CA) plate reader with Magellen6 software (Tecan, version 6.4). The software provides a convenient methodology for reading plates with many different numbers of wells and subsequent saving of absorbance values to Microsoft Excel. Absorbance measurement is read at 450 nm with a reference of 650 nm.

All data are reported as the means ± standard deviation of at least triplicate samples. Analysis of covariance (ANCOVA) was used to test for mean intercept

differences and differences in the slopes of the linear dose-response curves in cell viability analysis between wild-type and a series of mutant cells. A Student's *t*-test was utilized to determine the significant differences ($p<0.05$) between means of two groups.

Test with cisplatin

To determine the dosage range to assure toxicity for cisplatin, dosing in different ranges was performed until sufficient toxicity was observed to reduce survival of the wild type DT40 cells to below 50% at the highest dose used. That range was determined to be 0 – 100 nM for cisplatin. The cisplatin was serially diluted in DMSO and kept on ice until used. The method described above was then employed to determine the survival rates of the DT40 cell lines. DMSO was added to the blank wells.

Test with acrolein

The dosage range for acrolein (Acr) was determined as above for cisplatin and found to be 0 – 2 μM. Acrolein was serially diluted in PBS and kept on ice. PBS was used to treat the blank wells.

Statistical Methodology Development

<u>Model the relation between treatment dosage and survival proportion</u>

Let x and y be treatment dose and survival proportion, respectively. We modeled their relation by a linear model $y = a + bx$ or a quadratic model $y = a + bx + cx^2$ and compared the linear model and the quadratic model for each cell line by analysis of variance (ANOVA). With an ANOVA p-value cutoff at 0.05, we found the quadratic

model fit the data significantly better than the linear model in 14 of the 31 cell lines treated with Acr. (Fig. A-1). Thus we adopted the quadratic model in these 14 cell lines and linear model for the remaining 17 cell lines. We could employ more complicated models such as higher degree polynomials or a spline model. However, as shown in the scatter plots in Figure A-1, linear or quadratic models already fit the data reasonably well, and more complicated models may lead to over-fitting. In fact, as shown in the following results, our linear/quadratic method and some non-parametric approaches have given consistent results in terms of testing for trends and evaluating minimum effective dose (MED).

Trend test of the dose-response relations

We corrected the multiple tests by the Bonferroni's method, i.e., we used a p-value 0.05/31 = 0.0016 as the significance cutoff. Based on the p-values of linear/quadratic models (Fig. A-1), there are significant trends that the survival proportion decreases as the Acr dosage increases in all cell lines except for FEN1 (p=0.03). We also carried out a trend test based on an isotonic (monotonic) regression model $y = f(x)$, where f is an unknown monotone function (Barlow et al., 1972; Hu, et al., 2005). Besides the monotone relation, this isotonic regression model does not assume any specific model forms. We fitted the model using the "pool-adjacent-violators" algorithm (Barlow et al., 1972). Following Hu et al. (Hu et al., 2005), we used a permutation to evaluate the p-value of the trend test. The estimated permutation p-values (from 100,000 permutations) are smaller than 0.0016 for all cell lines except for FEN1 (p=0.01).

Minimum effective dose

Given a significance level α, the MED is the lowest dose such that the mean response is significantly different than the mean response of the zero dose. Based on linear/quadratic models, the MED is simply the lowest dose where the upper bound of the predicted 1 - α confidence interval (CI) reaches zero. The MED of each cell line when α=0.05 are indicated in Figure A-1.

We also employed a non-parametric method to identify MED (Williams, 1986), which is based on two well-known works: (Williams, 1972; Shirley, 1977). Different from our model-based approach, this non-parametric method answers the question whether the response for an *observed* dose is significantly different from the response for the zero dose. Therefore, based on the monotone relation assumption, we can infer whether the MED is between two observed doses, but not its exact value. As shown in Table A-1, overall, the results of this non-parametric method are consistent with the results of our parametric method, despite the fact that we only have a limited sample size. However, our linear/quadratic parametric approach has the advantage of pinpointing the MED at any dose levels in addition to the observed doses.

Difference across cell lines

Next we sought to evaluate the difference across cell lines. We are especially interested in the difference between the wild type DT40 cell line and all the other cell lines. Based on the linear/quadratic model fit, the difference of the predicted response from two cell lines follows a t-distribution. Thus we can calculate the corresponding prediction CI. As shown in Figure A-2 (predicted difference and the corresponding 95%

CI), nine cell lines, ASCIZ, ATM, BRCA1, FEN1, LIGIV, MSH2, POLK, RAD54, and WRN may have significantly different survival proportions compared with that of cell line DT40.

Results and Discussion

Reproducibility and sensitivity in the DT40 cell DNA damage response analysis using the XTT method

To address reproducibility of the response of DT40 parental cells to mutagens, we exposed DT40 cells to MMS, a strong DNA alkylating agent whose damage is repaired by BER, at different concentrations in three independent experiments. Figure 2-3A indicates the level of reproducibility achieved from those experiments. We tested for statistical difference between the three results for 50% lethal concentration (LC_{50}) values, slope and y-axis interception. These analyses provide no significant difference, suggesting a reasonable reproducibility of our assay for measuring the response in DT40 cells to MMS in terms of cell viability. We also compared DT40 cells and POLB (a BER polymerase) mutants exposed to MMS for cell viability (Fig. 2-3B). The statistically significant difference in dose-response relationship between two cell lines was detectable as low as 37.5 μM of MMS. This sensitivity appears to be similar to the results obtained by clonogenic analysis (Yoshimura et al., 2006).

Comparison of cisplatin exposure results between the DT40 XTT dye and clonogenic methods

Cisplatin

To assess for any difference of results between the DT40 XTT dye and the clonogenic methods, cisplatin, a DNA cross-linking agent has been chosen. For the purpose of this comparison, we have chosen to report on three DT40 mutant cells lines that are known to be involved in the repair of DNA interstrand crosslinks (ICL): the DNA helicase, BLM, and the enzymes, XRCC2 and FANCD2. Cells lacking these proteins are known to be hypersensitive to DNA cross-linking agents. In addition, we have chosen the flap endonuclease, FEN1, which should show little, if any, additional sensitivity to cisplatin beyond the *wt.* As depicted in Figure 2-4A the results for the BLM, XRCC2 and FANCD2 mutants indicate quite strong sensitivity for these mutants compared with the *wt,* while the FEN1 knockout showed little sensitivity. This result for XRCC2 shows very close agreement for relative sensitivity with the *wt* to that of Takata, et al., although our result indicated sensitivity for both the *wt* and the mutant at much lower concentrations due to continuous treatment (Takata et al., 1998). Takata, et al., using the clonogenic assay, report that XRCC2 mutants were approximately 8-fold more sensitive to cisplatin than the normal cells while we showed an approximate 5-fold increase (an LC_{50} of 22% compared with the *wt*) in sensitivity (Fig. 2-4B). This result for XRCC2 exposure also shows excellent agreement with Nojima, et al., who reported an approximate 25% D_1 survival compared with the *wt,* using the colony formation assay (Nojima et al., 2005). The result for the BLM mutant using the XTT method produced an LC_{50} of 22% compared with the *wt,* whereas Nojima, et al., were somewhat higher with a

D_1 survival at ~ 45%. For the FANCD2 mutant exposed to cisplatin, we found a profound sensitivity where the LC_{50} survival was only 9% that of the normal cells. This result agrees well with that obtained by Matsushita, et al., which were ~ 5% using the clonogenic method (Matsushita et al., 2005). As expected, the XTT method with FEN1 mutants indicated an LC_{50} of 87% compared with the *wt*, while Nojima, et al., was ~ 90% in their laboratories clonogenic assay (Nojima et al., 2005).

Further validation of the DT40/XTT assay with the genotoxic agent, acrolein

Acrolein

Acrolein (2-propenal), an α,β–unsaturated aldehyde, is a highly reactive bifunctional electrophile, and can react by Michael-addition with DNA bases at various positions with the exocyclic nitrogen (N^2) of deoxyguanosine (dG) providing the most common target (Minko et al., 2008) (Fig. 2-5). Human exposure to Acr may be divided into three main source groups including dietary, endogenous and environmental sources. Endogenous sources include products of lipid peroxidation and products of the metabolism of polyamines (Stevens and Maier, 2008). Acr is also ubiquitous in the environment with sources such as the incomplete combustion of petroleum, coal, wood and plastic, the frying of foods in oils, and the smoking of tobacco products (reviewed by Esterbauer et al., 1991). Exogenous Acr is absorbed by human cells with reasonable efficiency and can react with cellular molecules directly without metabolic activation. Acr has been identified in at least 32 hazardous waste sites that have been proposed for inclusion on the EPA National Priorities List (Foroon et al., 2008) and has been listed

thirty-seventh on the 2007 CERCLA Priority List of Hazardous Substances. Of all sources, the smoking of tobacco products provides at least as much human exposure as all other sources combined (Stevens and Maier, 2008). Indeed, Acr has been found in cigarette smoke at levels up to 1000-fold of those for benzo[a]pyrene, a known cancer inducer (Feng et al., 2006).

Acr is cytotoxic and some reports show it to be mutagenic, although the mutagenicity of its major adduct with DNA is controversial (Minko et al., 2008; Kim et al., 2007; Stevens and Maier, 2008). The major product of reaction between Acr and N^2-dG is γ-hydroxypropano-deoxyguanosine (γ-HOPdG) with α-hydroxypropano-deoxyguanosine (α-HOPdG) being formed in much lesser amounts (Chung et al., 1984) (Fig. 2-5). These lesions have been measured in various human tissues to be in the range from 1-200/10^8 guanine (reviewed in Nair, 2007). The ability of α-HOPdG to be mutagenic is fairly well established (Wang et al., 2009), but mutagenicity of the dominant adduct, γ-HOPdG, remains to be clarified.

Although the γ-HOPdG adduct may exist in equilibrium between the ring-open and ring-closed forms, it has been shown to prefer the ring-open form at neutral pH with the N^2-(3-oxopropyl) group protruding into the minor groove in double-strand DNA (dsDNA) (de los Santos et al., 2001). As with formaldehyde, Acr induced mutagenesis has been proposed to be the result of DPC caused by reaction of the propano-aldehyde, found in the ring-open γ-HOPdG adduct (Fig. 2-5, Rxn A), with nucleophiles such as the amine groups of amino acid side chains. Some reports have suggested that these γ-HOPdG-mediated DPC are repaired by nucleotide excision repair (NER) (Yang et al., 2001; Minko et al., 2005; Baker et al., 2007) and probably not base excision repair

although other DNA repair mechanisms have not been well studied and may be found to be involved. Also, there have been numerous reports involving TLS of Acr-DNA adducts and most but not all have indicated that such bypass is mostly error-free in mammalian cells (Kanuri et al., 2002; Minko et al., 2003; Yang et al., 2001; Yang et al., 2003; Washington et al., 2004).

Acrolein Results

Our purpose in this study has been to investigate the DNA repair mechanisms used by cells to alleviate damage caused by Acr along with the additional intention being to further verify our DT40 assay against a well studied chemical. To this end we have made use of the DT40 cell line including 28 isogenic mutants deficient in DNA repair pathways as well as some cell cycle checkpoint and DNA damage sensor mutants described in Chapter 1. In a another study using the same system we found that cells deficient in FANCD2, the breast cancer 1 (BRCA1), breast cancer 2 (BRCA2) and others of the HR repair proteins for which we have mutants, are hypersensitive to formaldehyde at a concentration normally found in human plasma (Ridpath et al., 2007). From these results we postulated that the FANC/BRCA pathway is required for the repair of DPC mediated by formaldehyde. For this study we hypothesized, based on our formaldehyde results, that acrolein adducts, which putatively also form similar DPC to formaldehyde would cause hypersensitivity in the HR mutants. Our data indicates that this may not be the case – especially with mutants of the FANC/BRCA pathway. The FANCD2, BRCA1 and BRCA2 mutants were not particularly sensitive to Acr when compared with the

DT40 isogenic parent. This outcome suggests that Acr may not induce DPC in vertebrate cells.

In the last several years a number of in vitro studies have been performed that suggest that DNA interstrand cross-links could be mediated by the γ-HOPdG adduct (Stone et al., 2008; Kozekov et al., 2003; Cho et al., 2005). DNA interstrand cross-links have been shown to require HR and the FANC/BRCA pathway for their repair (Panasci et al., 2002; Wang and D'Andrea, 2004; Pichierri and Roselli, 2004). Our results with FANCD2 along with our results here with BRCA1 and BRCA2 have indicated no particular sensitivity to Acr (Fig. 2-6) in these mutants (although some other HR mutants were mildly sensitive). Thus, these results suggest that, along with DPC, DNA interstrand cross-links are not induced by Acr in vertebrate cells.

Our findings do agree with those that suggest that TLS is responsible for dealing with DNA damage induced by Acr in vertebrate cells. In particular, the DNA TLS polymerase κ (POLK) and polymerase η (POLH) appear to be more sensitive than the *wt*, although, of the two, only the POLK results were found to be significantly different from the wt under the scrutiny of our statistical method. These results are in agreement with others that have indicated that TLS (Fig. 2-6) is involved (Minko et al., 2009; Minko et al., 2008; Minko et al., 2003; Yang et al., 2003).

Lastly, and perhaps most noteworthy, we must consider the Acr concentrations responsible for our observations when compared with previous studies. Whereas the *wt* DT40 cells produced an LC_{50} of 2.71 μmole/L upon exposure to Acr, LC_{50} POLK and POLH were 1.07 μmole/L (39.5% *wt*) and 1.00 μmole/L (36.9% *wt*). To our knowledge, these values represent the lowest ones found in vertebrate cell studies to date and are near

or below the normal levels of Acr found in human plasma (0.50 – 30 μmole/L), or well below the levels found in certain pathophysiological occurrences (Sakata et al., 2003; Garrett et al., 2008; Tomitori et al., 2005).

Advantages of the XTT dye method over the clonogenic method

As can be observed in Table 2-2, there is a dramatic reduction in materials, manipulation and cost required for the XTT dye method. Also, and just as importantly, much more data can be acquired per unit time. This should be especially attractive to government agencies that wish to quickly establish correct and effective biomarkers so that more meaningful regulations may be determined on a timely basis.

As far as we are aware, only two studies have performed multi-well based DT40 cell DNA damage response analysis using more than 12 different cell lines. One of these reports is our previous study regarding formaldehyde (Ridpath et al., 2007). The other recently reported using DT40 mutant cells with a 12/24-well format to analyze DNA damage responses to UV/ionizing radiation and $NaAsO_2$ (Ji et al., 2009). That particular investigation also compared their 12/24-well plate assay and the clonogenic assay with regard to sensitivity and produced very comparable results between the two methods. These data combined with results from the present study indicate that a multi-well plate-based DT40 cell assay is reasonably sensitive for the detection of genotoxicity induced by diverse agents such as formaldehyde, cisplatin, MMS, UV/ionizing radiation and $NaAsO_2$.

Additionally, the XTT dye method may lend itself readily to automation, whereas it is doubtful the same could be said for the clonogenic method. The advantage of

automation becomes obvious if one wishes to use the method as a screen to aid in the determination of the MOA of numerous environmental chemicals. In fact, the ability of the DT40 system to provide very specific information about the DNA repair pathway used to alleviate damage induced by a chemical agent could become one of the most valuable tools in the chest of those given the responsibility to ascertain the genotoxicity/mutagenicity of the agent. Also, and perhaps even more importantly, currently available DT40 mutant cell line has originated from a single parent and is therefore isogenic. This reduces greatly the uncertainty found with other systems where mutant cell lines have been created from different sources – which further increases the value of DT40 in the determination of the MOA.

Taking into consideration the forgoing we conclude that, while the DT40 clonogenic assay is very accurate, it is somewhat cumbersome, costly and time consuming for use as a screen for toxicological purposes where a higher throughput may be desired. We have shown that our DT40/XTT method can provide comparative accuracy and sensitivity with the clonogenic method while doing so with much less tedium, cost and especially time to produce results.

Chapter 2 Figures.

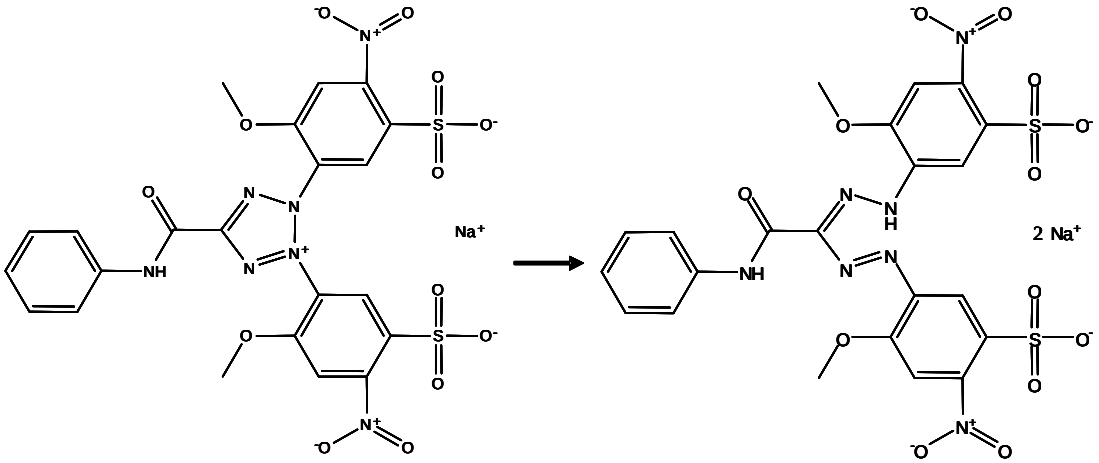


Figure 2-1. XTT metabolization to formazan salt

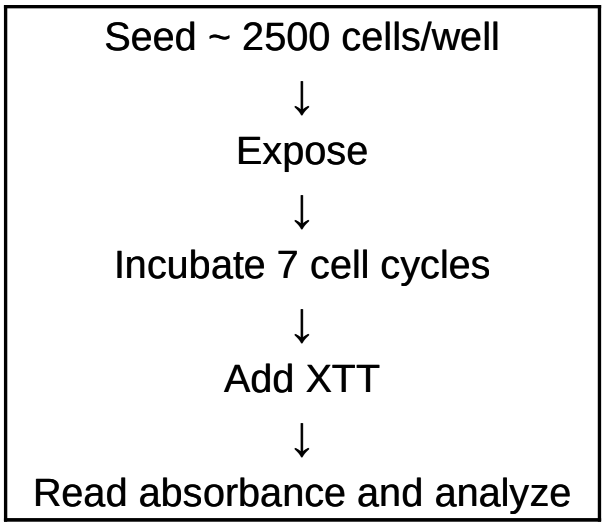


Figure 2-2. Protocol overview of XTT-based DT40 cell DNA damage response analysis using 24-well plate format.

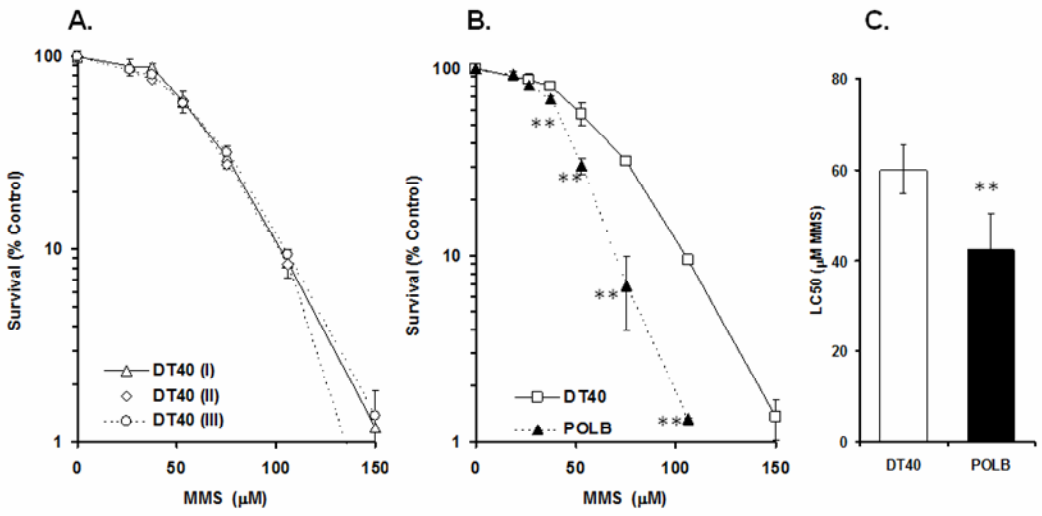


Figure 2-3. Cell survival results for DT40 cells and POLB mutants continuously exposed to MMS. (A) Results of three independent survival experiments in DT40 cells exposed to MMS. Survival data were log-transformed giving approximate normality. Analysis of covariance (ANCOVA) was used to test for mean intercept differences and differences in the slopes of the linear dose-response curves in cell viability analysis between experiments. No significant difference ($p<0.05$) was detected between experiments. (B) Survival percentage for DT40 and POLB mutant cells exposed to MMS compared with concurrent control. A Student's *t*-test was utilized to determine the significant differences between means of two groups (DT40 (II) vs. POLB exposed to MMS at same concentration, **P < 0.01). (C) Survival data from DT40 (II) and POLB cells exposed to MMS were log-transformed giving approximate normality. Each LC_{50} value was then calculated for each cell line. ANCOVA showed a significant difference for mean intercepts of the linear dose-response curves between wild-type and POLB cells (**P < 0.01). *Bars*: 95% confidence intervals for LC_{50} value for each cell line.

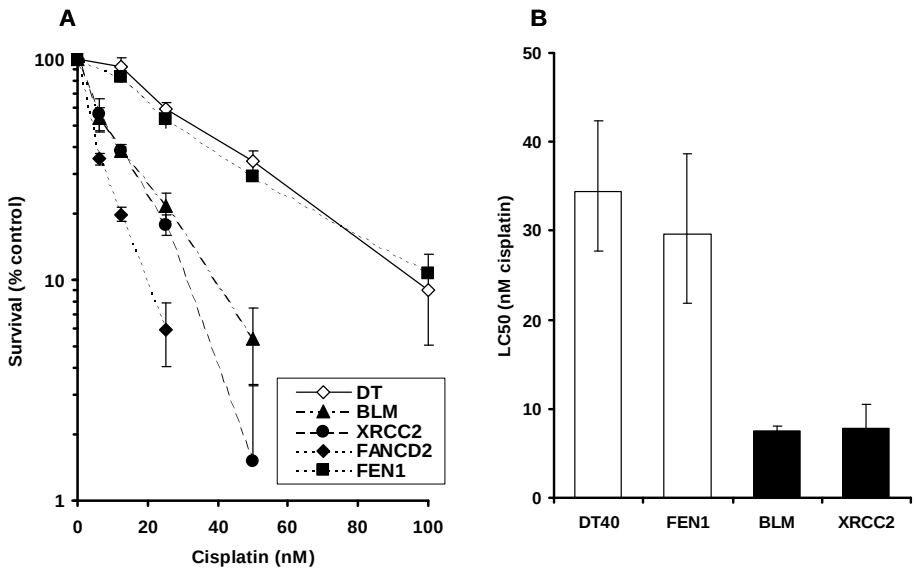


Figure 2-4. Cell survival results for DT40 cells and their isogenic mutants continuously exposed to cisplatin. (A) Survival percentage for each cell line exposed to cisplatin compared with concurrent control. (B) Survival data were log-transformed giving approximate normality. Black columns indicate significant differences analyzed by ANCOVA ($p<0.01$) between parental DT40 cells and mutants (BLM, XRCC2 and FANCD2 cells). In contrast, FEN1-deficient cells showed no significant difference by ANCOVA ($p>0.05$). *Bars*: 95% confidence intervals for LC_{50} value for each cell line.

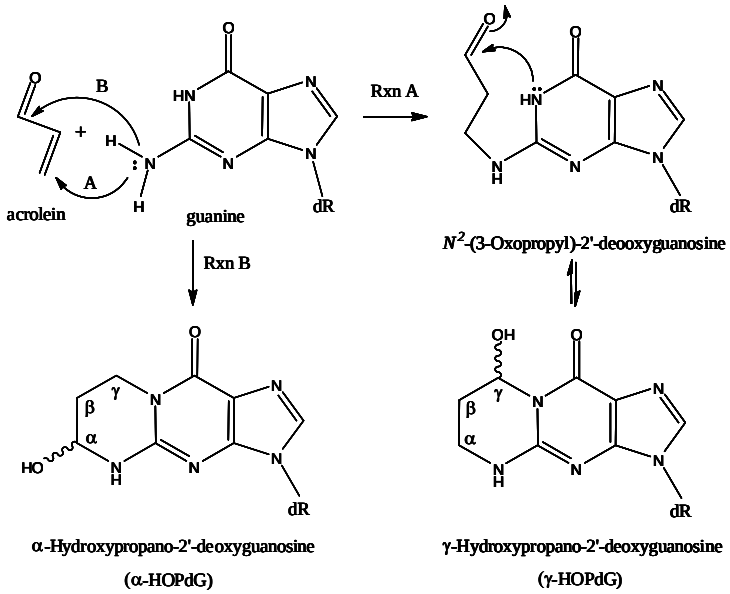


Figure 2-5. Reactions of acrolein with deoxyguanine.

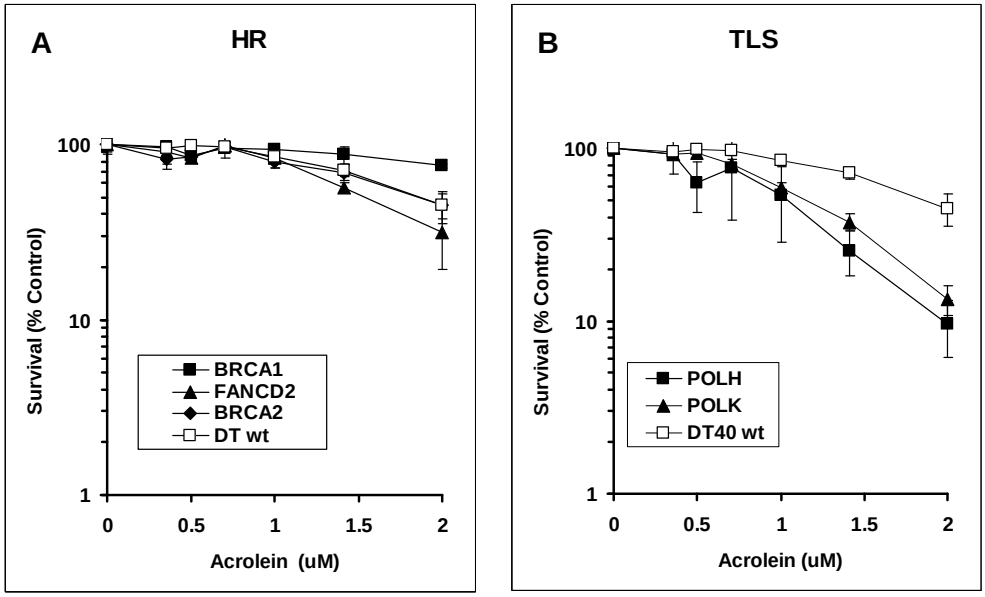


Figure 2-6. Cell survival results for DT40 cells and their isogenic mutants continuously exposed to acrolein. (A) Survival percentage for HR cell lines exposed to acrolein compared with concurrent control. (B) Survival percentage for TLS cell lines exposed to acrolein compared with concurrent control. Survival data were log-transformed giving approximate normality.

Chapter 2 Tables.

Table 2-1. Format for Treatment of 24-well Plate

1	1	1	5	5	5
2	2	2	6	6	6
3	3	3	C	C	C
4	4	4	C	Bl	Bl

Numbers represent dosing solutions: from highest concentration (1) to lowest (6); C: control (cells plus vehicle only); Bl: blank (complete medium without cells plus vehicle).

Table 2-2. Comparison of clonogenic method to XTT dye method

	Clonogenic method (Simpson and Sale 2006)	XTT dye method
Medium	Methylcellulose medium requiring overnight mixing, pH adjustment, etc.	Complete medium requiring about 5 min preparation time
Plates	One 6-well plate/cell line/dose totaling 20 plates for 4 cell lines and 5 doses	One 24-well plate/cell line with up to 6x3 doses on a single plate
Plating of samples	Separate pipetting of medium (with more stirring) and cell samples	A single addition of cell suspension to well
Incubation period	10 days to 2 weeks	3-4 days
Test method	Requires centrifugation, washing and resuspension of cells	No centrifugation/resuspension of cells
Measurement	Manual colony counting – one cell line/dose at a time. Plates should be counted promptly when ready	Absorbance reading on plate reader – one complete cell line with all doses at a time. Plates can be stored at 4 °C until convenient to read.
Estimated daily data accumulation	8 cell lines with 5 doses/cell line (40 plates)	80-100 cell lines with 6 doses/cell lines plus control

Chapter 3. Use of the DT40/XTT Assay in the Determination of the DNA Repair Pathway Responsible for the Tolerance of Formaldehyde Genotoxicity

Abstract

Formaldehyde is an aliphatic monoaldehyde and is a highly reactive environmental human carcinogen. While humans are continuously exposed to exogenous formaldehyde, this reactive aldehyde is a naturally occurring biological compound that is present in human plasma at concentrations ranging from 13 to 97 μM. It has been well documented that DPCs likely play an important role with regard to the genotoxicity and carcinogenicity of formaldehyde. However, little is known about which DNA damage response pathways are essential for cells to counteract formaldehyde. In the present study, we first assessed the DNA damage response to plasma levels of formaldehyde using chicken DT40 cells with targeted mutations in various DNA repair genes. Here we show that the hypersensitivity to formaldehyde is detected in DT40 mutants deficient in the BRCA/FANC pathway, homologous recombination or translesion DNA synthesis. In addition, FANCD2-deficient DT40 cells are hypersensitive to acetaldehyde, but not to acrolein, crotonaldehyde, glyoxal, and methylglyoxal. Human cells deficient in FANCC and FANCG are also hypersensitive to plasma levels of formaldehyde. These results indicate that the BRCA/FANC pathway is essential to counteract DPCs caused by aliphatic monoaldehydes. Based on the results obtained in this Specific Aim, we are

currently proposing that endogenous formaldehyde might have an impact on highly proliferating cells, such as bone marrow cells, as well as an etiology of cancer in FA patients.

Introduction

Formaldehyde is an aliphatic monoaldehyde and is a highly reactive environmental pollutant found in automobile emissions and tobacco smoke. Several studies have demonstrated genotoxicity and carcinogenicity due to formaldehyde (Monticello et al., 1996; Emri et al., 2004).Recently, formaldehyde has been re-evaluated by the IARC as a known environmental human carcinogen (Group 1) due to a positive association between the extent of formaldehyde exposure of workers and their death from nasopharyngeal cancer (Cogliano et al., 2005). While humans are continuously exposed to exogenous formaldehyde, this chemical is also a naturally occurring biological compound that is present in all tissues, cells and bodily fluids. Formaldehyde and its oxidation product, formic acid, function as key intermediates in the "one-carbon pool" (Neuberger 1981) utilized for the biosynthesis of purines, thymidine and some amino acids. It has been found that the concentration of endogenous formaldehyde in human plasma ranges from 13 to 97 μM (Szarvas et al., 1986; Heck and Casanova, 2004; Luo et al., 2001) with Heck, et al., reporting an average of 82 μM (calculated using 1.06 for the density of human blood). Formaldehyde is usually rapidly metabolized by reduction, oxidation and glutathione (GSH)-dependent pathways (Koivusalo et al., 1989). However, saturation in formaldehyde metabolism may lead to DNA damage. It has been well documented that cells exposed to formaldehyde exhibit, as a major form of DNA damage,

DPCs (Quievryn and Zhitkovich, 2000; Casanova et al., 1991) which are clastogenic due to their ability to arrest DNA replication (Hubal et al., 1997) (Fig. 3-1). Failure to remove the lesion or fully repair it before cell division can frequently cause cell death or chromosome aberrations. Cellular DNA is found in tight association with proteins involved in both structural organization of the genome and others that control cellular processes. In fact, DNA-histone crosslinks have been shown to have a strong correlation with formaldehyde-induced tumor incidence in animals. As a result, the level of formaldehyde-induced DPCs is considered to be a good molecular dosimeter for formaldehyde cancer risk assessment (USEPA, 1991; Casanova et al., 1991; Hubal et al., 1997). Although understanding the importance of DPCs with regard to the genotoxicity of formaldehyde is paramount, little is known about which DNA damage response pathways are essential for cells to counteract formaldehyde.

Fanconi anemia, an inherited disorder, is characterized by developmental abnormalities and is associated with progressive bone marrow failure and predisposition to malignant leukemia and solid tumors (D`Andrea and Grompe, 2003; Fanconi, 1967). FA is a rare disease with heterozygosity existing at a frequency of approximately 0.3% to 1% of the population (Joenje and Patel, 2001). It has been documented that cells from FA patients are hypersensitivity to DNA-DNA crosslinking agents (D`Andrea and Grompe, 2003), and there is increased chromosomal breakage induced by these agents. At least 13 FANC complementation groups (A,B,C,D1,D2,E,F,G,I,J,L,M,N) have been identified (Kennedy RD and D`Andrea AD, 2006). These proteins interact in a common pathway that activates FANCD2 via monoubiquitination (Kennedy and D`Andrea, 2006). Upon recognition of a stalled replication fork, for instance, by the nuclear E3 monoubiquitin

ligase core complex, activated FANCD2 is targeted to BRCA1 nuclear foci where it regulates DNA repair by possibly HR and TLS (Kennedy and D`Andrea, 2006). DT40 cells and their isogenic mutants have predominantly been utilized to investigate the function of various gene products (Yamazoe et al., 2004). In this Specific Aim, we assessed the DNA damage response to formaldehyde by the reverse genetic approach using the DT40 cell model system and isogenic human cancer cells deficient in the FANC pathway.

Materials and methods

Materials.

Fetal bovine serum, XTT, PMS, acetaldehyde, acrolein, glyoxal, and methylglyoxal were obtained from Sigma. Formaldehyde and crotonaldehyde were purchased from Fisher Scientific and Across Organics, respectively. The total glutathione (GSH) quantification kit was from Dojindo Molecular Technologies, Inc. RPMI-1640 culture medium, chicken serum and penicillin/streptomycin were obtained from Invitrogen.

Cell lines and cell culture.

All DT40 mutants were derived from isogenic DT40 parent cell lines (Table 1). The DT40 cells and their mutants were cultured as previously reported (Tano et al., 2007). Parental colorectal cancer cell lines (RKO) cells and isogenic clones harboring an engineered disruption of *FANCC* or *FANCG* (Gallmeier et al., 2006) were cultured in a humidified 5% CO_2 atmosphere at 37°C. The medium consisted of RPMI-1640 cell

culture medium containing 10% fetal bovine serum (heat inactivated), and 100 mg/ml penicillin and 100 mg/ml streptomycin.

Cell survival assay.

For DT40 cells and their mutants, suspended cells (approximately 600 cells/250 μL/well) were seeded into 24-well plates, exposed to formaldehyde (and other aldehydes) and allowed to divide for approximately ten cycles. The formaldehyde used was 37% aqueous and was serially diluted in sterile 1x PBS (Gibco) to obtain the appropriate concentrations in the plates. All formaldeyde dilutions were made fresh and kept on ice. (Initially, a colony formation assay was attempted, but discontinued as it appeared formaldehyde reacted adversely with the methylcellulose semisolid medium). After cultivation, cell viability was determined by the XTT assay (Scudiero et al., 1988). For RKO cells and their isogenic cells deficient in FANCC or FANCG, the adhesive cells (approximately 600 cells/250 μL/well) were seeded into 24-well plates and cultivated for two days before treatment. After changing to fresh medium, the cells were exposed to formaldehyde and allowed to divide for seven days. The medium was replenished at four days after treatment. The survival rates were determined as described above.

Determination of intracellular total GSH.

Total GSH levels were measured according to the manufacturer's directions using a commercially available kit. Briefly, 5,5'-dithiobis-(2-nitrobenzoic acid) and GSH react to generate 2-nitro-5-thiobenzoic acid. The concentration of 2-nitro-5-thiobenzoic acid in

the sample solution was determined by measuring absorbance at 412 nm and comparison to a standard curve.

Statistical analysis.

Survival data were log-transformed giving approximate normality. Analysis of covariance (ANCOVA) was used to test for mean intercept differences and differences in the slopes of the linear dose-response curves between wild-type and a series of mutant cells.

Results and discussion

HR repair and NHEJ repair of DNA damage induced by formaldehyde.

We assessed the contribution of each DNA damage response pathway to formaldehyde-induced DNA damage. Toward this end, we utilized a panel of isogenic DT40 cell mutants, each of which was defective in a particular DNA repair or cell-cycle checkpoint response pathway. Here we show that formaldehyde causes a reduction in survival of DT40-derived mutants that are deficient in HR repair. The HR repair pathway mutants showed sensitivity to formaldehyde in the following order: FANCD2 >> BRCA2 > BRCA1, XRCC2, RAD51C, RAD51D, XRCC3, RAD54 > RAD52 > parent DT40 cells (Figure 3-2). In contrast, cell survival rates were largely equivalent between NHEJ-deficient cells and parent DT40 cells (Fig. 3-3). One of the metabolic pathways used by cells to detoxify formaldehyde is GSH-dependent. Therefore, we tested whether there is an association between intracellular GSH levels and hypersensitivity of HR-deficient cells to formaldehyde. However, we found no correlation between GSH

concentrations and formaldehyde-induced cell toxicity in series of isogenic DT40 cells. These results revealed a requirement for the HR pathway, but not the NHEJ pathway in processing DNA damage induced by formaldehyde, strongly suggesting that the HR pathway is involved in repair of DPCs.

Role of excision repair pathways in the repair of DNA damage induced by formaldehyde.

Results of biochemical experiments have indicated that nucleotide excision repair (NER) is involved in the elimination of DPCs (Minko et al., 2002; Reardon and Sancar, 2006). However, the relative contribution of the various excision repair pathways to the removal of DPCs has not been fully characterized. Therefore, we tested each excision repair pathway (NER and BER) as to its effectiveness in removing DPCs caused by formaldehyde. Our results show hypersensitivity in the following order: PARP1 (BER/single and double strand break repairs) > XPA (NER) > POLβ (BER) > FEN1 (BER) (Fig. 3-4). The DT40 mutants for POLB and FEN1 showed slight to marginal hypersensitivity, indicating that BER is not heavily involved in DPC repair. PARP1 is used in many pathways such as BER, single strand break repair, stabilization of replication forks and possibly double strand break repair (Schreiber et al., 2006; Hochegger et al., 2006). Thus, since POLB and FEN1 sensitivities indicate that BER is probably not highly implicated in formaldehyde-induced DPC repair, the higher sensitivity of the *PARP1* mutant may be due to its role in DNA replication and double strand break repair in cells exposed to formaldehyde. In addition to PARP1, the xeroderma pigmentosum complementation group A protein (XPA), likely plays a moderate role for counteracting DNA damage induced by formaldehyde. These results

obtained from XPA-deficient DT40 cells agree with a previous study, which demonstrated that human fibroblasts deficient in NER (XPA and XPF) were slightly hypersensitive to formaldehyde in cell survival (Quievryn and Zhitkovich, 2000).

Role of translesion DNA synthesis and cell-cycle checkpoint pathways in the repair of DNA damage induced by formaldehyde.

In the DT40 cell model system, REV1, REV3, and RAD18-deficient cells have been reported to be hypersensitive to various ICL-inducing agents including cisplatin and mitomycin C (Nojima et al., 2005; Shen et al., 2006). To determine the role of TLS in the repair of DNA damage induced by formaldehyde we exposed DT40 and DT40 cells deficient in REV1, REV3, and RAD18 along with POLQ to formaldehyde. For the *REV1*, *REV3,* and *RAD18* mutants our results show good agreement with the ICL study while *POLQ* mutants were only marginally sensitive to formaldehyde (Fig. 3-4). Proliferating cell nuclear antigen (PCNA) has been reported to be partly ubiquitinated in a RAD18-dependent manner and this monoubiquitination of PCNA is necessary for carrying out TLS by polymerase η in yeasts (Haracska et al., 2004; Hoege et al., 2002). In addition, it has been proposed that ATR/CHK1 signaling is required for RAD18-mediated PCNA monoubiquitination against DNA damage induced by benzo[*a*]pyrene dihydrodiol epoxide (Bi et al., 2006). To further characterize the DNA damage response to formaldehyde, we exposed formaldehyde to cells deficient in the S-phase cell-cycle components ATM or CHK1. Formaldehyde sensitivity in these cells was similar to parental cells (Fig. 3-4). These results suggest that neither the ataxia telangiectasia

mutated (ATM) nor the CHK1 pathways participate in the DNA damage response to formaldehyde-induced DNA damage.

Role of FANCD2 in the repair of DNA damage induced by endogenously existing aldehydes other than formaldehyde.

Using DT40 cells deficient in FANCD2, the most sensitive cell line to formaldehyde, we addressed whether other major endogenously existing aldehydes cause similar hypersensitivity to FANCD2 cells. Among the endogenously existing aldehydes potentially capable of inducing DPCs (Kuykendall and Bogdanffy, 1992), acetaldehyde caused hypersensitivity in FANCD2 cells at millimolar levels (Fig. 3-5A). Methylglyoxal (Fig. 3-5B), glyoxal (data not shown), acrolein and crotonaldehyde (Fig. 3-5C,D) showed no hypersensitivity in FANCD2 cells (Fig. 3-5B to 5D). These results suggest that the FANC pathway may be essential to counteract DPCs induced by aliphatic short monoaldehydes, but not DPCs caused by either aliphatic dicarbonyl compounds or α,β-unsaturated aldehydes. Another possibility is that either α,β -unsaturated aldehydes or aliphatic dicarbonyl compounds may more efficiently introduce other types of deleterious DNA lesions, such as exocyclic base adducts or oxidative base lesions.

Endogenous DPC-inducing agents and Fanconi anemia.

Fanconi anemia, a rare disease with heterozygosity existing at a frequency of approximately 0.4% of the population (Schroeder et al., 1964), is an inherited disorder associated with progressive bone marrow failure and predisposition to malignant leukemia and solid tumors (Kennedy and D`Andrea, 2006). It has been documented that

cells from FA patients are hypersensitive to DNA interstrand crosslinking agents (Kennedy and D`Andrea, 2006), with an associated increase in chromosomal breakage. We know, however, of no report that has described a condition whereby endogenous reactive agents such as formaldehyde have induced DPCs with the further induction of toxicity in cells deficient in the FANC pathway. It has been reported that endogenous formaldehyde in human plasma is detectable at concentrations ranging from 13 to 97 μM (Szarvas et al., 1986; Heck and Casanova, 2004). The two most sensitive DT40 mutants are FANCD1 (BRCA2)- and FANCD2-deficient cells, which showed hypersensitivity to formaldehyde at concentrations between 10 and 15 μM. This observation raises a question about the relevance of data derived from the DT40 model system to the human cellular response to formaldehyde. Therefore, we exposed RKO cells and their isogenic cells disrupted in *FANCC* or *FANCG*. The human cells deficient in either FANCC or FANCG were hypersensitive to formaldehyde at concentrations 20 mM or higher (FANCC) or 38 mM or higher (FANCG) (Fig. 3-6). These results indicate that the FANC/BRCA pathway plays a critical function in not only DT40 cells, but in human cells. Therefore, we currently hypothesize that endogenous formaldehyde induces DPCs and plays a critical role in the initiation of progressive bone marrow failure or predisposition to malignant tumors in FA patients.

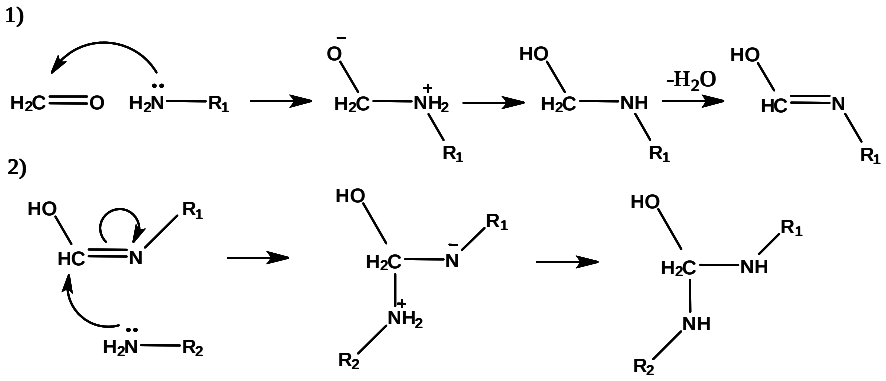
Possible DNA repair pathways for DPCs induced by formaldehyde.

It is widely accepted that formaldehyde predominantly introduces DPCs in cells. The DPC formation is believed to be related to formaldehyde-induced cancer in animals and humans; however, little information is available in terms of the DNA damage

response to formaldehyde. Interestingly, we recognized that the DNA damage responses to formaldehyde in the DT40 cell model system were similar to those of cisplatin (Nojima et al., 2005). For example, survival of isogenic DT40 mutants to cisplatin is in the following order: REV3 > FANCC > RAD18 > XRCC2 > XRCC3 > PARP1 > RAD54 > BRCA1 > XPA = ATM > BRCA2 = MSH3 > parental DT40 cells. Since both DNA-DNA crosslinks and DPCs have the potential to cause stalled DNA replication forks and may introduce double strand breaks, hypersensitivity of HR-deficient cells to formaldehyde is likely due to DPCs. Furthermore, cisplatin induces DPCs in addition to inter- or intra-strand crosslinks. The similarity of the DNA damage response in DT40 mutants to cisplatin and formaldehyde also raised the possibility that DPCs caused by cisplatin may have a role in the hypersensitivity of DT40 cells deficient in the FANC/BRCA and HR pathways. We also found hypersensitivity of PARP1-deficient DT40 cells to formaldehyde. Interestingly, PARP-1 also inhibits HR from interference by KU and LIGASE IV in DT40 cells (Hochegger et al., 2006). This report, combined with our results, suggests that without precise regulation of HR in the absence of PARP1, the HR repair pathway for the repair of double strand breaks induced by DPCs may not efficiently restore the integrity of genomic DNA. Although *XPA* mutants are more resistant to formaldehyde compared with HR-deficient cells (a characteristic shared with cisplatin) (Nojima et al., 2005), XPA-deficient cells were moderately hypersensitive to formaldehyde, suggesting a reasonable role for NER in the elimination of DNA damage caused by formaldehyde. Figure 3-7 shows a possible DNA repair pathway to counteract DPCs induced by formaldehyde. In the error-free DPC excision process, formaldehyde-induced DPCs could be first degraded to DNA-amino acid crosslinks (DAC) by a cellular

proteasome. The NER pathway may recognize and eliminate the DACs followed by DNA repair synthesis (Minko et al., 2002; Reardon and Sancar, 2006; Quievryn and Zhitkovich, 2000). In contrast, error-prone DPC excision processes could exist to tolerate formaldehyde-induced damage. After degradation of DPCs to DACs but before initiation of excision repair, DNA replication may start and TLS DNA polymerases may by-pass and extend DNA synthesis past the DACs. After DNA replication, the NER pathway may recognize and eliminate the DACs followed by DNA repair synthesis. Since HR-deficient cells are hypersensitive to formaldehyde, the requirement for HR repair for uncoupling of initiation of DNA replication and excision repair processes can be hypothesized. The NER pathway recognizes and eliminates DACs. Before the completion of excision repair, DNA replication could begin and lead to the formation of DNA double strand breaks with subsequent repair by a HR-dependent pathway. Another possibility is that DPCs formed during DNA replication cause stalled DNA replication forks, followed by the formation of DSBs. The HR-dependent pathway could then repair these DSBs.

Chapter 3 Figures.



R_1 = amino acid side chain group; R_2 = base amine group. Adapted from Barker et al., (2005) Mut Res 589; 111-135.

Figure 3-1. Formation of DNA-protein cross-link with formaldehyde

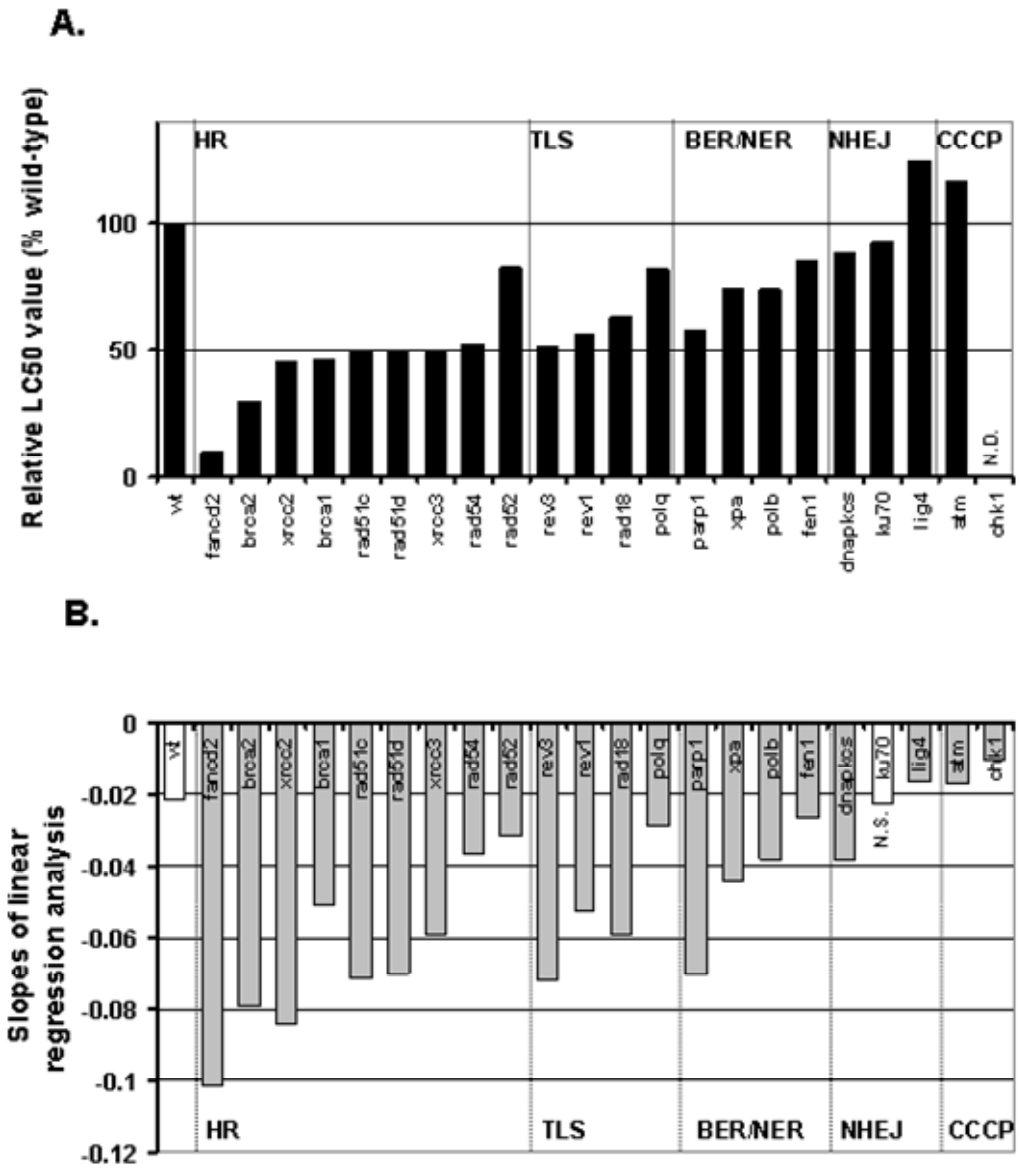


Figure 3-2. Relative LC50 values and linear regression analysis cell survival results in DT40 cells and their mutants exposed to formaldehyde. (A) Each LC50 value was calculated from results of cell survival data shown in Figures 2 to 4. Relative LC50 values were normalized according to the LC50 value of parental wild-type cells. (B) Survival rates were log transformed for ANCOVA analysis to compare slopes or interceptions between two lines generated by linear regression lines derived from DT40 wild-type cells and mutants. The value of the slopes was calculated from survival data and was plotted for each mutant. All mutants except *ku70* (one of the white columns, N.S.: no significance) showed statistically significant difference in slopes or interception ($p<0.05$). (Ridpath, et al., (2007) Cancer Res,67; 11117-11122.)

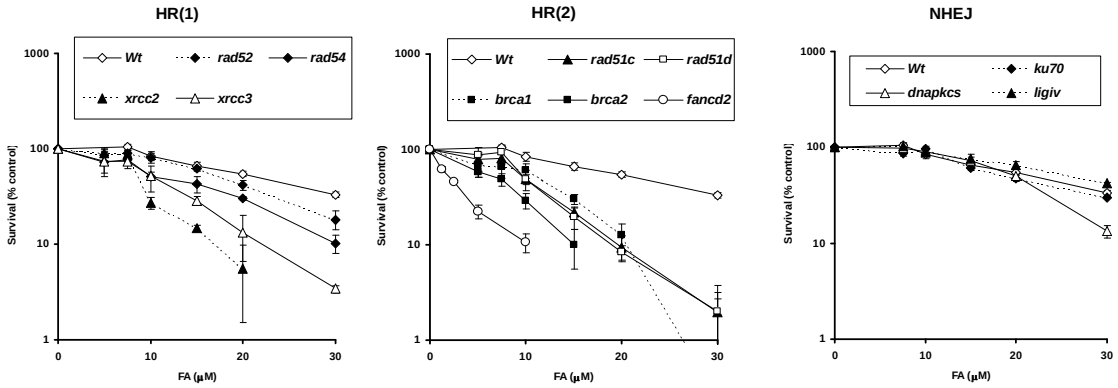


Figure 3-3. Sensitivity of *wild-type* (*wt*) and HR- and NHEJ- deficient cells after exposure to formaldehyde. The concentrations of formaldehyde are displayed on the x-axis on a linear scale, while the survival rates are displayed on the y-axis in a logarithmic scale. Error bars show the standard deviations (SD) of the mean for three or four wells. Reproducibility was confirmed at least three times. (Ridpath, et al., (2007) Cancer Res,67; 11117-11122.)

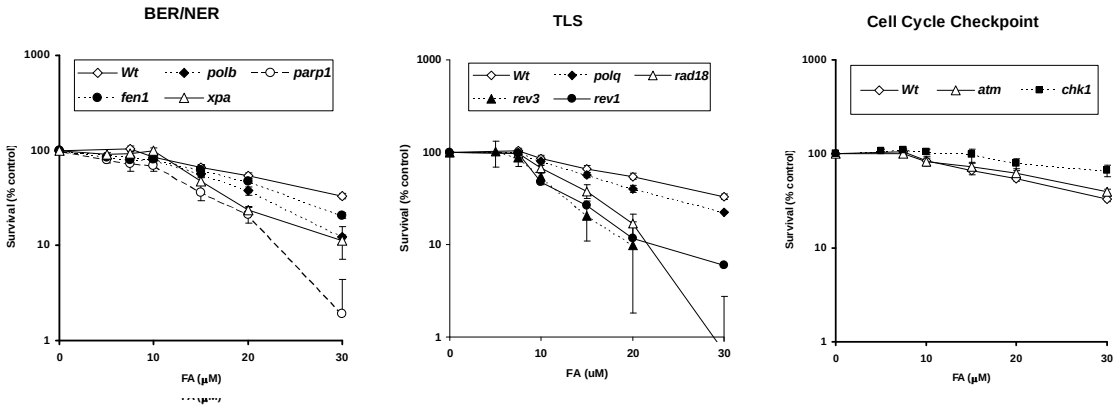


Figure 3-4. Sensitivity of *wild-type* (*wt*) and BERNER, TLS and Cell Cycle Checkpoint-deficient cells after exposure to formaldehyde. The concentra-tions of formaldehyde are displayed on the x-axis in a linear scale, while the survival rates are displayed on the y-axis in a logarithmic scale. Error bars show the SD of the mean for three or four wells. Reproduc-ibility was confirmed at least three times. (Ridpath, et al., (2007) Cancer Res,67; 11117-11122.)

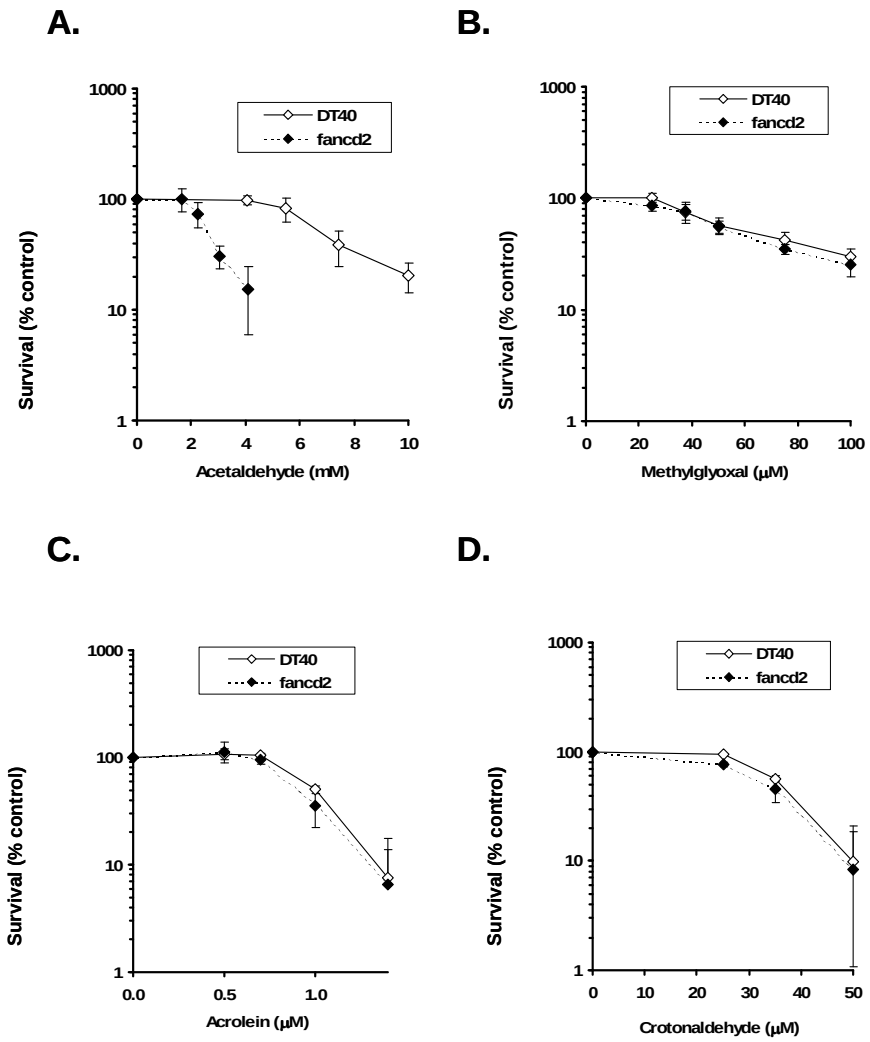


Figure 3-5. Sensitivity of DT40 cells and DT40-derived FANCD2-deficient cells to aldehydic agents. (A) Survival of DT40 cells and FANCD2-deficient cells after exposure to acetaldehyde, (B) methylglyoxal, (C) acrolein and (D) crotonaldehyde. The concentration of each aldehyde is displayed on the *x*-axis in a linear scale, whereas the survival rates are displayed on the *y*-axis in a logarithmic scale. *Point*, mean for three or four wells; *bars,* SD. Reproducibility was confirmed at least three times. (Ridpath et al., (2007) Cancer Res, 67; 11117-11122)

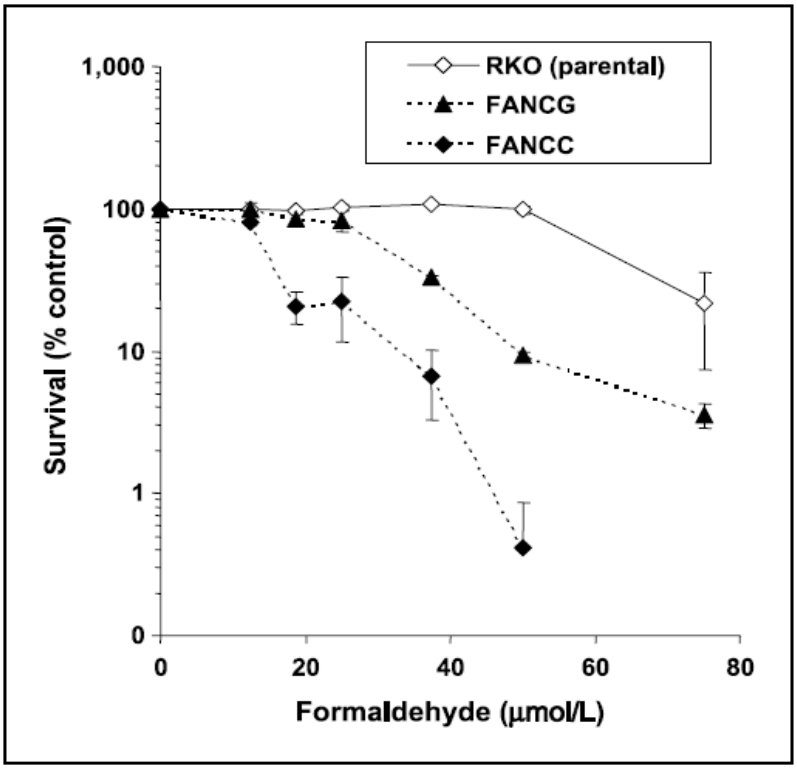


Figure 3-6. Sensitivity of parental RKO cells and their isogenic *FANCC*-/-/- and *FANCG*-/- cells after exposure to formaldehyde. The concentrations of formaldehyde are displayed on the x-axis in a linear scale, while the survival rates are displayed on the y-axis in a logarithmic scale. Error bars show the SD of the mean for three or four wells. Reproducibility was confirmed at least three times. (Ridpath, et al., (2007) Cancer Res,67; 11117-11122.)

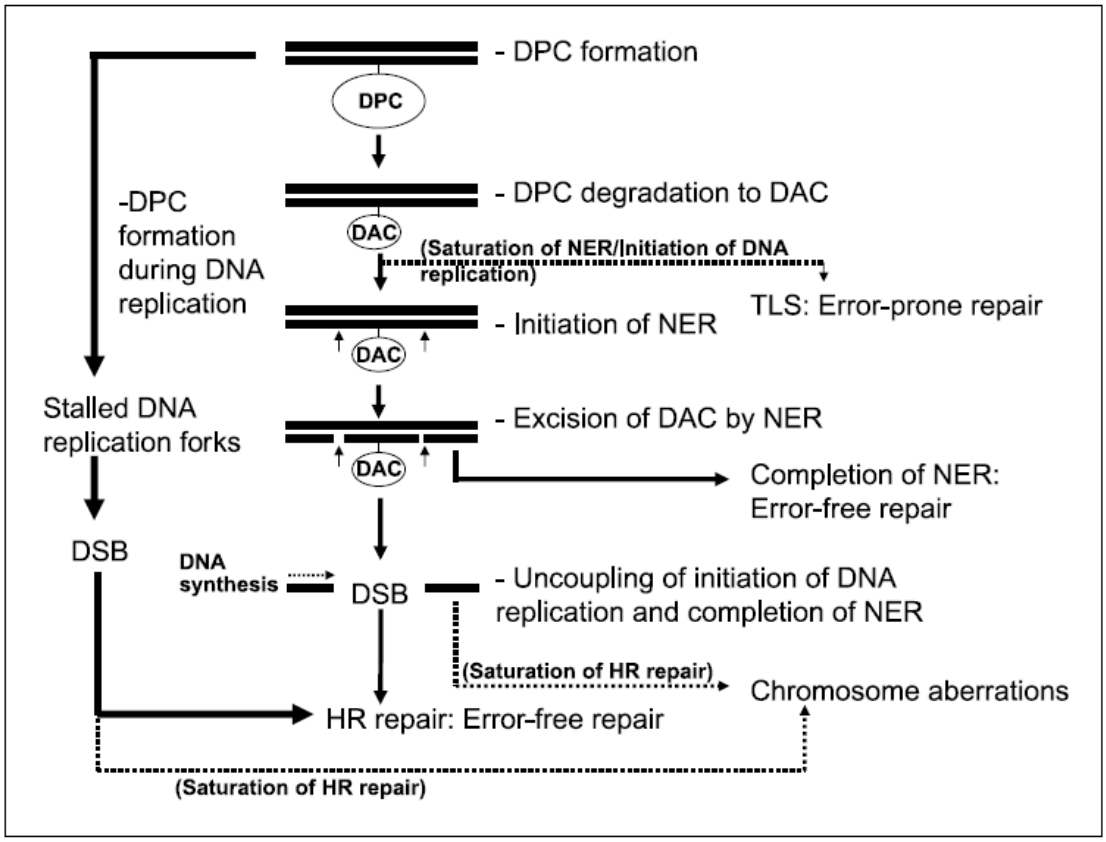


Figure 3-7. Hypothesized pathway for the repair of DPC. *DSB,* double-strand break. (Ridpath, et al., (2007) Cancer Res,67; 11117-11122.)

Chapter 4. Use of the DT40 Cell System for the Determination of the DNA Repair Pathway Responsible for the Tolerance of Hexavalent Chromium Genotoxicity

Abstract

Chromium has long been widely used industrially by-products of chromium usage pose an environmental health risk and is a known human carcinogen. Of the three oxidation states of chromium found in the environment Cr(VI) is the most relevant biologically. Cr(VI)-induced DNA damage has been reported, including; oxidative stress induced damage, DNA inter-strand crosslinks, DNA-protein crosslinks, ternary adducts of Cr with the DNA backbone phosphate, phosphate-base crosslinks, abasic sites and DNA single- and double-strand breaks. Since it is unlikely all of these damage forms are important to the ultimate mode of action of Cr(VI) toxicity, the subject of how Cr(VI) is mutagenic remains controversial. We have investigated the DNA damage response of DT40 cells to exposure to potassium chromate. Our findings indicated a response pattern similar to hydrogen peroxide exposure and also strong sensitivity of mutants in the homologous recombination repair pathway. The following assays were performed to investigate Cr(VI)-induced oxidative stress; NAD(P)H depletion, a neutral glyoxal gel electrophoresis assay, and the OTX-AGE assay – all for the determination of DNA SSB, as well as an MS assay for 8-oxo-dG. None of these assays indicated levels above their respective controls that would suggest chromate-induced oxidative stress in DT40 cells.

The exquisite sensitivity found in the HR mutants, BRCA1, BRCA2 and RAD54 along with a lack of sensitivity in the FANCD2 mutant, indicates the possibility of DSB due to replication fork stalling, but suggests ICL are not involved in CR(VI) genotoxicity. A strong sensitivity also seen in RAD9 and RAD17 but not ATM mutants, indicates that the ATR/ATRIP checkpoint pathway is stimulated when replication forks encounter Cr-DNA adducts – either ternary adducts or proposed microchelates of Cr with the phosphate and guanine. Since the error-prone TLS polymerase, POLZ, is known to participate after ATR-induced checkpoints, we propose that this may be the avenue to Cr(VI)-induced mutagenicity.

Introduction

Exposure to hexavalent chromium

Chromium is a naturally occurring element found in rocks, animals, plants, and soil. It can exist in several different forms. Depending on the form it takes, it can be a liquid, solid, or gas. The most common valence states are chromium(0), chromium(III), and chromium(VI) (Zhitkovich, 2005a; McCarroll et al., 2009). The metal chromium, which is the chromium(0) form, is used for making steel. Chromium(VI) and chromium(III) are of the most significance biologically (Fishbein, 1976; Cohen et al., 1993; Valko et al., 2005). Chromium compounds are used for chrome plating, dyes and pigments, leather tanning, corrosion inhibitors, fungicides, printing and reproducing and wood preserving (Fishbein, 1981; IARC Monograph vol.49, 1990). The most important use of chromium is for alloying with other metals to make products such as stainless steel (Westbrook, 1979). Chromium can be found in air, soil, and water after release from the

manufacture, use, and disposal of chromium-based products and is released from the burning of coal. The Agency for Toxic Substances and Disease Registry estimated that in the United States, ~2,700-2,900 tons of chromium are emitted annually into the atmosphere and of this ~35% is Cr(VI) (ATSDR, 2008). Chromium has been found in at least 1,127 of the 1,699 current or former hazardous waste sites on the EPAs National Priorities List (ATSDR, 2008). Chromium does not usually remain in the atmosphere, but is deposited into the soil and water. The forms of chromium in Superfund sites to which humans are most likely to be exposed are Cr(III) and Cr(VI) as dichromate ($Cr_2O_7^{2-}$), hydrochromate ($HCrO_4^-$) or chromate (CrO_4^{2-}) depending on pH (Fig. 4-1) (Zhitkovich, 2005a). Chromate is the primary anion at pH 7 whereas lowering the pH to 6 leads to the predominance of hydrochromate. Dichromate is found at negligible levels at physiological pH. Typical cations for Cr(VI) are sodium, potassium, calcium and ammonium (IARC Monograph vol. 49, 1990). Of the two valence states, Cr(III) is considered to be moderately toxic while Cr(VI) is both very toxic and carcinogenic. Environmental exposure to Cr likely impacts millions of people drinking Cr-containing water, residing in the vicinity of numerous toxic sites, and various industrial users of Cr products. In fact, Sedman, et al., have reported on a recent analysis that indicated that ~38% of the municipal drinking water systems in California have detectable levels of hexavalent chromium (Sedman et al., 2006).Also, the presence of Cr in urban particulate matter and emissions from automobile catalytic converters leads to exposure by very large segments of populations in densely populated areas (Salnikow and Zhitkovich, 2008).

The general population is exposed to chromium (generally Cr(III)) through the ingestion of food and water, and inhaling air that contains the chromium compounds (ASDTR, 2008). People residing near chromium waste disposal sites or chromium manufacturing and processing plants have a greater probability of elevated chromium exposure than the general population and these exposures are generally to mixed Cr(VI) and Cr(III) (ASDTR, 2008). The average daily intake from air, water, and food is estimated to be less than 0.2 to 0.4 µg, 2.0 µg, and 60 µg, respectively(ASDTR, 2008). Dermal exposure to chromium may occur during the use of consumer products that contain chromium, such as wood treated with copper dichromate or leather tanned with chromic sulfate or through handling Cr contaminated soil, either directly by absorption through the skin or orally by eating or smoking with improperly washed hands (ASDTR, 2008).

It has been previously reported that chromium induces cytotoxicity, oxidative stress, DNA damage, apoptosis and altered gene expression (Rudolf et al., 2006; Curtis et al., 2007). Chromium induced carcinogenicity was first observed more than 100 years ago (Stern, 1982). The United States Department of Health and Human Services, the United States Environmental Protection Agency, and the International Agency for Research on Cancer (IARC) have classified Cr(VI) compounds as human carcinogens based on increased incidences of lung cancers in workers in the chromium industry and its association with malignancies in lungs of rats exposed to the compounds by inhalation (IARC, 1990; Cohen et al., 1993; NTP, 1998). Recent epidemiological and risk assessment studies have indicated up to a 25% lifetime risk of dying of lung cancer under the 52 $\mu g/m^3$ permissible exposure limit (Gibb et al., 2000; Park et al., 2004). This

standard, adopted by OSHA in 1971 has been lowered to 5 $\mu g/m^3$ in 2006 (OSHA, 2006), but even with the new standard, an additional 10-45 deaths per 1000 exposed workers is expected.

Absorption, distribution, metabolism and excretion of hexavalent chromium

Hexavalent chromium is absorbed after oral, dermal or inhalation exposure (Wahlberg and Skog, 1965; Wahlberg, 1970; Kerger et al., 1997; Mancuso, 1997). Gastrointestinal absorption of Cr(VI) is estimated to be less than 5% (USEPA Tox Rev, 1998). It has been demonstrated that contact of Cr(VI) with gastric juices significantly reduces absorption of the metal in the intestine (Sedman et al., 2006). Contact with gastric juices has been found to reduce Cr(VI) to the trivalent form (Sutherland et al., 2000). A number of factors can influence the absorption of chromium following inhalation, including the size, oxidation state, and solubility of the chromium particles; the activity of alveolar macrophages; and the interaction of chromium with biomolecules following deposition in the lung (ATSDR, 1993). Water soluble chromate is absorbed rapidly by inhalation whereas insoluble compounds are less effectively absorbed. Cr(VI) is much more effectively absorbed into cells than Cr(III) due to its facilitated diffusion via non-specific anion channels since chromate ions, being tetrahedral, successfully mimic phosphate or sulfate ions (Zhitkovich, 2005a) .

Chromium has been found widely distributed in the body after exposure to Cr(VI) in both human and animal studies (Coogan et al., 1991; Cohen et al., 1993; Mancuso, 1997). Liver, kidney, spleen and bone were found to have the higher

concentrations (Sutherland et al., 2000). Chromium has also been demonstrated to cross the placenta (Saxena et al., 1990).

Ingested Cr(VI) is excreted primarily in the feces due to poor absorption while intravenously absorbed Cr(VI) is eliminated primarily in urine (Sayato et al., 1980). Hexavalent chromium is highly permeable into red blood cells where it binds with hemoglobin (Sayato et al., 1980;). Cr(III) is the biologically active form of the metal in the cell, although it is not readily absorbed. The more readily absorbed form is the chromate ion which is easily absorbed through the non-specific ion channel (Alexander and Aaseth, 1995). Human cells can accumulate Cr(VI) up to 30x the concentration existing outside the cell within 3 hr (Reynolds et al., 2007; Reynolds and Zhitkovich, 2007; Sehlmeyer et al., 1990). However, Cr(VI) itself is unreactive toward cellular macromolecules and must be actively reduced to the trivalent form (Salnikow and Zhitkovich, 2008). Cr(VI) metabolism in mammalian cells does not require any enzymes and relies on the direct electron transfer from ascorbate and nonprotein thiols, such as glutathione and cysteine (Zhitkovich, 2005a). Ascorbate accounts for about 90% of the reduction of Cr(VI) to Cr(III). Thus, the reduction of Cr(VI) to the thermodynamically stable Cr(III) (through Cr(V) and Cr(IV)) is the activating step of chromium toxicity (Fig. 4-2) . Variable amounts of Cr(V) and Cr(IV) may be present and the levels of each depend upon the reducing agent and ratio of reactants (Moghaddas et al., 1992; Stearn and Wetterhan, 1994; Lay and Levina, 1998). When ascorbate is present in ≥2-fold molar excess to Cr(VI), a direct two-electron reduction to Cr(IV) occurs with Cr(V) being found only if ascorbate is present at a non-physiologically low level (Stearn and Wetterhan, 1994; Lay and Levina, 1998;

Goodgame and Joy, 1987). Thus, some believe it is doubtful that exogenous exposure to Cr(VI) could produce significant levels of Cr (V) (Salnikow and Zhitkovich, 2008). Cysteine reduction of Cr(VI) occurs by two one-electron reductions to Cr(V) and then Cr(IV) (Quievryn et al., 2001). Reduction of Cr(VI) by glutathione occurs mainly by an initial two-electron progression (O'Brien et al., 1992). Cr(III) then readily forms coordination complexes with DNA and proteins (Salnikow and Zhitkovich, 2008). The coordination number of Cr(III) is six and all Cr(III) complexes have octahedral geometry (Zhitkovich, 2005a). The ability of Cr(III) to form hexa-coordinate complexes makes the molecule thermodynamically stable and allows it to complex with H_2O and other biologically important atoms (such as O, N and S) located on ascorbic acid, cysteine and glutathione which reduce Cr(VI) to Cr(III). The rapid coordination of Cr(III) with any of these reducing agents is responsible for maintaining the solubility of Cr(III) at physiological pH. Also, the ability to complex with N gives the ability to coordinate with DNA bases.

DNA damage induced by chromium

Cr(III) is a hard Lewis acid and strongly prefers coordination to negatively charged oxygen groups (such as the one found on the phosphate in the DNA backbone) (Zhitkovich, 2005a). The octahedral shape of the Cr(III) complex may have an influence on which DNA repair mechanism is chosen to repair interstrand cross-links caused by the complex. NER would normally be chosen to repair crosslinks caused by bulky molecules that are planar (such B[*a*]P) and those that intercalate into the DNA strand, however the octahedral shape of the Cr(III) complex may cause distortions to the DNA helix that are

not recognized by NER enzymes. The major adducts formed between DNA and chromium are ternary DNA-Cr-X cross-links with other molecules such as ascorbate, glutathione, cysteine and histidine (Fig. 4-3) (Zhitkovich et al., 1995; Quievryn et al., 2002). These ternary adducts are all formed via the attachment of Cr(III) to DNA backbone phosphates (Zhitkovich et al., 1996; Quievryn et al., 2002). Other phosphate-linked Cr adducts have been proposed in which the phosphate is linked by Cr(III) to the N^7 position of guanine forming a microchelate which has been suggested to be the mutagenic adduct while the ternary adduct may not be mutagenic (Fig. 4-4; Zhitkovich et al., 2001). DNA-protein cross-links have also been identified in chromate treated cells, however, only at acutely cytotoxic concentrations (Costa, 1990; Costa et al., 1996) and are estimated to be less than 1% of all Cr-DNA adducts (Salnikov and Zhitkovich, 2008).

Small amounts of Cr(III) induced ICL have been detected during in vitro experiments after reduction of Cr(VI) by ascorbate or cysteine, but not by glutathione and the development of these ICL had a major dependence on the ratio of reducing agent to Cr(VI) (Zhitkovich et al., 2000; O'Brien et al., 2002; Bridgewater et al., 1994;). However, ICL are difficult to rationalize from a steric standpoint due to the bulk of the octahedral Cr(III) complex, which is a highly polar moiety, and the need for the complex to invade the non-polar interior of the DNA helix (Zhitkovich, 2005a). Therefore, it is probable that ICL induced by chromate occur only in vitro (Salnikov and Zhitkovich, 2008).

Chromate-induced oxidative stress with resultant SSB have also been reported in the literature (Casadevall and Kortenkamp, 1995; Hassoun and Stohs, 1995; Bagchi et al., 1997; Costa et al., 2002; Lee et al., 2004). The theory is that under conditions of low

ascorbate, a single electron reduction of Cr(VI) could lead to significant levels of Cr(V). Cr(V) has been shown to act as a catalyst in Fenton-like reactions with H_2O_2 to produce hydroxyl radicals (Molyneux and Davies, 1995). These ROS can induce SSB either by direct attack at the sugar-phosphate backbone or indirectly as a result of BER of oxidative base damage (Friedberg et al., 2006).

Cell culture and in vivo studies of chromium toxicity have been reporting evidence, such as increased chromosomal breaks (Wise et al., 2003; Xie et al., 2009) and micronuclei (Seoane et al., 2001), implicating the existence of DSB. Only recently has more direct evidence of DSB been demonstrated (Reynolds et al., 2007; Ha et al., 2004). DSB are induced indirectly after cells pass through S-phase and in conjunction with mismatch repair (MMR) proteins (Salnikow and Zhitkovich, 2008). One aspect of Cr(VI) related cancers is the presence of microsatellite instability (Hirose et al., 2002) which suggests a loss of functional MMR (Kunkel et al., 2005; Modrich, 2006). This Cr(VI)-induced microsatellite instability was then associated with the loss of expression of the essential MMR protein MLH1 (Takahashi et al., 2005). This absence of a functional MMR pathway leads to the inability of cells to repair replication errors resulting in significantly increased mutation rates and consequently leading to the expression of a mutator phenotype caused by the inability to avoid future mutations, even without continued Cr exposure (Salnikow and Zhitkovich, 2008). Also, MMR-deficient human cells have been reported to be resistant to clonogenic lethality induced by Cr(VI) (Reynolds and Zhitkovich, 2007) as well as having an abrogated cellular ability to inhibit replication in the presence of Cr-DNA adducts (Reynolds et al., 2007; Peterson-Roth et al., 2005). Therefore, based on these findings, it is apparent that MMR may be required

for processing Cr-DNA adducts into DSB and this has led to the proposal of a model in which chronic exposure to toxic doses of Cr(VI) leads to the clonal selection of resistant cells deficient in MMR (Peterson-Roth et al., 2005; Zhitkovich et al., 2005b).

Our hypothesis in this aim has been that phosphate-linked Cr(III) adducts are causing replication fork stalling which, in turn, is leading to DSB which need HR for their repair and that oxidative stress induced by Cr(VI) is not important to the overall genotoxic effects of Cr(VI). To investigate this proposition we have endeavored to make use of the DT40 cell system using the multi-well plate based system developed in Specific Aim 1 to ascertain which DNA repair pathway(s) are used to tolerate damage induced by Cr(VI) (as potassium chromate). Also, to further assess the DNA damage response to Cr(VI), we have employed several other assays to evaluate the levels of oxidative stress and DNA SSB induced in cells after exposure to Cr(VI). These include measurement of 8-oxo-dG by ultra high pressure liquid chromatography-heat assisted electrospray ionization-tandem mass spectrometry (UPLC-HESI-MS/MS), an NAD(P)H depletion assay to assess SSBs, glyoxal gel electrophoresis (GGE) and *O*-(tetrahydro-*2H*-pyran-2-yl)hydroxylamine agarose gel electrophoresis (OTX-AGE), also to measure SSBs, and a DNA fiber spread methodology whereby the state of cellular replication can be monitored.

Materials and Methods

Cell culture

DT40 cells were cultured and maintained as described in Chapter I. Exponentially growing cells with an approximate concentration of $1x10^6$/mL were used for each experiment described hereafter in this chapter.

DT40 multi-well cell survival assay

The DT40/XTT multi-well plate survival assay was performed as described in Chapter I and nearly the full complement of mutants was used. Cells were exposed to a continuous treatment of potassium chromate (K_2CrO_4) dissolved in molecular grade sterile water. Chromate concentrations were 0, 0.094, 0.188, 0.375, 0.75, 1.5 and 3 μM and the dilutions were kept at room temperature until used. Control and blank wells received sterile water. Growth was continued under the prescribed conditions until the cells in the control wells were nearly confluent (3 – 4 days). The plates were then exposed to XTT, allowed to incubate 3 – 4 h and read on a plate reader either immediately or after storage in plastic bags at 4 °C.

Larger-scale exposure method

When it has become necessary to expose DT40 cells on a larger scale for the purpose of DNA extraction, the exposures have been performed in 20 mL complete medium in 10 cm sterile dishes. Cells for these experiments were grown overnight so as to reach a cell concentration of $1x10^6$/mL and also be in an exponential growth phase. After the desired treatment, cells were collected, centrifuged at 1100g for 5 min and washed 3x in ice cold PBS or ice cold 20 mM 2,2,6,6-tetramethylpiperidine-*N*-oxyl

(TEMPO) if the assay being performed was investigating oxidative stress or DNA SSB. Although DT40 cells grow suspended in the medium, it was found that some cells will attach and that scraping the dish will significantly increase the number of cells collected. After the last wash, cells were pelletized and stored in a small amount of PBS or TEMPO at -80 °C prior to DNA extraction.

DNA extraction

DNA extractions were performed using the PureGene DNA extraction kit with a previously described modification (Nakamura et al., 2000). Briefly, thawed cell pellets were lysed in lysis buffer supplemented with 20 mM TEMPO as a radical scavenger. Protein was precipitated with the protein precipitation solution provided in the kit after which the DNA/RNA mixture was precipitated from the supernatant with isopropyl alcohol. The DNA/RNA pellet was then resuspended in lysis buffer containing 10 mM TEMPO and incubated with RNase T1 (50 unit/mL) at 37 °C for 30 min, followed by precipitation of protein and DNA. The DNA pellet was resuspended in sterilized distilled water with 1 mM TEMPO and stored at -80 °C. DNA concentration was determined using a UV spectrophotometer.

Potassium chromate acute Toxicity analysis in DT40 cells

To analyze the level at which acute toxicity occurs after treatment with Cr(VI) we exposed DT40 parental cells to 0, 9.4, 18.8, 37.5, 75, 150 and 300 μM concentrations of potassium chromate for 4 h under standard DT40 incubation conditions. Exposure took place in 10 cm dishes with ~$0.6x10^6$ cells/mL in 20 mL complete medium. After 4 h, cell

counts to determine the level of cell proliferation were performed on each sample dish using a hemacytometer and trypan blue dye. Results were reported as a percentage increase in cell count over the initial seeding.

Assay for level of 8-oxo-dG

The UPLC-HESI-MS/MS method previously described (Boysen et al., 2010) has been used for the determination of 8-oxo-dG contained in DT40 cells after exposure to potassium chromate at concentrations of 0, 30 and 60 μM for 4 h using the larger-scale exposure method described above. DT40 parental cells were also exposed to 80 μM H_2O_2 for 0.5 h for use as a positive control. The DNA was extracted by the method described above, quantitated by UV spectrometry and stored at -80 °C until used for the assay.

Briefly, after addition of 500 fmol of [$^{15}N_5$]8-oxo-dG the DNA is hydrolyzed with DNAse I followed by phophodiesterase I with alkaline phosphatase. The enzymes were removed by ultra-filtration using pre-washed YM-10 microcentrifuge filters (Millipore, Bedford, MA). The 8-oxo-dG was then purified from the DNA filtrates by reverse phase HPLC using an Ultrasphere ODS C18 4.6mm×250mm 5 μm column (Beckman, Fullerton, CA) on an Agilent 1200 HPLC system (Agilent, Santa Clara, CA). Solvents were removed with a SpeedVac concentrator and samples were stored at −20 °C until analysis by UPLC–HESI–MS/MS. The quantitative analysis of 8-oxo-dG was performed with a UPLC (Waters, Milford, MA) coupled to a TSQ-Quantum Ultra triplequadrupole mass analyzer (ThermoFinnigan, San Jose, CA) using an HESI source.

Glyoxal Gel Electrophoresis

DT40 parental cells were exposed using the larger-scale exposure method for 4 h at an initial cell concentration of $1x10^6$ cells/mL. Concentrations of potassium chromate were 0, 30, and 60 μM. The cells were also exposed to 1 mM methyl methanesulfonate (MMS) to provide for a positive control. The Lambda DNA-HindIII Digest (New England Biolabs, Ipswich, MA) was used as a marker (5 μg).

The neutral GGE assay was used to fractionate genomic DNA SSB according to a previously described method, albeit with modification (Drouin, et al., 1996; Pachkowski, et al., 2009). Briefly, equal amounts of DNA (3 – 10 μg) from samples to be compared were denatured in 1.5 M glyoxal, DMSO (50% v/v) and 10 mM sodium phosphate buffer (pH 7) for 1 h at 50 °C. Loading buffer, consisting of 50% glycerol, 0.01% bromophenol blue, 0.01% xylene cyanol and 10 mM sodium phosphate buffer (pH 7), was added to each sample prior to loading onto 0.7% agarose gel in 10 mM sodium phosphate running buffer (pH 7). All reagents with the exception of the loading buffer were made fresh for each day of an experiment. The gels were electrophoresed at -4 °C for 15 h at 25v. Gels were stained with acridine orange (5 μg/mL) for 1 h and de-stained with de-ionized water until background staining was reduced to an acceptable level. Imaging was performed on a Kodak Image Station 440CF system.

The gel lane images obtained were similar to the images acquired from a Comet assay. Thus, the GGE images were assessed using the image analysis procedure normally used for the Comet assay (CometScoreVersion 1.5, Tritek). The high molecular weight DNA appearing above the 23.1 kb marker in the GGE analysis was equated with the DNA retained in a comet head. Correspondingly, the DNA smear produced by the

GGE represents the comet tail and the extent of the DNA migration represents the amount of SSB present in each case. The SSB content is represented by a tail moment calculated as the product of the tail length and percentage of DNA in the tail. Higher tail moment values suggest a greater amount of DNA damage in the form of SSB.

OTX-AGE Electrophoresis

DT40 cells with identical exposures to the GGE were used for this assay. A modified AGE protocol (Drouin, et al., 1996) was performed to assess SSB in DNA from cells exposed to potassium chromate and MMS. Agarose gel (0.7%) was prepared in 50 mM NaCl and 1 mM EDTA (pH 7). After setting, the gel was immersed in mild running buffer (30 mM NaOH, 1 mM EDTA, pH 12.4) for at least 30 min. Equal quantities of DNA (3-5 μg) in 50 mM HEPES buffer were incubated for 1 h at 37 °C in the presence or absence of OTX (Aldrich). Afterward, the samples were denatured with alkaline buffer (100 mM NaOH, 30 mM OTX, 50 mM HEPES, pH 12.8) for 20 min at 37 °C. Loading buffer (10 mM NaOH, 95% fomamide (Fluka), 0.05% bromophenol blue, 0.05% xylene cyanol), was added to the samples which were then loaded onto the agarose gel. The gels were electrophoresed at -4 °C for 15 h at 30v after which the gel was treated with neutralization buffer (400 mM Tris, pH 7.5) for 20 min at room temperature. The gels were stained with acridine orange (5 μg/mL) for 1 h followed by de-staining in de-ionized water. Imaging of the gels was performed on a Kodak Image Station 440CF system. The gels were then analyzed using the same method as described for the GGE assay above.

DT40 DNA fiber spread analysis

Cell preparation and treatment

The DT40 cells parental cells were used and maintained in logarithmic growth to obtain a concentration of approximately $1x10^6$ cells/mL on the day of the experiment. Extra dishes of cells were grown so that those cells could be removed by centrifugation and the used medium collected and stored at 39.5 °C for use in the experiment. Cells were added in the amount of 3.6 mL to the wells of a 6-well plate after which was added 3.6 μL (1000x dilution in medium) of iodo-deoxyuridine (IdU). The cells were incubated for 5 min at 39.5 °C for 5 min and then centrifuged for 5 min at 1100g (10 min total exposure time). The medium was then aspirated from the cells. Used medium plus potassium chromate (30 μM in a total of 4 mL) was then added to the cell pellet which was then re-suspended and incubated as above for a total of 15 min (the last 5 min being during the centrifugation). The medium was then aspirated, the cells washed in PBS and then quickly centrifuged (~2 min) and the PBS aspirated. The cells were then re-suspended in used medium with a final pulse of chloro-deoxyuridine (CldU) for a 20 min incubation (once again including a 5 min incubation). The medium was aspirated and the cells were re-suspended in 5 mL ice cold PBS. After a final centrifugation of 5 min the cells were re-suspended to obtain a count of 100 – 200 cells/μL.

DNA Fiber Spread Analysis

The DNA fiber-labeling procedure used was originally described by Merrick, et al., and modified by Chastain et al. and Unsal-Kacmaz et al. (Merrick et al., 2004; Chastain et al., 2006; Unsal-Kacmaz et al., 2007). Briefly, 2 μL cells were applied in a

line across silane-prep slides and allowed to dry to tackiness. The cells were overlaid with lysis buffer for 10 min after which the slides were placed at a 25° angle and allowed to dry for a minimum of 4 h. The slides were then fixed in 3:1 methanol:acetic acid for 2 min and stored at -20 °C for a minimum of 24 h. The slides were then treated with 2.5M HCl, washed 1x with PBS/Tween and 2x with PBS, and blocked in 3% BSA in PBS for 30 min at RT. Next, the slides were incubated with 100 μL of the 1° antibodies (mouse α-BrdU, rat α-BrdU) for 1 h at room temperature. After being placed in stringency buffer for 10 min, the slides are washed 3x in PBS for 30 min at RT and blocked in 3% BSA once more. A second incubation with 100 μL of the 2° antibodies (Alexaflour 594-conjugated rabbit α-mouse, Alexaflour 488-conjugated chicken α-rat) was performed after which the slides were washed 3x in PBS followed by blocking in 2% goat serum in PBS for 30 min. Incubation with 100 μL of the 3° antibodies (Alexaflour 594-conjugated goat α-rabbit, Alexaflour 488-conjugated goat α-chicken) for 30 min at room temperature came next. The slides were washed 3x in PBS and cover-slipped with anti-fade from Invitrogen. After staining, the slides are imaged on a confocal microscope after which the labeling patterns on the combed DNA are marked and scored.

Determination of intracellular NAD(P)H after treatment with potassium chromate

The level of intracellular NAD(P)H was measured by a method originally described by Nakamura et al., (Nakamura et al., 2003) and later modified (Takanami et al., 2005). Cells (5×10^3) in complete medium were seeded into a 96-well plate and allowed to incubate over night. The cells were then treated with potassium chromate or MMS at the indicated concentrations. The NAD(P)H level was determined at time 0 and

every one half hour after for 4 h. At the same time the cells were also incubated with 0.5 mM XTT and 0.02 mM PMS. At the prescribed times, the formazan dye formation was read on a plate reader at 450 nm referenced to 650 nm. The absorbance values for the exposed wells were then compared with those of controls. Each test was performed with and without the PARP inhibitor 3-aminobenzamide (3-AB; 20 mM).

Statistical analysis

For the determination of statistical significance between controls and test sample values, a Student's *t* test was performed for each of the mutant results in each of the DNA repair response tests and for the potassium chromate acute toxicity analysis in DT40 cells.

Results and Discussion

Role of homologous recombination repair and non-homologous end-joining repair of DNA damage induced by potassium chromate.

The DNA damage induced by Cr(VI) (as potassium chromate) has been evaluated for the contribution each DNA repair pathway devotes to tolerance of that damage. The DT40/XTT cell survival assay previously described was used for this evaluation. The data indicate that DT40 cells deficient in HR repair proteins display strong sensitivity when exposed to Cr(VI). The HR repair pathway mutants showed sensitivity to Cr(VI) in the following order: BRCA1 >> RAD54 > BRCA2 >> FANCD2 ~ DT40 parental cells (Fig 4-5). The hypersensitivity of BRCA1 along with the strong sensitivity of RAD54 and BRCA2 indicate that the HR pathway plays an important role in the repair of damage induced by chromate and would appear to indicate the possibility of DNA double-strand breaks induced by chromate which is in agreement with a recent report (Stackpole et al.,

2007). The lack of sensitivity of FANCD2 would seem to rule out DNA inter-strand crosslinks or DNA-protein crosslinks. The NHEJ deficient cells exposed to Cr(VI) exhibited mixed results with the following sensitivities: Ku70 > LIGIV > DNAPKcs = DT40 parent cells (Fig 4-5). The ATM knockout showed no sensitivity ($p = 0.23$) to exposure to chromate. ATM is a DNA damage sensor protein responsible for sensing DNA double-strand breaks and, along with the Mre11/Rad50/Nbs1 (M/R/N) complex and BRCA1, is indispensable for the activation of the intra-S checkpoint (Sancar et al., 2004). Taken together, these results reveal that while DSB may not be involved in this process (lack of ATM sensitivity), HR proteins are, nonetheless, protecting the cell from damage by chromate. The significant sensitivity of the NHEJ mutants also suggests a role for that damage pathway in the tolerance of chromate damage. The NHEJ deficient cell sensitivity agrees with a recent report of similar sensitivity in yeast, however, it should be noted that the concentrations used in that study were approximately 10,000 fold the concentrations used here (~25 mM vs. ~2 μM; Santoyo and Strathern, 2008). Thus, our evidence represents the first report of NHEJ deficient cell sensitivity at biologically relevant concentrations.

The contribution of the excision repair (BER/NER) pathways to the repair of DNA damage induced by potassium chromate.

Several reports have indicated that Cr(VI) induces adducts with the phosphate group of DNA (Zhitkovich et al., 1995). As mentioned above, these adducts are mostly ternary DNA-Cr-X adducts where X = glutathione, cyteine, histidine or ascorbate. These adducts form through attack on DNA by preformed ligand-Cr(III) bi-dentate complexes

(Zhitkovich et al., 1995), and probably constitute about 90% of all Cr-DNA adducts. The DNA-Cr-X adducts have been reported to be nonmutagenic (Salnikov and Zhitkovich, 2008). The remaining 10% of the adducts are reported to be mutagenic and are postulated to be phosphate-N^{7}-dG microchelates (Zhitkovich et al., 2001; Arakawa et al., 2000; Fig 4-4) which would be likely substrates for NER. To investigate this reported aspect of Cr adduction we once again employed our DT40 mutants deficient in both BER and NER. Our results show sensitivity in the following order: FEN1 > PARP1 >> XPA > POLB ~ DT40 parental cell (Fig. 4-6). The POLB mutant consistently showed survival rates nearly identical to the parental cells suggesting the possibility BER plays no role in chromate DNA damage tolerance, however, this cannot completely rule out BER as a chromate induced damage repair pathway due to the existence of backup polymerases for POLB (such as POLL and POLQ; Tano et al., 2007; Yoshimura et al., 2006). Our result for POLB agrees with O'Brien, et al., who found no inhibition of Cr(III) repair synthesis after treatment with the POLB inhibitor, 2,3-dideoxythymidine triphosphate (O'Brien et al., 2005). The higher sensitivity of the PARP1 deficient cells may be explained by the participation of this enzyme in DNA replication and double-strand break repair. Although FEN1 has traditionally been thought of as a BER long-patch pathway 5′-endonuclease, studies have found that the protein is involved in the degradation of RNA primers in Okazaki fragments during replication (reviewed in Liu et al., 2004, and Burgers, 2009). Therefore, we may be expected to find sensitivity in FEN1 mutants because this cell line spends so much time in S-phase. Indeed, we have found this protein deficiency to show sensitivity in other studies (such as with acrolein) we have done for which no BER activity was suspected. Surprisingly, our results for the XPA mutant show only mild but

significant sensitivity to Cr(VI) exposure, with an LC50 rate of 76% of that for the parental cells. This lack of hypersensitivity in XPA mutants may be attributable to the finding mentioned previously that ~90% of Cr adducted DNA is in the form of preferentially bound Cr(III) to the phosphate backbone that only create 1-2° distortions in DNA duplexes (Blankert et al., 2003). Such small distortions may not be readily recognized by the NER pathway that normally requires distortions greater than 20%. Therefore, the sensitivity we have observed may be due to the remaining 10% of Cr-DNA adducts which are microchelates between the phosphate and N^7 of dG.

Result of acute toxicity test of DT40 parental cells exposed to potassium chromate.

We needed to determine the level at which sub-lethal but significant sensitivity occurs with Cr(VI) treatment of DT40 cells. This was necessary so that DNA could be extracted from these cells for the purpose of performing other tests such as those for the determination of oxidative stress and SSB. Cells were continuously exposed to increasing doses of potassium chromate for 4 h as described above. As a result, it was determined that the lowest of our potassium chromate concentrations showing significant sensitivity was 37.5 μM ($p > 0.0004$) (Fig. 4-7).

The role of oxidatively induced genotoxicity due to exposure to Cr(VI) in DT40 cells.

There have been several reports espousing the notion that Cr(VI) induces oxidative stress-related DNA damage, mostly due to the production of oxygen radicals during the intracellular reduction of Cr(VI) to Cr(III) (Hassoun and Stohs, 1995; Casadevall and Kortenkamp, 1995; Costa et al., 2002; Lee et al., 2004; Slade et al., 2005;

Hailer et al., 2005). Other reports have shown that an increase of DNA SSBs occurs after treatment with Cr(VI) (Sugiyama et al., 1986; Bagchi et al., 1997; Hodges et al., 2001; Dana Devi et al., 2001; Gambelunghe et al., 2003;) which further implicates oxidative stress as at least one route of genotoxicity generated by Cr(VI). In fact, our own DNA damage response data resulting from DT40 BER and NHEJ mutants exposed to H_2O_2 reveal a pattern similar to that obtained with hexavalent chromium. Consequently, we elected to investigate further the role of oxidative stress induced genotoxicity in DT40 cell. To assess the level of oxidative stress, we made use of four assays at our disposal: 8-oxo-dG levels determined by mass spectrometry to directly look for those lesions which are typically associated with oxidative stress and three DNA single-strand break assays; the NAD(P)H depletion assay, the GGE assay, the OTX-AGE assay. Every report we have encountered to date indicating SSB induction by Cr(VI) have made use of assays (either an alkaline Comet or alkaline elution) that require alkaline conditions which are known to cause artifactual DNA SSB in the presence of adducts involving the DNA backbone (such as the DNA-Cr-X adducts that are thought to make up the majority of all adducts of Cr with DNA). Thus, the choice of the two electrophoretic agarose gel assays: 1) the GGE assay performed at physiological pH; and, 2) the OTX-AGE assay which is performed at alkaline pH but uses the hydroxylamine, OTX, to protect abasic sites from β-elimination at the deoxyribose which would result in artifactual strand breaks if not protected. The OTX-AGE assay has the added benefit of providing the ability to indirectly assess the presence of abasic sites by operating the test with and without OTX. The NAD(P)H depletion assay serves as a surrogate for NAD^+ consumption, an indicator of PARP1/2 activation resulting from SSB formation.

Result of 8-oxo-dG assay after exposure to chromate.

Results from the mass spectrometric assay for 8-oxo-dG exposed to chromate are depicted in Fig. 4-8. Statistical analysis has indicated no significant difference in the response between the 30 and 60 μM chromate exposed cells and the controls whereas the H_2O_2 exposed cells showed a very significant increase in the 8-oxo-dG level.

Result of NAD(P)H depletion assay for SSB

Poly(ADP-ribose) polymerase-1 (PARP-1) is a protein modifying enzyme used in BER that seeks out and binds to SSB and subsequently synthesizes polymeric adenosine diphosphate-ribose (PAR) that is appended to both PARP-1 itself and other proteins involved in DNA metabolism. This adduction of PAR to DNA metabolic proteins, such as topoisomerase, histones and DNA replication factors, has been proposed to be an important function in the repair of SSB as well as in BER and may limit genotoxic stress during these repair processes (Boothman et al., 1994; Trucco et al., 1998; Woodhouse et al., 2008;). During the production of PAR, significant amounts of NAD^+ are consumed as a source of ADP-ribose (Schreiber et al., 2006). Thus, the NAD(P)H depletion assay has been developed to serve as a surrogate for NAD^+ consumption and an indicator of PARP-1 activation due to SSB (Nakamura et al., 2003).

As has been previously shown and we have repeated here, exposure of DT40 parental cells to MMS triggers the accumulation of SSB as intermediates of BER which leads to PARP-1 activation with NAD^+ consumption and depletion of NAD(P)H (Fig. 4-9B; Pachkowski et al., 2009). Co-exposure to both MMS and the PARP inhibitor, 3-AB, protected against NAD(P)H depletion indicating that PARP-1 is active in response to

MMS exposure (Fig. 4-9B). The same experiment, repeated with potassium chromate at exposures up to and including 120 μM, a concentration which is significantly genotoxic, demonstrated no significant difference between 3-AB treated and untreated cells (Fig. 4-9A). This result presents a strong indication that SSB are not being induced in significant measure by Cr(VI) exposure in DT40 cells.

Result of GGE assay for SSB

Methods utilizing alkaline conditions have been traditionally used for the purpose of quantitating SSB. However, these methods are not suitable for separating direct strand breaks from those induced during the procedure due to the lability of abasic sites and oxidatively modified bases in alkaline conditions (Drouin et al., 1996). The glyoxal agarose gel electrophoresis method, initially described by Carmichael and McMaster for use on single-stranded RNA, is performed in neutral conditions using glyoxal and DMSO for denaturation of genomic DNA (Carmichael and McMaster, 1980). Glyoxalation of DNA allows the strands to remain denatured during electrophoresis and the electrophoretic mobility of glyoxalated DNA is due to frank strand breaks and not to the presence of abasic sites or modified bases (Drouin et al., 1996). For the GGE assay, we treated parental DT40 cells with 0, 30, and 60 μM concentrations of potassium chromate for 4 h. As indicated previously by our acute toxicity assessment, 60 μM exposure should be significantly toxic for the purpose of inducing SSB if this type of damage is caused. Parental cells treated for 3 h with 1 mM MMS were used as a control. As can be seen in Fig. 4-10, there is no significant indication of SSB at the 30 and 60 μM level of

chromate treatment above that of the controls. Thus, Cr(VI) does not produce inducement of SSB when assayed under neutral conditions.

Result of OTX-AGE assay for SSB

DT40 cells for this assay were exposed in the same manner as in the GGE assay. The results (Figs. 4-11,12) indicate no significant difference between the 30 and 60 μM chromate treatment and the control regardless of the addition of OTX in the assay. The average of the percentages of DNA in the tails of the control and 60 μM chromate treated samples were 22% and 21%, respectively. Also, the average results for the OTX- and OTX+ treated 60 μM chromate exposed samples were nearly identical, indicating a lack of a significant numbers of abasic sites induced by chromate. Interestingly, in every sample pair but one on our gels, the OTX untreated sample did show an increase of SSB development during the assay indicating that alkaline conditions can overestimate the level of SSB formation if the DNA is not protected.

Result of DNA fiber spread analysis of DNA exposed to potassium chromate

Our DT40 survival data that relates to the HR and NHEJ DNA damage repair pathways indicates the possibility DSBs as a result of replication forks stalling upon contact with damage caused by chromate. To assess the affect of chromate exposure on DNA replication we conducted a process known as the DNA fiber spread (aka "combing") analysis which allows the visualization of the different stages of replication. Logarithmically growing DT40 cells were pulsed with IdU, exposed to potassium chromate at various concentrations (0, 15, 30, 60 and 120 μM), and then pulsed with

CldU. Approximately 400 cells were applied to a glass slide and treated with lysis buffer after which the slides were placed at an angle causing the DNA fibers to form straight rows (combing). The slides were then fixed. Immunostaining with fluorescent antibodies (AlexaFlour 594 for IdU and Alexaflour 488 for CldU) was then performed to detect the presence of the halogenated uracil with red staining indicating IdU and green staining CldU. The replication tracks were then detected by fluorescence microscopy and scored manually (Fig. 4-12). This technique allows for the determination of the status of replication by making it possible to distinguish between ongoing replication forks (elongation, labeled red/green), newly fired replicons (initiation, labeled green only), and prematurely stalled replication forks (labeled red only; Fig. 4-13; Merrick et al., 2004).

As can be observed in Figures 4-14,15 exposure to chromate appears to have a distinct effect on replication in *wt* DT40 cells, especially with respect to the inhibition of new origin firing at the higher Cr(VI) concentrations. A more modest effect was observed for replication terminations after Cr exposure and this effect was directly dose dependent. The most striking result from the DNA combing assay may be noticed in Figure 4-16, which indicates the slowing of replication forks, as evidenced by the decreasing relative length of green to red in red/green tracks. Replication fork slowing also was seen as dose dependent. Two distinct possibilities may be considered for these observations. First, the replication terminations and the slowing of the replicons are most likely due to the same cause – replication forks encountering Cr-DNA adducts. The observed terminations in the assay represent stalled forks at locations where repair of the adducts has not taken place while the slowing most likely is evidence of a fork that has stalled but continued after a repair action and replication fork recovery. Second, the

reduction in green-only tracks is an indication that an intra-S checkpoint has been triggered by the higher Cr concentrations.

Discussion

As depicted in Figure 4-17, hexavalent chromium has been proposed in the literature to induce numerous types of damage involving several different metabolic pathways. Still, the process by which Cr(VI) is mutagenic is hotly debated with a copious amount of new reports in the literature each year. Of all types of damage indicated in Figure 4-17, the two receiving the most attention in recent reports are oxidative stress induced damage and adducts to the DNA backbone. We felt that we could make a useful contribution to this body of work by exploiting the isogenic DT40 cell system and other modern techniques such as neutral single-strand break analysis, DNA fiber spread analysis and the NAD(P)H depletion assay for single-strand breaks, as well as others.

We first employed the DT40 survival method to determine which DNA repair and checkpoint pathways are involved in the tolerance of damage induced by potassium chromate. These data have revealed an exquisite sensitivity in mutants, with the exception of FANCD2, of the HR repair pathway as well as a more moderate sensitivity in the NHEJ pathway – both of which are employed by cells to repair DNA DSBs. These outcomes would suggest strongly that chromate is inducing DSBs if it were not for the result obtained from the ATM DT40 knockout which failed to reveal any significant sensitivity to Cr. ATM is a checkpoint-specific damage sensing protein required for

detecting DSB. Lack of sensitivity in the ATM mutant would therefore lead us away from a conclusion that Cr genotoxicity is a result of DSB.

Interesting, we did find that other damage sensors, Rad17 and Rad9, are quite sensitive and moderately sensitive respectively. This data implies that an alternative intra-S checkpoint, the ATM and Rad3 related/ATR-interacting protein (ATR/ATRIP)-mediated pathway has been activated in response to chromate exposure. When DNA is damaged by UV or experiences base damage by chemicals that create bulky base lesions (such as the proposed DNA-Cr-X ternary lesions) the chief damage sensor is the ATR/ATRIP heterodimer (Abraham, 2001; Cortez et al., 2001) which is recruited to RPA-coated ssDNA in the vicinity of the stalled replication fork and becomes activated. Other complexes which are recruited to this region include the checkpoint clamp loader complex, 9-1-1 (Rad9/Rad1/Hus9) complex, the Rad17-RFC complex, and the protein claspin. The presence of these complexes together leads to the phosphorylation and activation of the proteins Chk1, BRCA1 and Nbs1 by ATR (Tibbets et al., 2000; reviewed in Osborn et al., 2002). Activated Chk1 then phosphorylates Cdc25A which leads to the inactivation of the Cdk2/Cyclin2 E complex resulting in inhibition of replication origin firing and S-phase delay (Heffernan et al. 2002). Our DNA fiber spread results showing the slowing of replication, an increase in terminations and a decrease in new origin firing tends to support the notion that this process has been initiated. Phosphorylation of BRCA1 and Nbs1 by ATR is necessary for the recovery of stalled, regressed or collapsed replication forks (Sancar et al., 2004), therefore, the hypersensitivity of our BRCA1 mutant should not be surprising. Studies in yeast have shown that certain recombination proteins, such as Rad51 and the Mre11/Rad50/Nbs1

(M/R/N) complex, and TLS polymerases such as polymerase ζ (REV3), are involved in the recovery of stalled replication forks (Haber and Heyer, 2001; Kai and Wang, 2003). Polζ is known to be responsible for both error-free and error-prone translesion bypass and can induce mutagenesis resulting from the processing of UV damage, abasic sites and TLS through chemically damaged bases (reviewed by Prakash et al., 2005). O'Brien, et al., in a recent study in yeast, found that TLS (Polζ or Polη) deficiency had little impact on clonogenic cell survival or cell growth, but *rev3* yeast displayed attenuated Cr(VI)-induced mutagenesis (O'Brien et al., 2009). Not surprisingly, our REV3 DT40 mutants also showed little sensitivity to Cr(VI). Thus, one possible scenario for the mutagenicity of hexavalent chromium can be suggested from our results: 1) DNA-Cr-X ternary adducts are formed during the S-phase of the cell cycle; 2) replication forks encounter these lesions and stall, regress or collapse requiring HR proteins to aid in restarting; 3) the intra-S checkpoint is initiated by the ATR/ATRIP pathway resulting in inhibition of replicon firing and delay of replicating forks; 4) in the meantime, Polζ, in an error-prone process, may be called upon resulting in a mutagenic progression leading to a mutator phenotype and eventually to cell transformation.

We next wished to assess the possibility that Cr(VI) exposure induces oxidative stress as an alternative route to genotoxicity. As 8-oxo-dG is considered to be an important marker of oxidative stress, we assayed for that adduct using a state of the art mass spectrometric method developed in our laboratory (UPLC-HESI-MS/MS). No significant level of 8-oxo-dG was found above background in this analysis. To further investigate the possibility of oxidative stress, we employed an NAD(P)H depletion assay, also developed in our laboratory. This assay represents a very sensitive, albeit indirect,

method for the determination of SSB (which should be present as a result of oxidation of DNA bases). This assay failed to reveal any significant difference of NAD(P)H depletion between the control and the Cr(VI) treated cells. Finally, we employed two different types of agarose gel electrophoresis methods, also in an attempt to find SSB induced by Cr(VI) exposure. The GGE assay, performed under neutral conditions, indicated no increase SSB above the control. Correspondingly, the OTX-AGE electrophoresis again showed no significantly higher levels of SSB above controls. Based on these findings, we are inclined to propose that the genotoxicity induced by Cr(VI) in DT40 cells is not due to oxidative stress.

Chapter IV Figures.

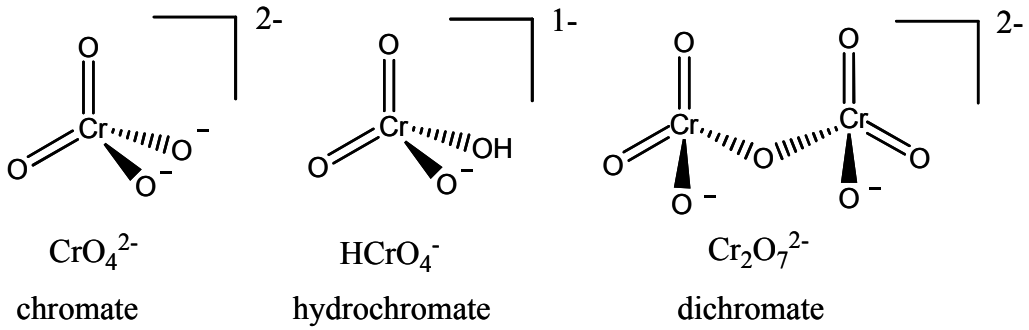
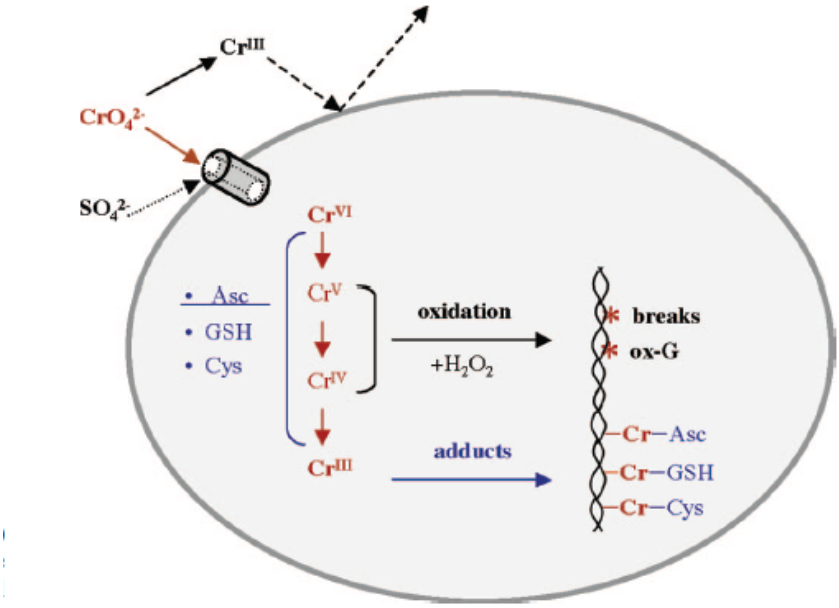


Figure 4-1. Forms of chromium(VI) in aqueous solution



Salnikow and Zhitkovich, (2008) *Chem. Res. Toxicol.*, 21, 28-44.

Figure 4-2. Major steps in the uptake, metabolism and formation of DNA damage caused by Cr(VI).

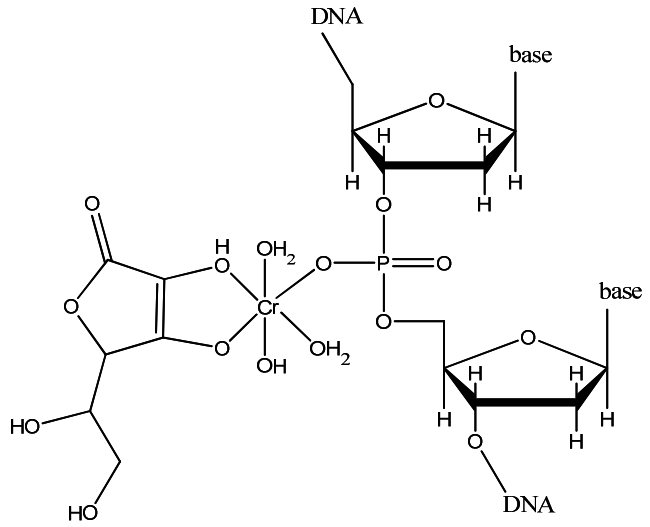


Figure 4-3. Putative Asc-Cr-DNA ternary adduct. (Adapted from Zhitkovich et al., 2001)

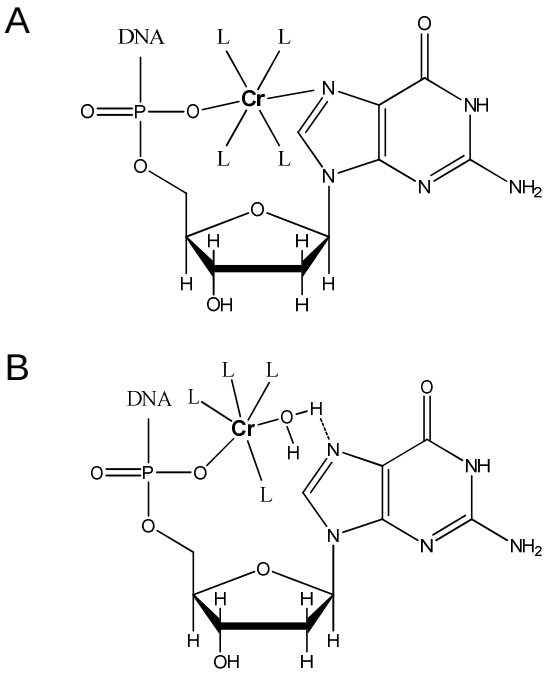


Figure 4-4. Formation of microchelate adducts of Cr(III) with DNA. (A) Direct coordination of Cr(III) to 5'-phosphate and N-7 of dG. (B) Direct coordination of Cr(III) to 5'-phosphate with hydrogen bonding to N-7 of dG. (Adapted from Zhitkovich et al., 2001)

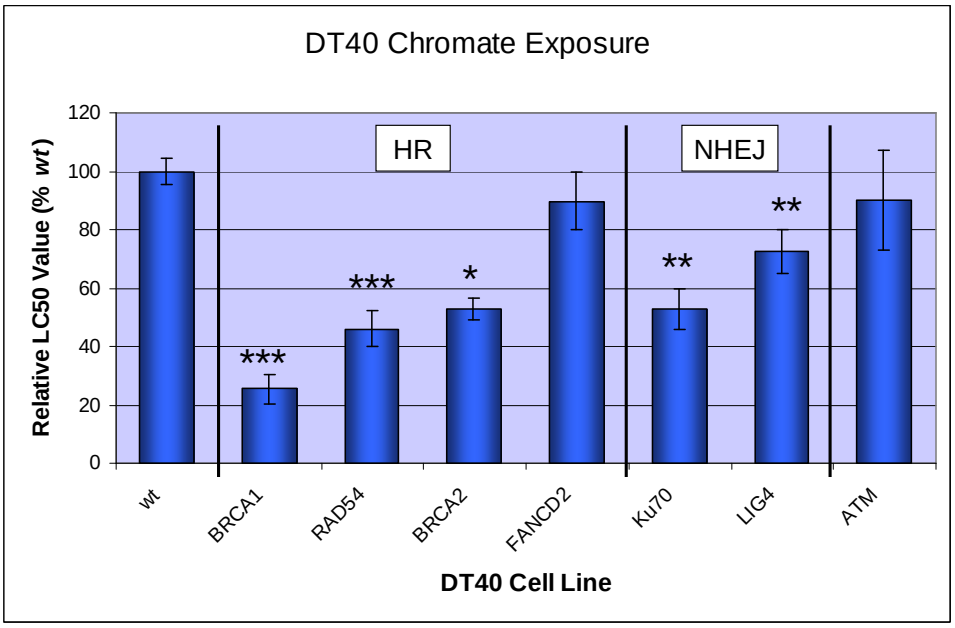


Figure 4-5. Relative LC50 values of cell survival results in DT40 parental and HR or NHEJ mutant cells exposed to potassium chromate. Each LC50 value was calculated from cells survival results and were normalized to the LC50 value of the parental DT40 cells (wt). Significance calculated using Student's *t* test.

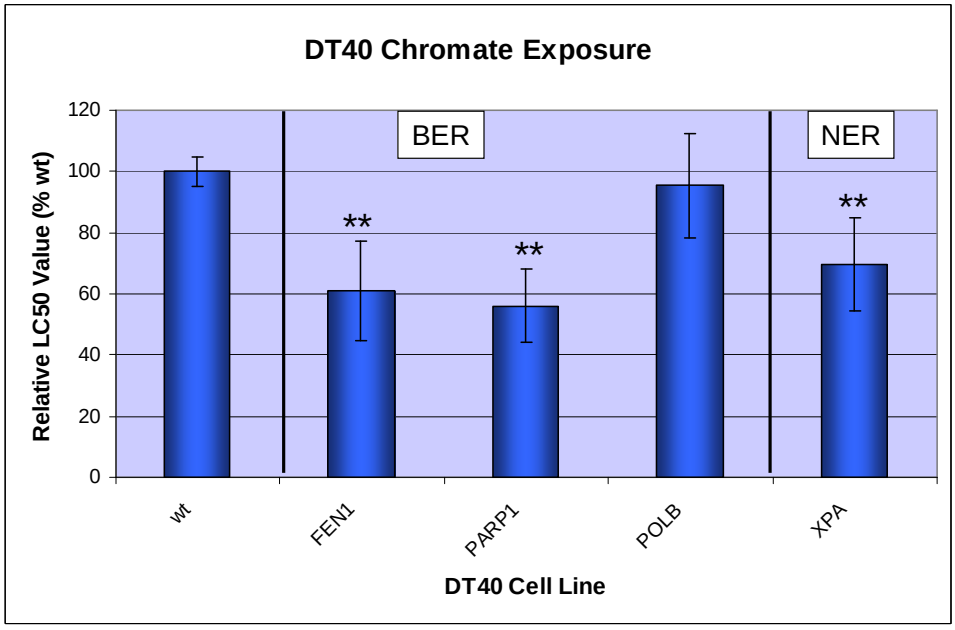


Figure 4-6. Relative LC50 values of cell survival results in DT40 parental and BER or NER mutant cells exposed to potassium chromate. Each LC50 value was calculated from cells survival results and were normalized to the LC50 value of the parental DT40 cells (wt). Significance calculated using Student's *t* test.

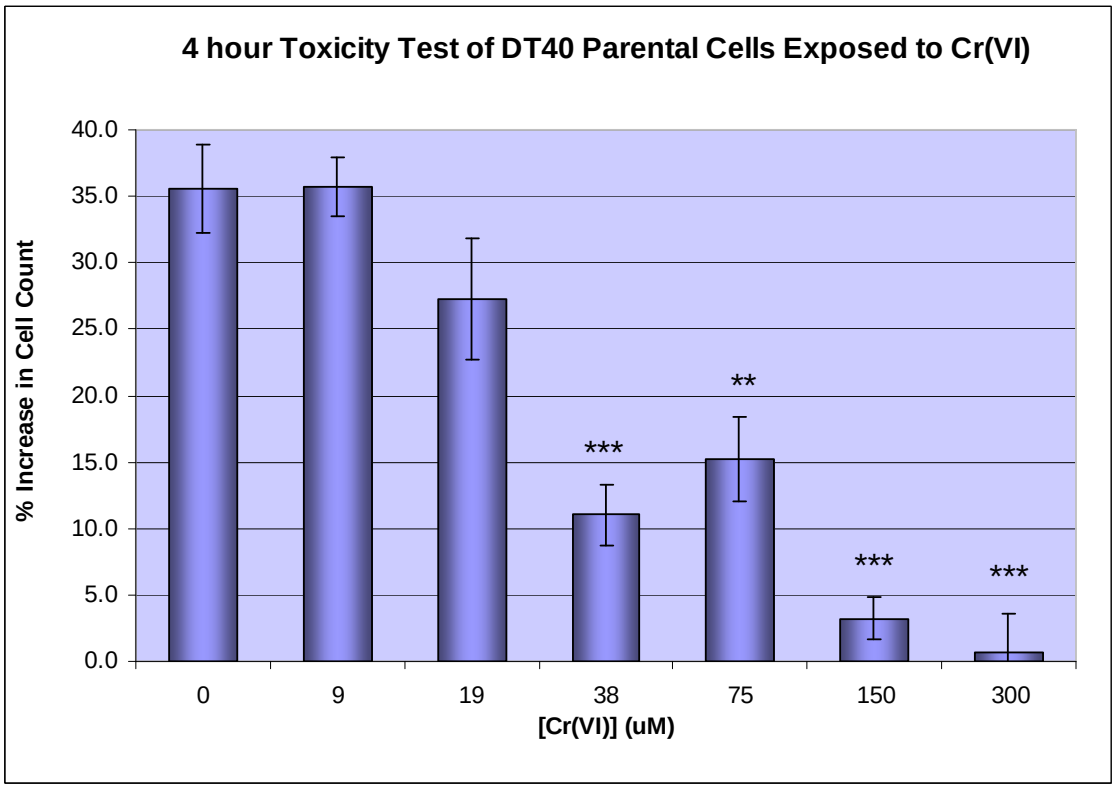


Figure 4-7. Result of 4-hour continuous exposure of parental DT40 cells to the indicated Cr(VI) concentration. Bars represent percentage increase in cell count performed by hemacytometer. Significance calculated using Student's *t* test.

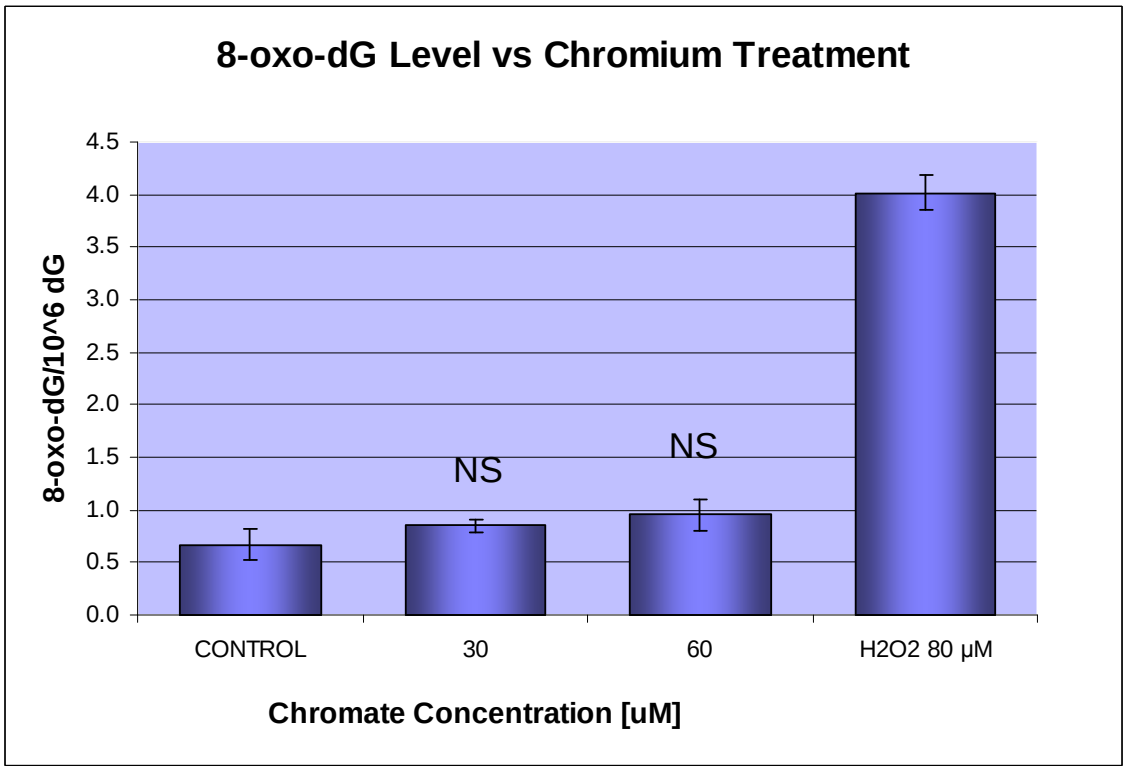


Figure 4-8. Result of assay to determine level of 8-oxo-dG due to exposure to potassium chromate. DT40 *wt* cells where exposed to the indicated concentrations of chromate for 4 h. Result of exposure to H_2O_2 for 0.5 h is shown as a positive control. A Student's *t* test indicated no significant difference between the samples from the chromate control, 30 µM and 60 µM treatments. NS: no statistical significance from control (Student's *t* test).

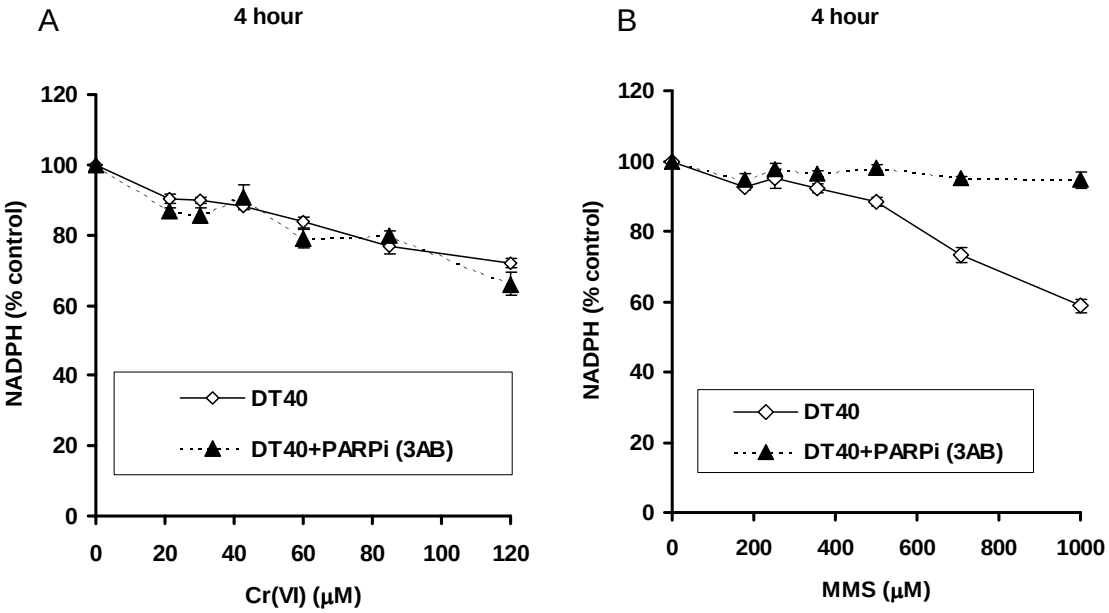


Figure 4-9. Result of NAD(P)H depletion assay performed with DT40 parental cells. (A) Cells continuously exposed to increasing concentrations of potassium chromate or potassium chromate plus the PARP inhibitor 3-AB for 4 h. (B) Cells continuously exposed to MMS or MMS plus 3-AB for 4 h.

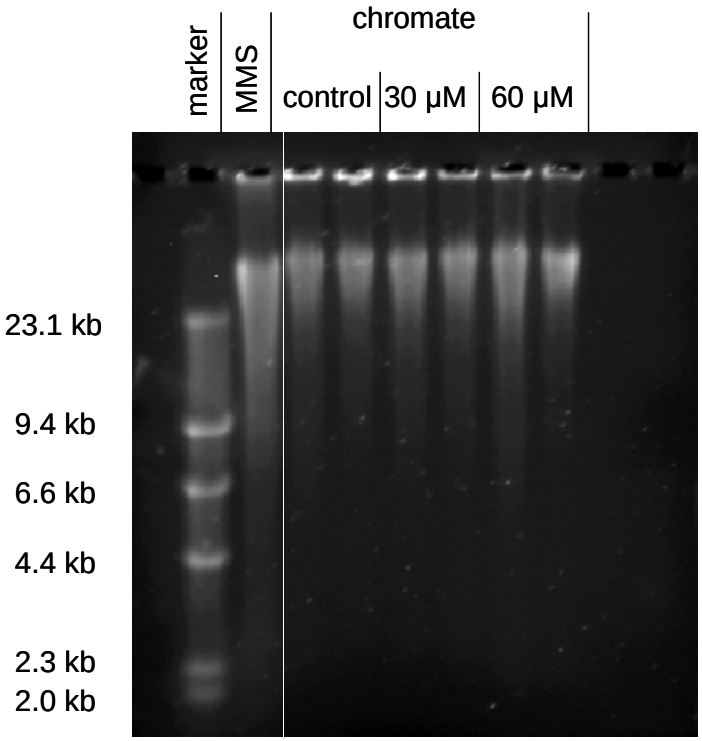


Figure 4-10. Result of GGE SSB assay for DT40 parental cells exposed to 0, 30 and 60 uM (two samples each) potassium chromate for 4 h.

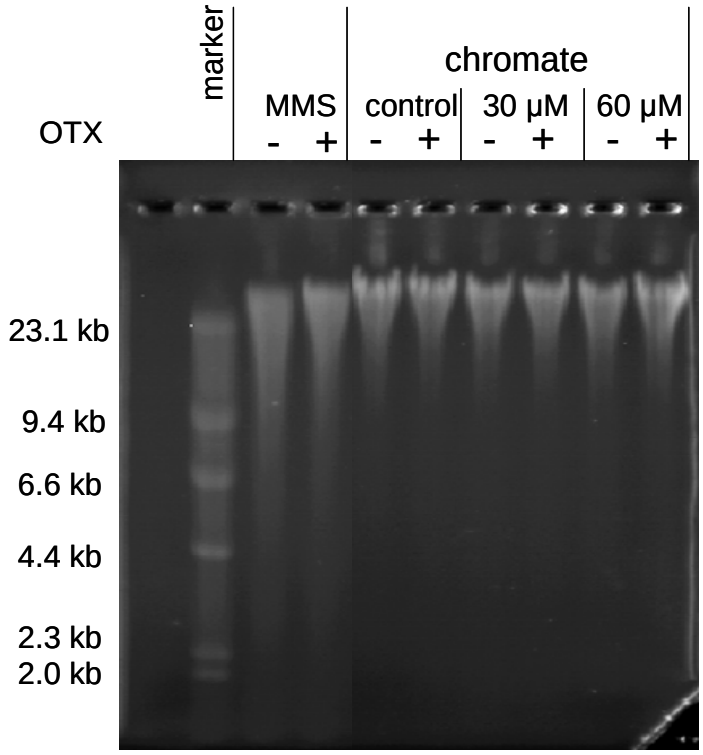
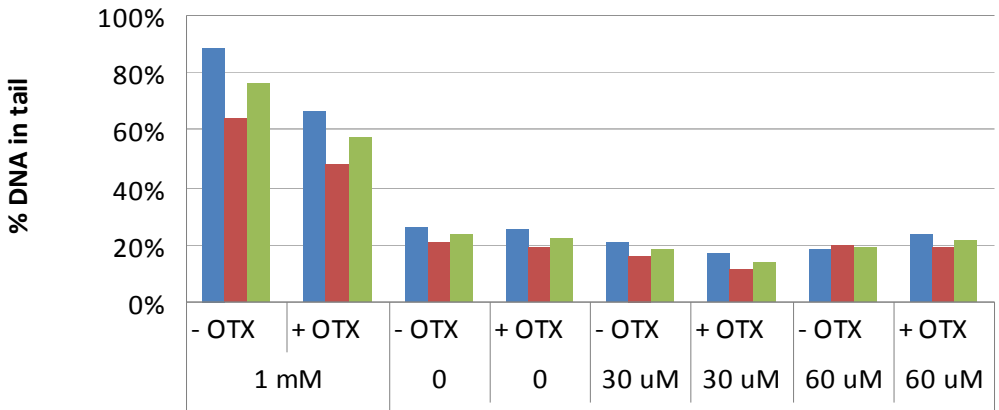


Figure 4-11. Result of OTX-AGE SSB assay for DT40 parental cells exposed to 0, 30 and 60 uM potassium chromate for 4 h.

Figure 4-12 OTX-AGE Assay of Chromate Exposure



	- OTX	+ OTX	- OTX	+ OTX	- OTX	+ OTX	- OTX	+ OTX
	1 mM MMS		0 K2CrO4		30 uM K2CrO4		60 uM K2CrO4	
Gel1	89%	66%	26%	26%	20%	16%	18%	24%
Gel1	64%	48%	21%	19%	16%	12%	20%	19%
Average	76%	57%	23%	22%	18%	14%	19%	21%

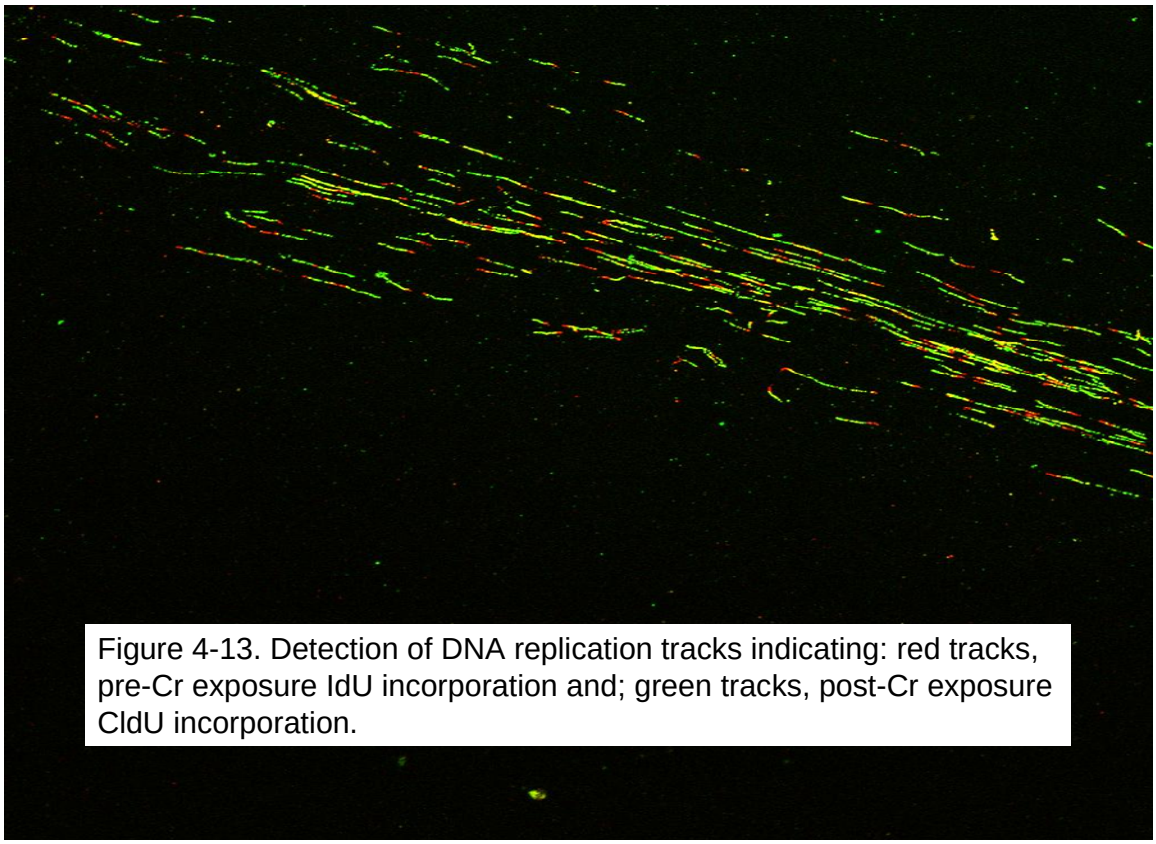


Figure 4-13. Detection of DNA replication tracks indicating: red tracks, pre-Cr exposure IdU incorporation and; green tracks, post-Cr exposure CldU incorporation.

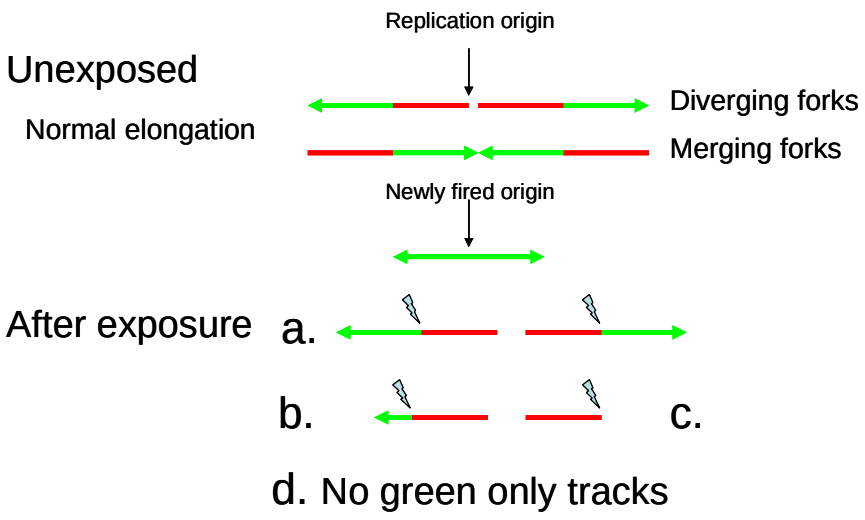


Figure 4-14. Visualization of replication using DNA combing. Red tracks indicate IdU incorporation, green tracks indicate CldU incorporation. Possible effects of exposure (indicated by lightning bolt): a. No effect; b. fork progression slowed; c. premature termination; d. replicon initiation inhibited

Figure 4-15. Chromium Slightly Increases Terminations

Log Population
of DT40 Cells

↓

Add 10 μM IdU (**red label**/first pulse), 10 min

↓

+/- Cr(VI)
(0, 15, 30, 60, 120 μM)

↓

Add 10 ml of reserved medium containing 100 μM CldU (**green label**/second pulse), 20 min

↓

Count new origins (CldU only tracks) and **IdU only tracks**

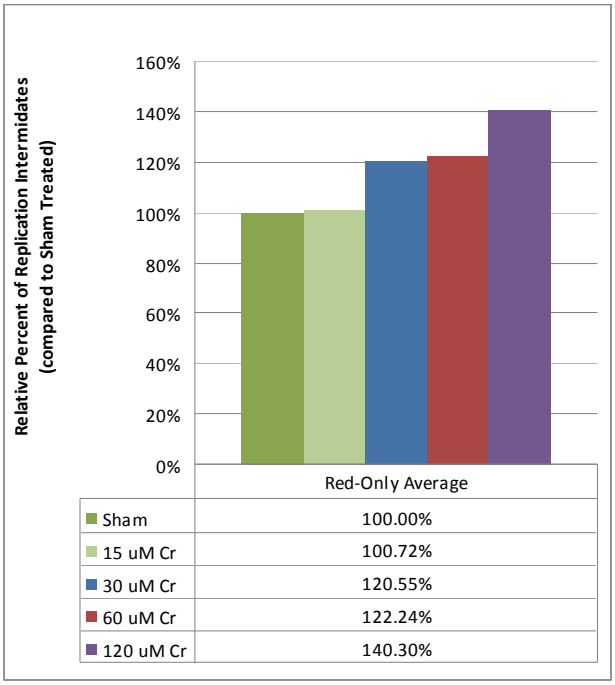


Figure 4-16. Chromium Alters Origin Initiation

Log Population
of DT40 Cells

↓

Add 10 μM IdU (**red label**/first pulse), 10 min

↓

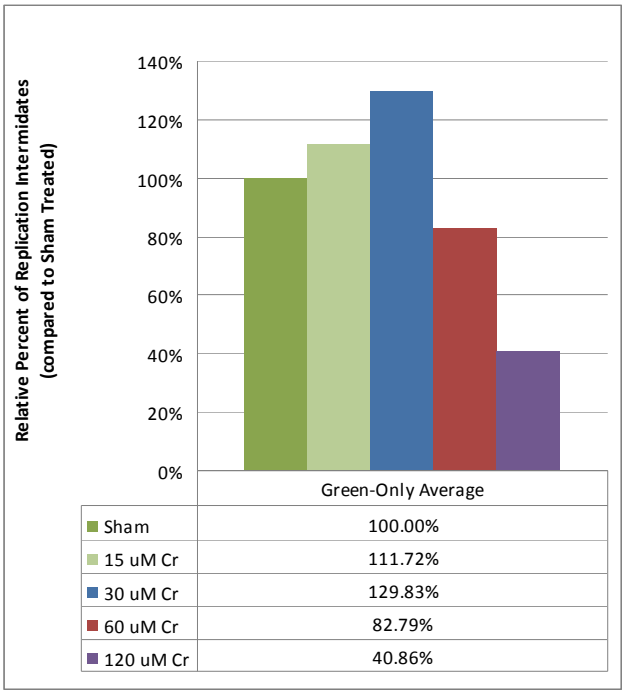
+/- Cr(VI)
(0, 15, 30, 60, 120 μM)

↓

Add 10 ml of reserved medium containing 100 μM CldU (**green label**/second pulse), 20 min

↓

Count new origins (CldU only tracks)
and IdU only tracks



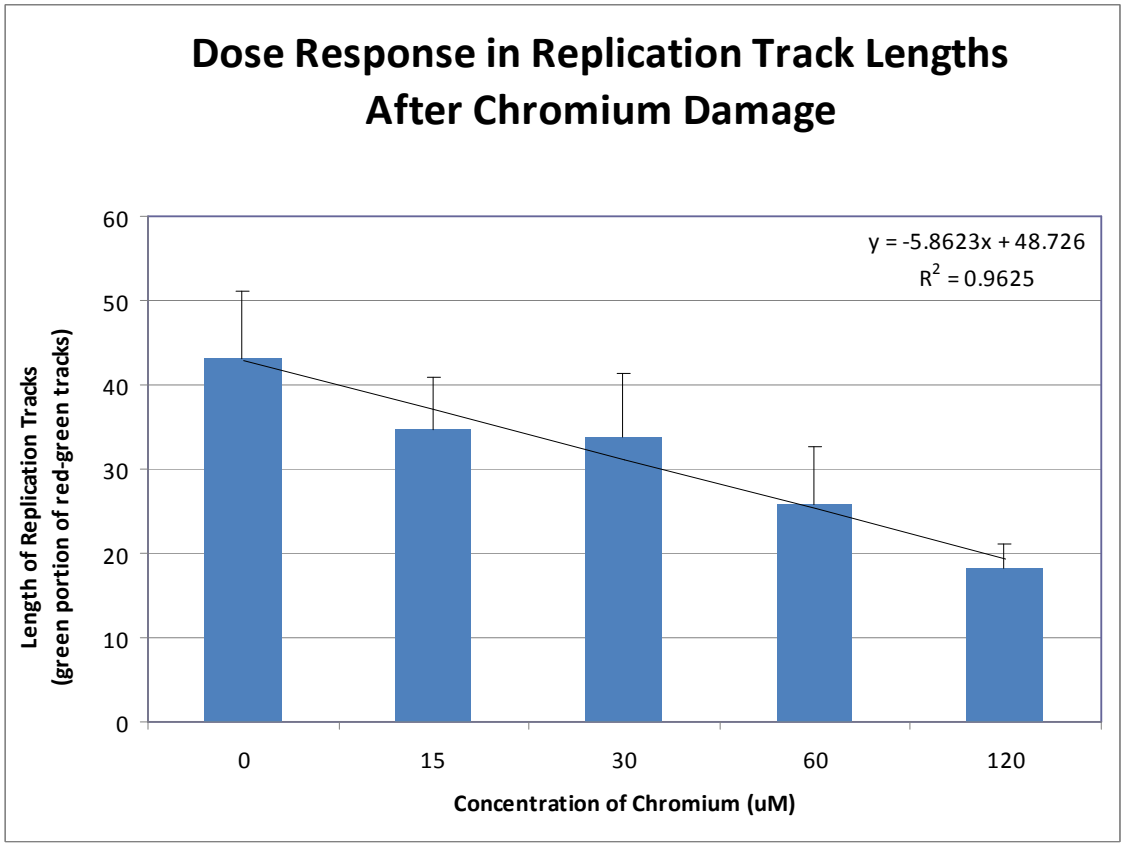


Figure 4-17. Effect of chromate exposure on replication rate in *wt* DT40 cells. The ratio of red/green track lengths should be approximately 1:1 as shown here for the sham dose. Exposure to chromate has increased the red/green ratio in a dose dependent manner indicating slowing of replication fork progression.

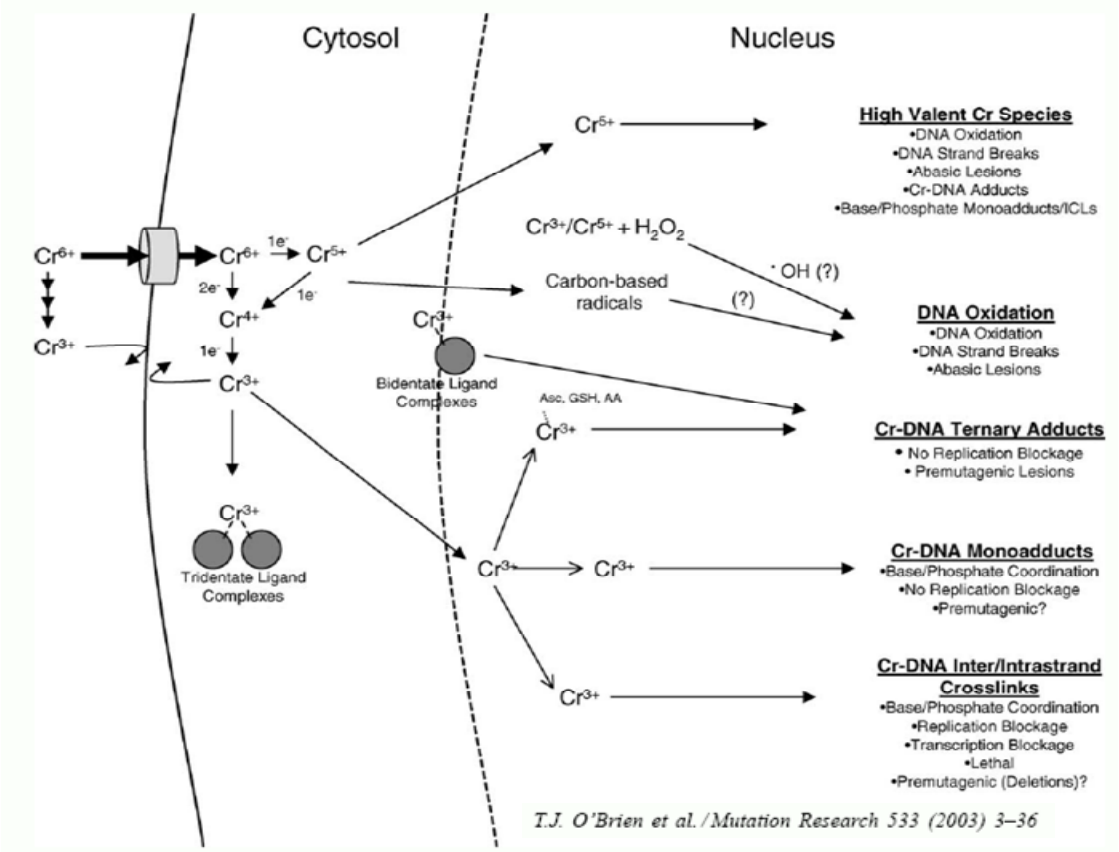


Figure 4-18. Proposed and reported possible types of DNA damage induced by hexavalent chromium.

Chapter 5. General Discussion

Summary and Conclusions

Considering the sizeable number of new chemicals that are being synthesized each year, when added to the huge number that already exists, it is a simple matter to understand the daunting task that regulatory bodies and others around the world have when determining the safe levels of exposure for humans and the environment. To aid in these determinations, scientists have made use of a variation of methodologies including the Ames test, animal toxicity studies, yeast reverse-genetic assays, studies with mammalian cells, various in vitro procedures and others. In this work we have made use of a very modern approach which takes advantage of the dozens of mutants available in the chicken B-lymphocyte, or DT40, cell system. This system presents a considerable advantage over others in that the complete system has been generated from a single parent and is thus isogenic. One obvious benefit from such a system is that it removes uncertainties inherent when several mutants are used for the determination of DNA damage response, but each mutant is from different cell lines with dissimilar genetic backgrounds. Conclusions made as a result of responses across these different cell lines are thus made more tentative due to unknown reactivities of proteins in the pathways being studied and particularly those of associated pathways. Other advantages of the DT40 cell line, such as the comparatively very high targeting efficiency of transfected DNA, its stable karyotype over long periods of culturing and its lack of demonstrable p53

activity have combined to make this cell line very attractive indeed for studies of not only metabolic pathways, but now, with our work, for DNA damage response assessments of toxicants as well.

This project has endeavored to develop, assess and validate a new high-throughput applicable methodology for the use of the DT40 cell system for the determination of the DNA damage response to exogenous and endogenous agents. The method developed here has dispensed with the more cumbersome, time consuming and expensive clonogenic assay which normally suffices quite well for metabolic pathway analyses and replaced it with a convenient, less expensive and much more rapid analysis which we feel has definite potential for automation. This would thus create a high-throughput process regulatory agencies could use to screen new chemicals in a much more timely manner.

Development of this assay has been performed during Specific Aim 1 of this project and consisted of combining two existing systems, the DT40 cells system, and the XTT cell proliferation assay to determine the response of the cells to chemicals such as MMS and cisplatin. These tests demonstrated that the assay could provide excellent reproducibility (as exemplified by the MMS result) as well as strongly comparable results with the clonogenic method as demonstrated by our cisplatin results. We were then able to further verify the viability of our system by assessing the DNA damage response to acrolein – the first environmental pollutant to be tested with this system. Our results with Acr generally agreed very well with the literature reports concerning damage pathway responses to Acr.

To further assess the validity of our assay we chose, in Specific Aim 2, to use our method to evaluate the DNA damage response to formaldehyde – a chemical shown to induce DPC. At the time, little was known about how cells deal with these lesions. We were able to show for the first time that cells deficient in the FANC/BRCA pathway are not only hypersensitive to formaldehyde, but the sensitivity is detectable at concentrations of formaldehyde that commonly found in human blood plasma. To help further verify our findings, we exposed two human cell mutants that are members of the FANC/BRCA pathway – FANCC and FANCG – and found they are also quite sensitive to formaldehyde. Based on these findings we were able to conclude that the FANC/BRCA pathway is essential for the removal of DPC and that endogenous levels of formaldehyde may have an effect on rapidly proliferating cell, such as bone marrow cells, and that an etiology of cancer due to formaldehyde exposure in Fanconi anemia patients may exist. In Specific Aim 3 we chose to further assess the value of the assay to toxicologists by evaluating the response of our DT40 system to another, completely different sort of chemical, the inorganic salt of the metal chromium – potassium chromate. Hexavalent chromium is a known human carcinogen for which a substantial number of reports have suggested several different types of possible damage to DNA that may be responsible for its mutagenicity. Nevertheless, even after decades of study, the avenue to chromate-induced mutagenicity is still not completely understood. After repeated trials with chromate against our DT40 mutants, we found the system to give very consistent results which indicated agreement as well as disagreement with some of the conclusions of others. Our DNA damage response results indicated that chromate presents a block to replication forks resulting in fork stalling and error-prone repair by a TLS bypass

polymerase. This conclusion is in agreement with accumulated works of others. Also, many studies have, using various techniques, espoused the idea that oxidative-stress related damage is induced by chromate. Our DNA damage response analysis also indicated a pattern consistent with that caused by exposure to hydrogen peroxide – a strong generator of oxidative stress. In this situation, however, we determined to use the DT40 cells for follow-up studies to further investigate the possibility of chromate-induced oxidative stress. These studies, the evaluation of 8-oxo-dG, and the level of DNA single-strand breaks as determined by the NAD(P)H depletion assay, the GGE and the OTX-AGE tests, indicated that chromate-induced oxidative stress is unlikely in DT40 cells.

Thus, with this chromium study we have been able to demonstrate another tremendous value of this assay – the ability to lead us in the direction of expanded studies to further characterize the genotoxicity of a toxicant, and just as importantly, to possibly deter us from following paths that are most likely to prove unproductive.

Future Directions

Although the method we have developed here has provided a convenient and relatively speedy assay for determining the DNA damage response to exogenous as well as endogenous agents, for the method to gain the most value to regulatory agencies the assay needs to be automated. We believe this is possible with existing instrumentation. Equipment is available which is capable of culturing and maintaining the cells as well as automatically seeding and exposing them in multi-well plates. Automation should allow for many more tests to be performed daily with relatively little human intervention and

should also lessen the variability due to pipetting variation inherent with human involvement.

We also envision a continuation of our study of chromium toxicity although we currently do not have the resources to perform the work ourselves. Although we found no significant increase in the 8-oxo-dG levels above controls, it has been reported that 8-oxo-dG may be susceptible to further oxidation to the compound spiroiminodihydantion (Sp) as well as other possible advanced oxidation products (Slade et al., 2005). In this study the authors found that the lesion formed on double-stranded DNA was trapped nearly exclusively by the BER protein mNEIL2 rather than hOGG1 indicating that Sp was the major form of the oxidative damage induced by chromate (Sp lesions were approximately 20-fold more numerous than 8-oxo-dG). As the Slade experiment was conducted in vitro on naked DNA, we think it would be interesting to clarify the importance of this advanced oxidation of dG in DT40 and human cells.

Another interesting aspect of our chromium research concerns the path by which chromate is reduced in DT40 cells. It has been determined that chromate is reduced in the cytoplasm via a two-electron reduction by ascorbate or one-electron reduction by glutathione or cysteine. The two-electron reduction would bypass the most likely culprit for inducing oxidative stress – Cr(V). Thus, it would be very interesting to ascertain the level of ascorbate existing in DT40 cells to determine if the lack of observed markers of oxidative stress may be due to relatively high levels of ascorbate in chicken cells.

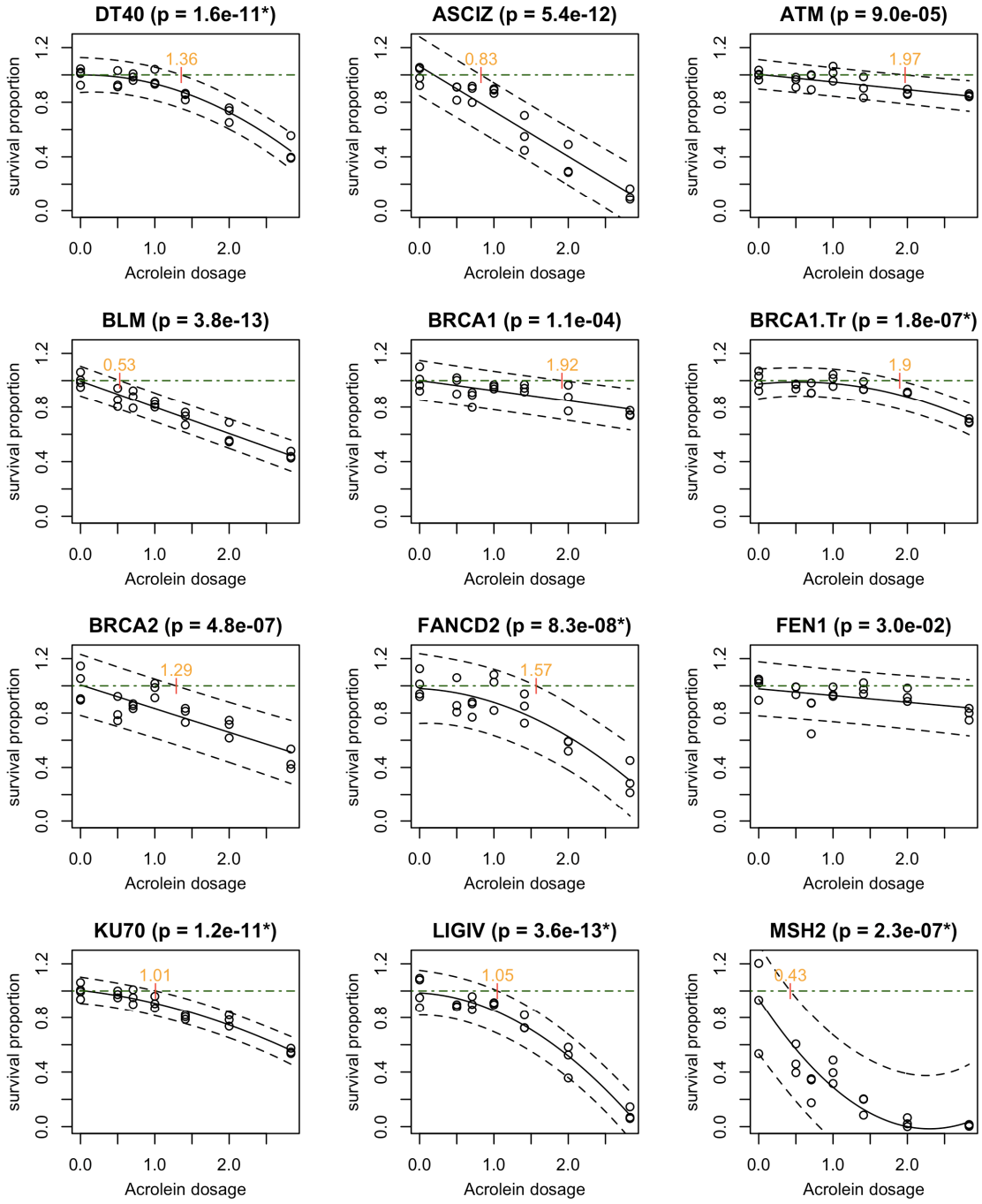
Another interesting possibility would be to perform the DNA fiber analysis on some chosen DT40 mutants. The preliminary data we have gathered using this assay have been the result of exposure of chromate to parental DT40 cells only. Since the data

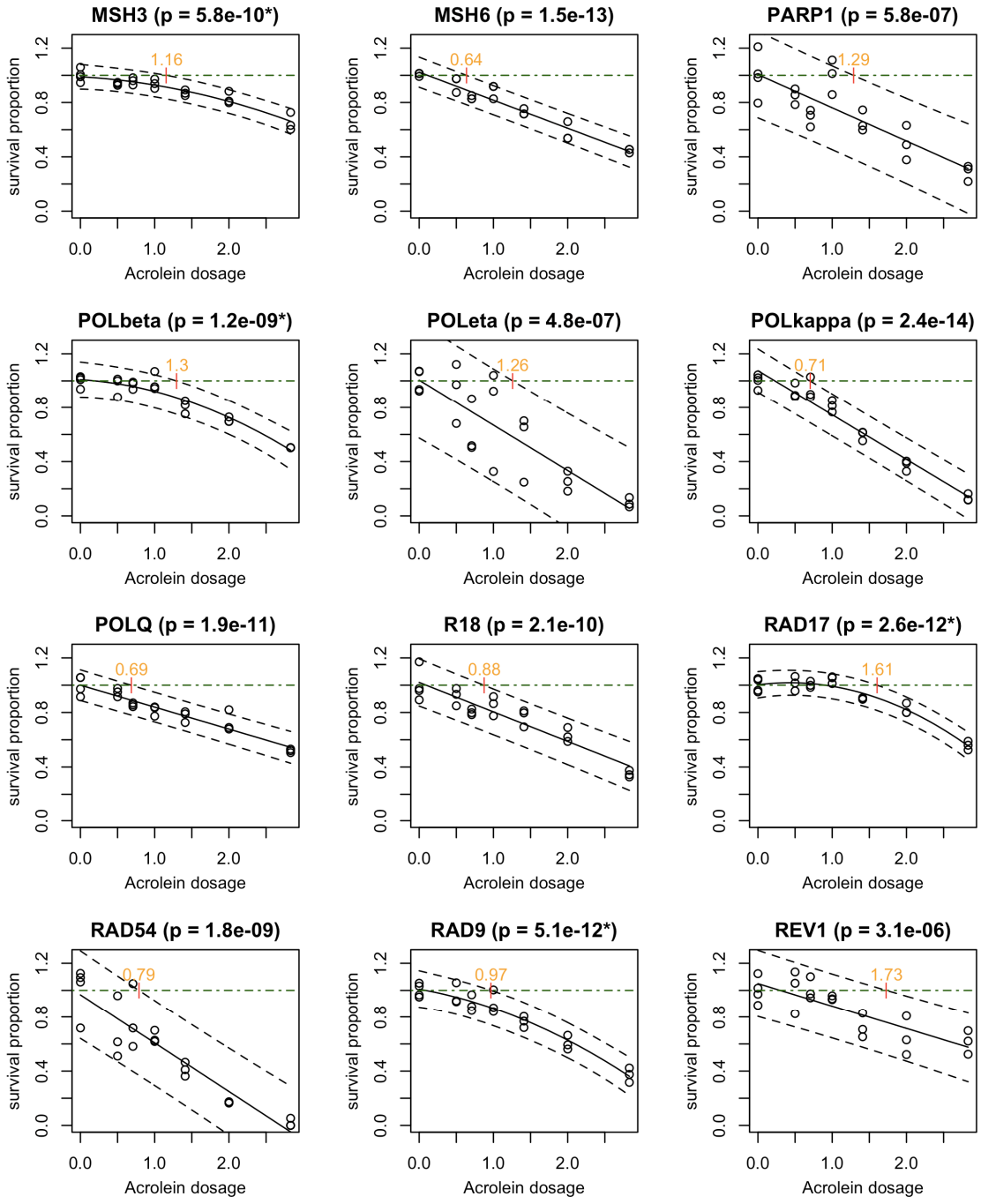
show a strong indication that replication forks are stalling due to chromate exposure, performing the same experiments with DT40 mutants deficient in double-strand break repair, for instance, may provide more clarification in regard to this phenomenon.

Finally, we feel quite strongly that we have developed what may become a very important new assay to aid in the determination of how chemicals are inducing genotoxicity in cells. Although we understand that much more validation with many more chemicals is necessary, we look forward to a time when this assay could take its place among the array of other assays that regulators and others use in the determination of the modes of action of the multitude of agents for which not enough is known about their means to cause harm to the genome.

Appendix A. Statistical Analysis

Figure A-1. Parametric (linear or quadratic model) fit of the relation between Acrolein dosage and survival proportion (black solid line), the corresponding 95% prediction CI (dash line), and the MED (the intersection of the upper bound of 95% CI and the horizontal line: survival proportion = 0). The star (*) next to a p-value indicates the model is a quadratic model, otherwise the model is linear model.




MSH3 (p = 5.8e-10*)
1.16
MSH6 (p = 1.5e-13)
0.64
PARP1 (p = 5.8e-07)
1.29
POLbeta (p = 1.2e-09*)
1.3
POLeta (p = 4.8e-07)
1.26
POLkappa (p = 2.4e-14)
0.71
POLQ (p = 1.9e-11)
0.69
R18 (p = 2.1e-10)
0.88
RAD17 (p = 2.6e-12*)
1.61
RAD54 (p = 1.8e-09)
0.79
RAD9 (p = 5.1e-12*)
0.97
REV1 (p = 3.1e-06)
1.73
survival proportion
Acrolein dosage
0.0
0.4
0.8
1.2
1.0
2.0

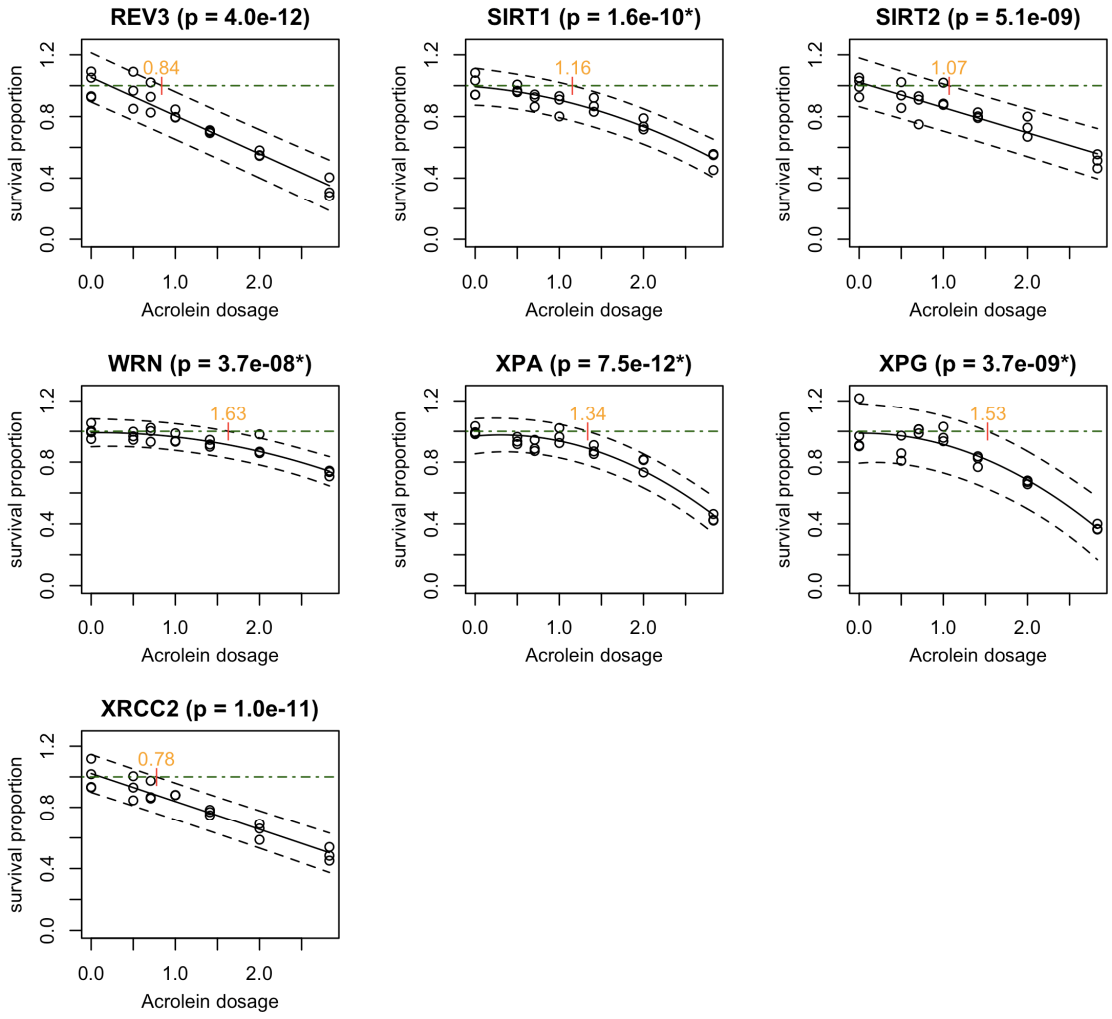
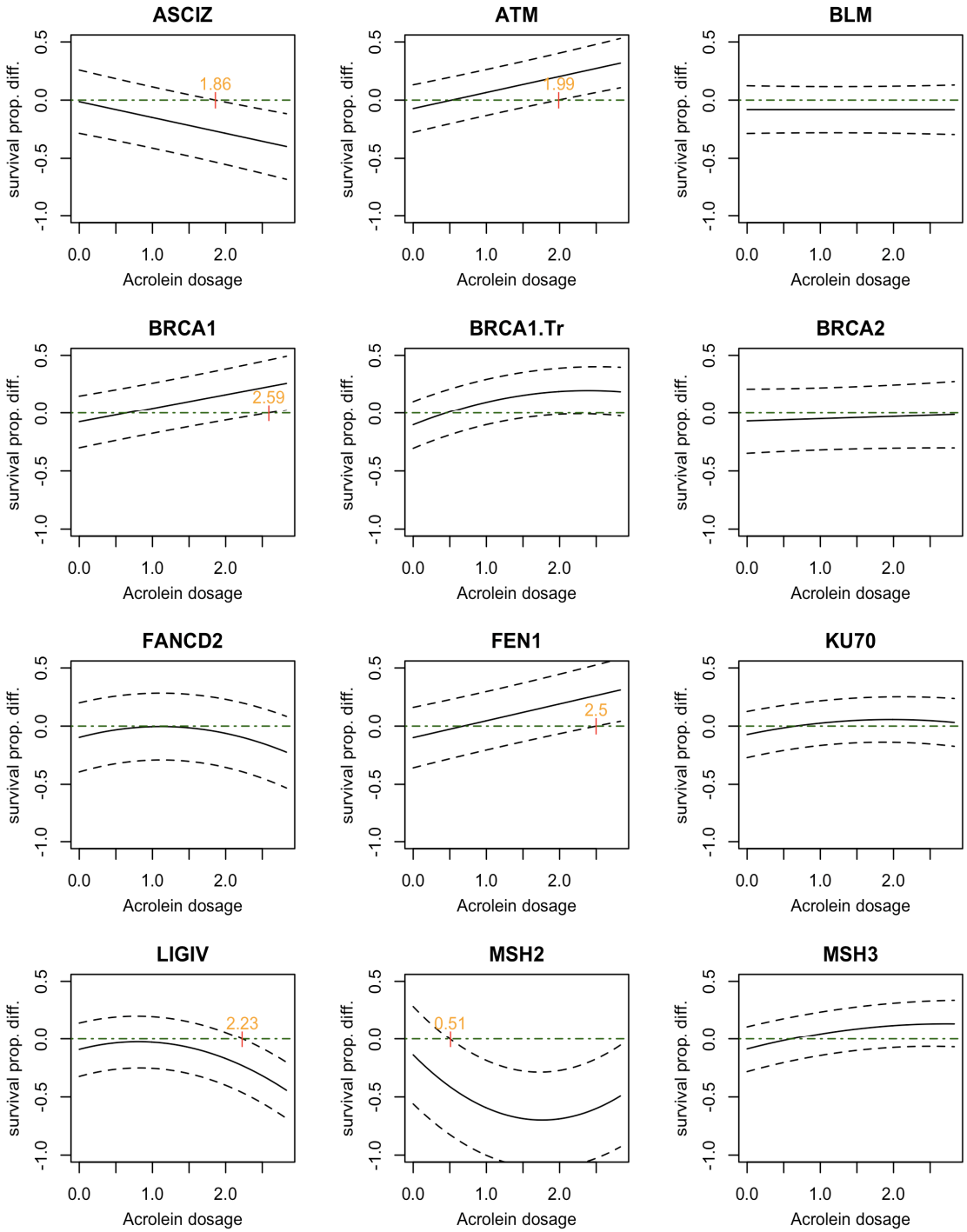
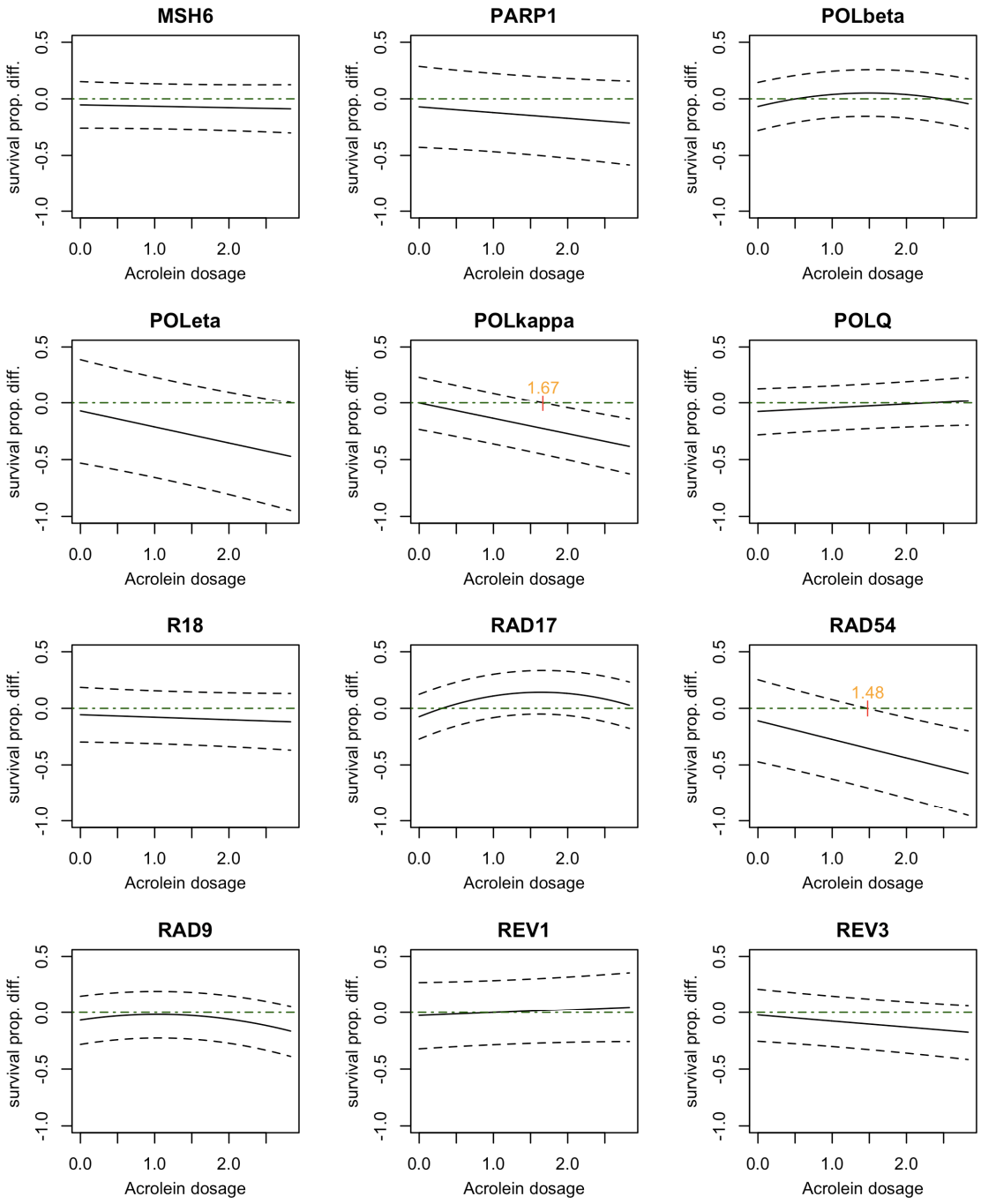

REV3 (p = 4.0e-12)
0.84
SIRT1 (p = 1.6e-10*)
1.16
SIRT2 (p = 5.1e-09)
1.07
WRN (p = 3.7e-08*)
1.63
XPA (p = 7.5e-12*)
1.34
XPG (p = 3.7e-09*)
1.53
XRCC2 (p = 1.0e-11)
0.78
survival proportion
Acrolein dosage
0.0
0.4
0.8
1.2
1.0
2.0

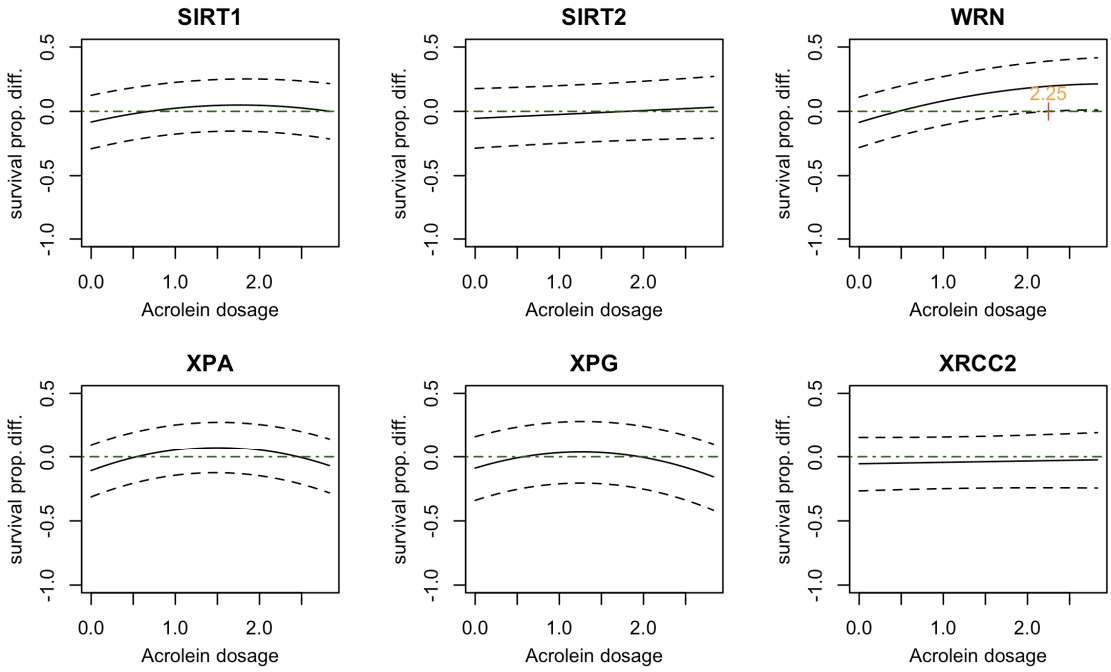
Table A-1. Contingency table comparison of the MED estimated from our parametric approach based on linear/quadratic models and a non-parametric approach (Williams 1986).

MED from linear or quadratic model	MED estimated by Williams (1986)					
	(0,0.5]	(0.5,0.71]	(0.71,1.0]	(1.0,1.41]	(1.41,2.0]	(2.0,2.83]
(0,0.5]	0	1	0	0	0	0
(0.5,0.71]	1	2	0	0	0	0
(0.71,1.0]	0	1	2	4	0	0
(1.0,1.41]	0	0	0	11	0	0
(1.41,2.0]	0	0	0	2	5	1
(2.0,2.83]	0	0	0	0	0	0

Figure A-2. The 95% prediction CIs of the difference between different cell lines and cell line DT40 and the minimum dose where two cell lines have significant different responses (if it exits).




MSH6
PARP1
POLbeta
POLeta
POLkappa
1.67
POLQ
R18
RAD17
RAD54
1.48
RAD9
REV1
REV3
survival prop. diff.
Acrolein dosage
-1.0
-0.5
0.0
0.5
0.0
1.0
2.0


SIRT1
SIRT2
WRN
XPA
XPG
XRCC2
survival prop. diff.
Acrolein dosage
-1.0
-0.5
0.0
0.5
0.0
1.0
2.0
2.25

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