GENOTOXICITY INVESTIGATION OF ORGANIC N-CHLORAMINES

Somprasong Laingam (MBiotech)

Discipline of Pharmacology

The University of Adelaide

(Faculty of Health Science)

July, 2009

A thesis submitted for the degree of Doctor of Philosophy

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABSTRACT

Organic N-chloramines have long been recognised as disinfection by-products (DBPs) found in both chlorinated and chloraminated water, but have gained little attention from water authorities in the past. However, in recent years studies have shown that organic N-chloramines are molecules involved in inflammation and several chronic diseases including cancers. A recent study (Bull *et al.*, 2006) has suggested that organic N-chloramines can be potential health risks but due to a lack of available toxicological information toxicity studies of compounds in this group have been recommended as a priority in DBPs research.

The aim of this study was to investigate genotoxicity of individual organic N-chloramines utilising a mammalian cell-based genotoxicity assay to help determine which compound(s) should be subject to further *in vivo* studies. The flow cytometry-based micronucleus (FCMN) assay was optimised and validated for use as a rapid screening for genotoxicity of organic N-chloramine candidates. A number of assay validations were conducted on two mammalian cell lines (WIL2-NS and L5178Y) using model genotoxicants with various modes of action. Comparative studies on these two cell lines showed that WIL2-NS cells were suitable for the FCMN assay and therefore selected for use in all studies described in this thesis.

For the genotoxicity investigation of organic N-chloramines, 16 compounds were synthesised by chlorination of amine precursors. At least 3 concentrations (in μM range) were subjected to screening for genotoxicity using the validated FCMN assay and confirmed by microscopic counting of micronuclei. This study found that of the 16 compounds, 4 were genotoxic to WIL2-NS cells by both FCMN and microscopy based MN

assay. Oxidative stress was hypothesised as a possible genotoxic mechanism of these compounds and also was investigated in this study. Following exposure to the 4 genotoxic organic N-chloramines, it was found that although there was a small reduction of cellular glutathione the change in lipid peroxidation was not observed. This suggested that oxidative stress is unlikely to be a mechanism involved in genotoxicity of these organic N-chloramines.

The final part of this research demonstrated an application of using the optimised FCMN assay to identify genotoxic DBP precursors in Australian water. We collaborated with Curtin University, Western Australia on this aspect. Highly coloured surface water was collected, concentrated, and fractionated based on molecular weight (MW) of the organic contents by researchers at Curtin University. Eight MW fractions (pre- and post chlorination) were tested for genotoxicity using the FCMN assay. No genotoxicity was observed in all pre-chlorinated MW fractions while significant genotoxicity was seen in chlorinated products of several fractions of medium to high MW. This result indicated that these fractions contain materials that are precursors to genotoxic DBPs and may lead to future studies such as characterisation of the genotoxic DBP precursors for their removal prior to the disinfection process.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968. The author acknowledges that copyright of published works contained within this thesis (as listed on page IV) resides with the copyright holder(s) of those works.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Somprasong Laingam

Singed .....

Date .....

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr Andrew Humpage and Dr Suzanne Froscio from the Australian Water Quality Centre for their guidance and support throughout my candidature. Their knowledge and experience in the field of toxicology as well as their constructive criticisms have made the completion of this thesis possible. I would also like to thank my co-supervisor, Dr Ian Musgrave from the discipline of Pharmacology, who has always ensured that my candidature outside the university ran smoothly.

Thanks to all researchers and staff at the Australian Water Quality Centre for their help and support during my study. Special thanks go to Stella and Melody, who have helped with the smooth running of the cell culture laboratory and Rebecca for all her encouragement and sharing her PhD journey, particularly during late hours and weekend work. Similar thanks go to all researchers and staff in the Applied Chemistry Research and Water Treatment who shared their knowledge and frequently help me with the chlorination work.

A sincere thank-you to the CRC for Water and Quality Treatment and the Discipline of Pharmacology, Faculty of Health Science who provided my scholarship and the funding for this project. It has been an honour being a CRC PhD candidate. Finally, I would like to thank my family and friends for their encouragement and support during my study. You have had a big influence on the completion of this thesis for which I am truely thankful.

PUBLICATIONS IN SUPPORT THIS THESIS

Laingam, S., Froscio, S.M. and Humpage, A.R. (2008). Flow-cytometric Analysis of *in vitro* Micronucleus Formation: Comparative Studies with WIL2-NS Human Lymphoblastoid and L5178Y Mouse Lymphoma Cell Lines. *Mutatation Research*, 656 (1-2): 19-26.

Laingam, S., Froscio, S.M. and Humpage, A.R. (2009). Toxicity and Genotoxicity of Disinfection By-products, Organic N-chloramines. *In preparation.*

Laingam, S., Froscio, S.M. and Humpage, A.R. (2009). Use of the Flow Cytometry-based Micronucleus Assay to Determine Genotoxic DBP Precursors from Australian Water. *In preparation.*

ABBREVIATIONS AND SYMBOLS

ANOVA	Analysis of variance
ATCC	American type culture collection
ADWG	Australian drinking water guidelines
AWQC	Australian water quality centre
BA	Bromoacetic acid
BaP	Benzo[a]pyrene
BrO_3^-	Bromate
$CHCl_3$	Chloroform
CHO	Chinese hamster ovary
CI	Confident interval
Cl_2	Chlorine
ClO_2	Chlorine dioxide
ClO_2^-	Chlorite
DBP(s)	Disinfection by-product(s)
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
DON	Dissolved organic nitrogen
DPD	N,N-diethyl-p-phenyl diamine
dsDNA	Double stranded deoxyribonucleic acid
DTNB	5, 5'- dithiolbis-2-nitrobenzoic acid
EC30	Effective concentration at 30% of the untreated control
EC50	Effective concentration at 50% of the untreated control
ECL	Enterochromaffin-like
EDTA	Ethylenediaminetetraacetic acid

EPA	Environmental protection agency
ETOPO	Etoposide
FACS	Fluorescence-activated cell sorting
FAS	Ferrous ammonium sulphate
FBS	Foetal bovine serum
FCMN	Flow cytometry based micronucleus
$FeSO_4$	Ferrous sulphate
FSC	Forward scatter
GSH	Glutathione (Reduced form)
GSSG	Glutathione (Oxidised form)
H_2O_2	Hydrogenperoxide
HAAs	Haloacetic acids
HANs	Haloacetonitriles
HBSS	Hank balanced salt solution
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HOCl	Hypochlorous acid
HPLC	High pressure liquid chromatography
HPSEC	High performance size exclusion chromatography
IARC	International agency for research on cancer
LOAELs	Lowest observed adverse effect levels
MDA	Malondialdehyde
MMC	Mitomycin C
MMS	Methyl methanesulfonate
MN	Micronucleus
MW	Molecular weight

MX	3-chloro-4(dichloromethyl)-5-hydroxy-2(5H) furanone
N	Nucleus
NCl_3	Trichloramine
NCP	N-chloropiperidine
NDMA	N-nitrosodimethylamine
NH_2Cl	Monochloramine
NH_3	Ammonia
$NHCl_2$	Dichloramine
NOM	Natural organic matter
O_3	Ozone
OCl^-	Hypochlorite ion
OECD	Organization for economic co-operation and development
OR	Odds ratio
PBS	Phosphate buffered saline
PI	Propidium iodide
QSTR	Quantitative structure toxicity relationship
RO	Reverse osmosis
RPMI	Roswell park memorial institute medium
r	Spearman's coefficient
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SSC	Side scatter
SUCRO	Sucrose
TB	Trypan blue
TBARS	Thiobarbituric acid reactive substances

TDN	Total dissolved nitrogen
THMs	Trihalomethanes
US	The United States
VINB	Vinblastin
WHO	World health organization