

Development of an *in vitro* mechanistic toxicity screening model using cultured hepatocytes

by
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A thesis submitted in partial fulfilment of the requirements for the
degree

Doctor of Philosophy
in
Pharmacology

in the
Faculty of Health Sciences
at the
University of Pretoria

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Pretoria
November 2011

Declaration

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Acknowledgments

- Prof. Vanessa Steenkamp and Dr. Duncan Cromarty for their guidance in the scientific process and taking the necessary steps to safeguard the quality of the results from this study. Teaching me the importance of objectivity and proving that my observations are accurate. For their patience and commitment.
- Prof. Mary Gulumian for her time and effort, ensuring that the interpretation of the results were accurate.
- Tracy Snyman, Clinical Chemistry at WITS, for her generous help regarding the propagation of the HepG2 cell line.
- Prof. Francois Steffens for pointing me in the right direction concerning statistical analyses and interpretation.
- My wife, Alet van Tonder, for her support, patience and understanding.
- God, for His faithfulness.

"Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science."

Henri Poincare

Abstract

In vitro testing includes both cell-based and cell-free systems that can be used to detect toxicity induced by xenobiotics. *In vitro* methods are especially useful in rapidly gathering intelligence regarding the toxicity of compounds for which none is available such as new chemical entities developed in the pharmaceutical industry. In addition to this, *in vitro* investigations are invaluable in providing information concerning mechanisms of toxicity of xenobiotics. This type of toxicity testing has gained popularity among the research and development community because of a number of advantages such as scalability to high throughput screening, cost-effectiveness and predictive power. Hepatotoxicity is one of the major causes of drug attrition and the high cost associated with drug development poses a heavy burden on the development of new chemical entities. Early detection of hepatotoxic agents by *in vitro* methods will improve lead optimisation and decrease the cost of drug development and reduce drug-induced liver injury. Literature highlights the need for a cell-based *in vitro* model that is capable of assessing multiple toxicity parameters, which assesses a wider scope of toxicity and would be able to detect subtle types of hepatotoxicity.

The present study was aimed at developing an *in vitro* procedure capable of mechanistically profiling the effects of known hepatotoxin dichlorodiphenyl trichloroethane (DDT) and its metabolites, dichlorodiphenyl dichloroethylene (DDE) and dichlorodiphenyl dichloroethane (DDD) on an established liver-derived cell line, HepG2, by evaluating several different aspects of cellular function using a number of simultaneous *in vitro* assays on a single 96 well microplate. Examined parameters have been suggested by the European Medicines Agency and include: cell viability, phase I metabolism, oxidative stress, mitochondrial toxicity and mode of cell death (apoptosis vs. necrosis). To further assess whether the developed method was capable of detecting hepatoprotection, the effect of the known hepatoprotectant, *N*-acetylcysteine, was determined.

Viability decreased in a dose-dependent manner yielding IC_{50} values of 54 μ M, 64 μ M and 44 μ M for DDT, DDE and DDD, respectively. Evaluation of phase I metabolism showed that

cytochrome P4501A1 activity was dose-dependently induced. Test compounds decreased levels of reactive oxygen species, and significantly hyperpolarised the mitochondrial membrane potential. Assessment of the mode of cell death revealed a significant elevation of caspase-3 activity, with DDD proving to be most potent. DDT alone induced dose-dependent loss of membrane integrity.

These results suggest that the tested compounds produce apoptotic death likely due to mitochondrial toxicity with subsequent caspase-3 activation and apoptotic cell death. The developed *in vitro* assay method reduces the time it would take to assess the tested parameters separately, produces results from multiple endpoints that broadens the scope of toxicity compared to single-endpoint methods. In addition to this the method provides results that are truly comparable as all of the assays utilise the same batch of cells and are conducted on the same plate under the exact same conditions, which eliminates a considerable amount of variability that would be unavoidable otherwise. The present study laid a solid foundation for further development of this method by highlighting the unforeseen shortcomings that can be adjusted to improve scalability and predictive power.

Keywords: *Apoptosis, CYP1A1, DDD, DDE, DDT, hepatotoxicity, mechanistic profiling, mitochondrial hyperpolarisation, necrosis, organochlorine, reactive oxygen species.*

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List of Abbreviations

%	percentage
°C	degrees Celsius
•O ₂ ⁻	superoxide
•OH	hydroxyl radical
7-ER	7-ethoxyresorufin
AAPH	2',2'-azobis(2-methylpropionamide) dihydrochloride
Ac-DEVD-AMC	Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
ADP	adenosine diphosphate
AhR	aryl hydrocarbon receptor
AIDS	acquired immune deficiency disease
AMC	7-amino-4-methylcoumarin
Apaf-1	apoptotic protease activating factor-1
Arnt	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
Bad	Bcl-2 associated death promoter
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2 protein
BH3	Bcl-2 homology domain 3
Bid	BH3 interacting-domain death agonist
Ca ²⁺	calcium
Cas-3	caspase 3
CAT	catalase
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	centimeter
CO ₂	carbon dioxide
Cu ²⁺	copper(II)
Cu ³⁺	copper(III)
CYP	cytochrome P450
cyt C	cytochrome C
d	days
DCFDA	2',7'-dichlorofluorescein diacetate
DDA	bis(<i>p</i> -chlorophenyl)acetic acid
DDD	dichlorodiphenyl dichloroethane
DDE	dichlorodiphenyl dichloroethylene
DDT	dichlorodiphenyl trichloroethane
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
Δψ _m	mitochondrial membrane potential

EDTA	ethylenediaminetetraacetic acid
EMEA	European Medicines Agency
EMEM	Eagle's minimum essential medium
EROD	ethoxyresorufin- <i>O</i> -deethylase
FADH ₂	flavin adenine dinucleotide (reduced)
FCS	foetal calf serum
Fe ²⁺	iron(II)
Fe ³⁺	iron(III)
g	gram
<i>g</i>	gravity
G-6-PD	glucose-6-phosphate dehydrogenase
GR	glutathione reductase
GSH	glutathione
GSH-Px	glutathione peroxidase
GSSG	glutathione disulfide
h	hour
H ⁺	proton
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	human immunodeficiency virus
HSP90	heat shock protein 90
IC ₅₀	concentration at which 50% of cells are not viable
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'- tetraethylbenzimidazolylcarbocyanine iodide
KH ₂ PO ₄	potassium dihydrogen orthophosphate
l	litre
LD ₅₀	dose at which 50% of the population does not survive
LDH	lactate dehydrogenase
M	molar
m	milli-
MEIC	Multicentre evaluation of <i>in vitro</i> cytotoxicity program
Mg ²⁺	magnesium (II)
min	minutes
MMO	microsomal monooxygenase system
mPTP	mitochondrial permeability transition pore
mRNA	messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
<i>n</i>	sample size
Na ⁺	sodium
NAC	<i>N</i> -acetylcysteine
NADH	nicotinamide adenosine dinucleotide (reduced)
NADPH	nicotinamide adenosine dinucleotide phosphate (reduced)
NFI	nuclear factor I



NRU	neutral red uptake
O ₂	oxygen
OH ⁻	hydroxyl
p	probability
p53	tumour protein 53
PBS	phosphate buffered saline
pH	negative logarithm of the hydrogen ion concentration
PI	propidium iodide
ppm	parts per million
Puma	p53 up-regulated modulator of apoptosis
r ²	coefficient of determination
RFI	relative fluorescence intensity
RLW	relative liver weight
RNS	reactive nitrogen species
ROS	reactive oxygen species
RSA	Republic of South Africa
SEM	standard error of the mean
SOD	superoxide dismutase
t	test statistic
TCDD	2,3,7,8-tetrachlorodibenzo- <i>p</i> -dioxin
U	units
UK	United Kingdom
US\$	US dollars
USA	United States of America
v	volume
V	volts
w	weight
XRE	xenobiotic response element
β	beta
γ-GCS	γ-glutamylcysteine synthase
λ _{em}	emission wavelength
λ _{ex}	excitation wavelength
μ	micro

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The relationship between drug metabolism and toxicity. Toxicity may occur through accumulation of parent drug or via metabolic activation, through formation of a chemically reactive metabolite, which, if not detoxified, can effect covalent modification of biological macromolecules. The identity of the target macromolecule and the functional consequence of its modification will dictate the resulting toxicological response.

Figure 1.2. _____ 12

Cooperation between various antioxidant enzymes and cofactors. SOD – superoxide dismutase, CAT – catalase, GSH – glutathione, GSSG – glutathione disulfide, GSH-Px – glutathione peroxidase, GR – glutathione reductase, γ -GCS – γ -glutamylcysteine synthase, GS – glutamine synthase, G-6-PD – glucose-6-phosphate dehydrogenase.

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Illustration of the two routes of metabolism of DDT to produce either DDE or DDD, showing the structure of each of the test compounds utilised in this study.

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Diagram illustrating the scope of toxicity that the chosen parameters are expected to evaluate. As the concentration of test compound increases one would expect to see different cellular responses in an attempt to restore homeostasis. Initially, cells should respond by trying to eliminate xenobiotics through metabolic inactivation (Phase I metabolism). At higher concentrations ROS generation may occur, originating from either mitochondria or from CYP activity. If the mitochondria are affected, they may release factors that will initiate apoptotic death via caspase-3. On the other hand, excessive ROS may result in deactivating caspase activity and lipid peroxidation, which will lead to cell death by necrosis. The degree of toxicity can then be assessed utilising an assay that enumerates viable cells. EROD - ethoxyresorufin-*O*-deethylase, JC-1 - 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, DCFDA - 2'7'-dichlorofluorescein diacetate.

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Diagram illustrating the plate setup. Plates were divided into six duplicate column sets, one for each of the six different assays to be performed and into eight rows, for the blanks, controls and various concentrations of test compound. Only one compound was tested on a single plate.

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Histogram density plots of the observed viability data of HepG2 cells exposed to DDT demonstrating the distributions of the collected data. The control group is a good example of a normal distribution. The observations did not always follow a normal distribution, especially in the lower ranges of viability. X-axis represents observed values and Y-axis, the count.

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Shapiro-Francia test normality results of the observed Cas-3 data after removal of outliers detected with Grubb's test. Values given in the table are p -values. Instances where $p < 0.05$ are significantly non-normal. * indicates $p < 0.05$.

Table 7.3. _____ 94

Active Cas-3 in HepG2 cells following 6 h exposure to DDT, DDE, DDD and Staurosporine (positive control). Results (% of Control) are presented as mean \pm SEM. ** indicates $p < 0.01$ and *** $p < 0.001$ as determined by Mann-Whitney and Student's t -tests.

Table 7.4. _____ 97

Relative Cas-3 activity in HepG2 cells after exposure to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ as determined by Mann-Whitney and Student's t -tests.

Table 7.5. _____ 98

Grubb's test results for detecting outliers in the data from PI staining. Values given in the table are p -values. Instances where $p < 0.05$ (*) indicates the presence of outliers.

Table 7.6. _____ 98

Shapiro-Francia test normality results of the observed PI data after removal of outliers detected with Grubb's test. Values given in the table are p -values. Instances where $p < 0.05$ are significantly non-normal. * indicates $p < 0.05$.

Table 7.7. _____ 99

PI staining in HepG2 cells following 3 h exposure to DDT, DDE, DDD and Triton X-100 (positive control). Results (% of Control) are presented as mean \pm SEM. * indicates $p < 0.05$ and ** $p < 0.01$ as determined by Mann-Whitney tests.

Table 7.8. _____ 100

PI staining in HepG2 cells due to DDT, DDE, DDD, with or without 1 h pre-treatment with NAC. * indicates $p < 0.05$ as determined by Mann-Whitney tests.