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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

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Declaration

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Subject of the work: Development of an *in vitro* mechanistic toxicity screening model using cultured hepatocytes

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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 µM, 64 µM and 44 µM 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
$\bullet O_2^-$	superoxide
•OH	hydroxyl radical
7-ER	7-ethoxyresorufin
AAPH	2',2'-azobis(2-methylpropionamidine) 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'-dchlorofluorescein diacetate
DDA	bis(*p*-chlorophenyl)acetic acid
DDD	dichlorodiphenyl dichloroethane
DDE	dichlorodiphenyl dichloroethylene
DDT	dichlorodiphenyl trichloroethane
DISC	death-inducing signalling complex
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
$\Delta\psi_m$	mitochondrial membrane potential

EDTA	ethylenediaminetetraacetic acid
EMEA	European Medicines Agency
EMEM	Eagle's minimum essential medium
EROD	ethoxyresorufin-*O*-deethylase
$FADH_2$	flavin adenine dinucleotide (reduced)
FCS	foetal calf serum
Fe^{2+}	iron(II)
Fe^{3+}	iron(III)
g	gram
g	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_2O	water
H_2O_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_2PO_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 *in vitro* 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
n	sample size
Na^+	sodium
NAC	*N*-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-*p*-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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Changes in $\Delta\psi_m$ in HepG2 cells following a 1 h exposure to various concentrations of DDT, DDE and DDD relative to untreated controls. Dark green bars represent cells exposed to test compounds alone and light green bars those with a 1 h pre-treatment with NAC (mean ± SEM). * = ***p*** < 0.05, ** = ***p*** < 0.01 and *** = ***p*** < 0.001 as determined by Mann-Whitney tests. Dashed horizontal lines represent Y = 100%.

Active caspase-3 in HepG2 cells following 6 h exposure to Control *vs.* Staurosporine (11 µM) (mean ±SEM). Staurosporine significantly induced caspase-3 activity with ***p*** < 0.001 (***).

Caspase-3 activity in HepG2 cells following 6 h exposure to DDT (A), DDE (B) and DDD (C) (mean ±SEM). Caspase-3 activity was used as a measure of cell death by apoptosis. Graphs (D), (E) and (F) represent the PI staining of cells exposed to DDT, DDE and DDD, respectively. Propidium iodide was used as a measure of membrane integrity and cell death by necrosis. Dashed horizontal lines represent untreated control values. Results are given as mean ± SEM. ** indicates ***p*** < 0.01 and *** ***p*** < 0.001 as determined by Mann-Whitney and Student's ***t***-tests, where applicable.

Propidium iodide staining as a measure of membrane integrity in HepG2 cells following treatment with 0.5% (v/v) Triton X-100 (TX-100) (mean ±SEM). *** = ***p*** < 0.001.

Caspase-3 activity in HepG2 cells following 6 h exposure to DDT (A), DDE (B) and DDD (C) (mean ±SEM). Graphs (D), (E) and (F) represent the PI staining of cells exposed to DDT, DDE and DDD, respectively. Caspase-3 activity and propidium iodide were used as a measure of cell death by apoptosis and necrosis, respectively. Dashed horizontal lines represent Control values. Results are given as mean ± SEM. * indicates ***p*** < 0.05, ** indicates ***p*** < 0.01 and *** ***p*** < 0.001 as determined by

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