Annexure A: Ethical approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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Faculty of Health Sciences Research Ethics Committee

DATE: 23/06/2011

TO:
Prof V Steenkamp
Dept of Pharmacology

Best Prof V Steenkamp

RE.: Commercial Lines: The use of Commercial lines ~ Mr J J van Tonder

During the meeting held on 22/06/2011, the use of Commercial Lines were discussed.

The Faculty of Health Science Ethics Committee approved the use of the cell lines for the various assays.

With regards

Dr R Sommers; MBChB; MMed (Int); MPharMed.
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

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Annexure B: Reagents

Bovine foetal calf serum (FCS)

FCS was procured from PAA (Pasching, Austria). Any host serum complement that may have been present was inactivated prior to use by incubation at 56□C for 45 min (Soltis *et al.*, 1979). This was added to cell culture media to a concentration of 10%.

Penicillin/streptomycin

A solution containing 10 000 U of penicillin and 10 000 µg streptomycin per millilitre was supplied by BioWhittaker (Walkersville, USA). This was added to the culture medium to a concentration of 1%.

Eagle's Minimum Essential Medium (EMEM)

EMEM powdered medium was purchased from Sigma-Aldrich (St Louis, USA). A mass of 48 g of medium powder was dissolved in 5 l sterile, deionised water. To this solution 11 g of sodium bicarbonate was added to adjust the pH to 7.4. The solution was filter sterilized twice using 0.22 µm cellulose acetate filters, dispensed into sterile 500 ml bottles, supplemented with 1% penicillin/streptomycin and stored at 4°C.

Medium was fortified with 10% (v/v) FCS prior to use.

Phosphate buffered saline (PBS)

PBS powder was obtained from BD Biosciences (Sparks, USA). A solution containing 9.23 g/l of purchased PBS powder in de-ionised water was prepared and stored at 4°C until use. Prior to use, the solution was filter-sterilised through a 0.22 µm pore size filter.

Trypsin/Versene

A trypsin/versene solution containing 0.25% (w/v) trypsin and 0.1% (w/v) ethylenediaminetetraacetic acid (EDTA) in Ca^{2+} and Mg^{2+} free PBS was obtained from Highveld Biological (Johannesburg, RSA) and stored at 4°C.

Dimethyl sulfoxide (DMSO)

DMSO was procured from Sigma-Aldrich (St Louis, USA) and used undiluted.

Trypan blue counting solution

Trypan blue in powder form was purchased from BDH Laboratories Supplies (UK). Powder (200 mg) was dissolved in 50 ml PBS to obtain a 0.4% (w/v) solution, which was then filtered through a 0.45 µm syringe filter to remove any insoluble particles.

Culturing consumables

Cellstar tissue culture flasks (25 cm^2 and 75 cm^2) were purchased from Greiner BioOne (Austria). For experimental assays, 96 well sterile white plates suitable for fluorescence were used (Nunc, Denmark).

Hepatotoxins

DDT, DDE and DDD were purchased from Supelco Analytical (Bellefonte, USA). Stock solutions (30 mM) of each toxin was prepared in DMSO and stored at -70°C.

Hepatoprotective agent

NAC was purchased from Sigma-Aldrich (St Louis, USA). A 30 mM stock solution was prepared in DMSO and stored at -70°C.

Neutral red dye

Neutral red dye was purchased from Sigma-Aldrich (St Louis, USA) and prepared fresh prior to use. The dye was made up to a final concentration of 100 µg/ml in EMEM, without FCS. To aid solubility, the solution was sonicated for 1 min, after which the pH was adjusted to 6.4 using 5 M KH_2PO_4 (Merck , Darmstadt, Germany).

Neutral red elution buffer

Elution buffer, used to dissociate bound dye, contained ethanol (Ilovo, Durban, RSA), acetic acid (Merck, Darmstadt, Germany) and distilled water in a ratio of 50:1:49 (v/v/v), respectively.

7-Ethoxyresorufin

7-Ethoxyresorufin (7-ER), NADPH and omeprazole were purchased from Sigma-Aldrich (St Louis, USA). A 100 µM 7-ER stock solution was prepared in 100% methanol. Aliquots were stored at -70°C. NADPH working solution was prepared fresh in PBS prior to use.

Omeprazole

Omeprazole stock solution (30 mM) was prepared in DMSO. Aliquots were stored at -70°C.

2',7'-Dichlorofluorescein diacetate (DCFDA)

DCFDA in powder form was procured from Sigma-Aldrich (St Louis, USA). A 1 mg/ml stock solution (2 mM) was prepared in methanol (Merck, Darmstadt, Germany) and stored at -20°C. Prior to use, the stock solution was diluted to 20 µM in PBS, 40 µl of which was added to 100 µl of medium to obtain a final concentration of 5.7 µM in each of the relevant wells.

2',2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH)

AAPH in powder form was purchased from Sigma-Aldrich (St Louis, USA). An 8 mg/ml stock solution (30 mM) was prepared in DMSO and stored at -70°C. Prior to use, the stock solution was diluted to 300 µM in PBS, 50 µl of which was added to 50 µl of PBS in each well to obtain a final concentration of 150 µM.

JC-1

JC-1 in powder form was purchased from Sigma-Aldrich (St Louis, USA). A 1 mM stock solution was prepared in DMSO and stored at -70°C. Prior to use, the stock solution was diluted to 20 µM in EMEM. Of this working solution a 100 µl was added to the relevant wells to load cells prior to exposure. Cells were loaded in a final concentration of 10 µM for 30 min at 37°C.

Tamoxifen

Tamoxifen, which causes mitochondrial membrane hyperpolarisation in HepG2 cells, was used as positive control (Donato *et al.*, 2009). Tamoxifen citrate in powder form was obtained from Sigma-Aldrich (St Louis, USA). A 30 mM stock solution was prepared in DMSO and stored at -70°C. Prior to use, the stock solution was diluted to 300 µM in PBS of which 50 µl was added to 50 µl of PBS to obtain a final concentration of 150 µM in each well.

Propidium iodide (PI)

PI in powder form was purchased from Sigma-Aldrich (St Louis, USA). A 3 mM stock solution was prepared in PBS and stored at 4°C.

t-Octylphenoxypolyethoxyethanol (Triton X-100)

Triton X-100 in liquid form was obtained from Sigma-Aldrich (St Louis, USA). Prior to use, Triton X-100 was diluted to 1% (v/v) in EMEM and stored at 4°C.

Caspase assay lysis buffer (pH 7.4)

The lysis buffer consisted of 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), 2mM 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM phenylmethylsulfonyl fluoride (All purchased from Sigma Aldrich, St Louis, USA) and 5 mM β-mercaptoethanol (Merck, Darmstadt, Germany). Phenylmethylsulfonyl fluoride and β-mercaptoethanol were added 30 min prior to use of the buffer.

Caspase assay buffer (pH 7.4)

The buffer consisted of 20 mM HEPES, 2 mM EDTA, 5 µM Cas-3-specific substrate coupled to a fluorescent probe (Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC)) (All purchased from Sigma Aldrich, St Louis, USA) and 5 mM β-mercaptoethanol (Merck, Darmstadt, Germany). β-mercaptoethanol was added 30 min and Ac-DEVD-AMC immediately prior to initiating the assay.

Staurosporine

Staurosporine in powder form was purchased from Sigma-Aldrich (St Louis, USA). A 1 mg/ml (2.14 mM) stock solution was prepared in DMSO and stored at -70°C. Prior to use, the stock solution was diluted into EMEM to obtain a 21 µM working solution, which yielded a final concentration of 11 µM in the assay well.

Annexure C: Research outputs

National conference presentation:

Van Tonder JJ, Cromarty AD, Gulumian M, Steenkamp V. Development of an *in vitro* toxicity screening model using hepatocytes. South African Congress of Pharmacology and Toxicology, Cape Town. 3-6 October 2010. (Oral presentation)

International conference presentation:

Van Tonder JJ, Cromarty AD, Gulumian M, Steenkamp V. A microplate method for multiparametric hepatotoxicity screening. 6th International Conference of Pharmaceutical and Pharmacological Sciences. Durban, South Africa, 25-27 September 2011. (Oral presentation)

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