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Isoproturon

Isoproturon Tech – Enzyme and Cell Proliferation Induction in Cultured Male Wild Type Rat Hepatocytes, Male CAR -/- Rat Hepatocytes and Male Human Hepatocytes

Report

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AUTHOR(S): [redacted]

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PERFORMING LABORATORY: CXR Biosciences
2 James Lindsay Place,
Dundee Technopole
Dundee, DD1 5JJ
Scotland, UK

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PO Box 262, Northern Industrial Zone
Ashod 77102
Israel

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This study was not conducted according to Good Laboratory Practice Standards as defined by 40 CFR part 160. No claim of GLP compliance was made for this study.

Author [redacted] 03 November 2014
Date

Sponsor [redacted] 05 Nov 2014
Date

Submitter _____

Date

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QUALITY ASSURANCE STATEMENT

This study has not been subjected to any study specific Quality Assurance procedures. Study activities at the Test Facility were conducted according to relevant SOPs. There were no deviations from the protocol, or from company procedures, to affect the quality, integrity or achievement of the study objectives.

GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Director
	Senior Scientific Officer
	Director
	Sponsor's Representative

Study dates

Study initiation date: 26th May 2014
Experimental start date: 26th May 2014
Experimental termination date: 16th September 2014

Deviations from the guidelines

N/A

Retention of samples

The data was held in a Study File according to CXR Biosciences SOPs 0003 and 0005.

Other

On completion of the study, the following items will be retained in the CXR Archive for 2 years, then in the CXR outsourced archive for 8 years:

- Study protocol and possible amendments
- Study Report and possible amendments
- Study File
- Relevant correspondence

All items will be kept for ten years from the date of signing the Study Report. At the end of the ten-year period, the Study Sponsor will be contacted and the items either sent to the Study Sponsor or retained by CXR Biosciences under a separate agreement.

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

Chronic peroral exposure of rats to Isoproturon technical (IPU) led to increased incidences of hepatocellular tumours. The observed tumour profile- and histopathology, together with information on liver toxicity from other apical studies, suggested that a receptor-mediated rodent-specific mode of action is the cause of the rat liver tumours: sustained liver activation as a consequence of binding to the constitutive androstane receptor (CAR), leading to hyperplasia and finally tumours.

This mode of action study investigated the ability of Isoproturon technical to induce typical CAR-dependent cytochrome P450 activity (CYP2B/3A isoforms) and cell proliferation in hepatocyte cultures. This study compared isolated primary male wild type (WT) Sprague Dawley rat hepatocyte cultures, male CAR -/- (CAR KO) rat hepatocyte cultures and male human hepatocyte cultures. Phenobarbital sodium salt (PB) and epidermal growth factor (EGF) were included as positive controls.

Hepatocytes were exposed to vehicle (0.1% dimethyl sulfoxide), IPU at 4 concentrations (0.5, 5, 50 and 500 µM) or PB at 1 concentration (500 µM) for 96 h. 500 µM IPU was the highest technically achievable concentration (limit of solubility). There were 3 replicates for each concentration in 25 cm^2 flasks for CYP2B/3A activity measurements (pentoxyresorufin-*O*-depentylation [PROD] and benzyloxyquinoline-*O*-debenzylation [BQ]), 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity measurements (measured as the change in cellular adenosine-5'-triphosphate [ATP]).

1.2 Results

Treatment with IPU up to the solubility limit resulted in no cytotoxicity in WT or CAR KO rat hepatocytes. The highest test concentration (500 µM IPU) was cytotoxic to human hepatocytes.

Treatment with 500µM IPU resulted in statistically significant increases in PROD activity in WT and CAR KO rat hepatocytes to 1.5- and 2.7-fold control, respectively. There were no statistically significant increases in PROD activity following treatment with IPU at any concentration in human hepatocytes.

Treatment with 0.5, 5 and 50 µM IPU resulted in statistically significant decreases in PROD activity in WT rat hepatocytes to 46.7, 56.4 and 59.9% of control respectively. In human hepatocytes treatment with 0.5 and 5 µM IPU resulted in statistically significant decreases in PROD activity to 72.3 and 74.5% of control respectively.

Treatment with 0.5 µM IPU resulted in a statistically significant increase in BQ activity in WT rat hepatocytes to 1.4-fold control, whilst treatment with 500 µM IPU resulted in a

statistically significant increase in BQ activity in CAR KO rat hepatocytes to 3.5-fold control and treatment with 50 µM IPU resulted in a statistically significant increase in BQ activity in human hepatocytes to 1.8-fold control.

Statistically significant increases in cell proliferation were noted at all concentrations of IPU tested in WT rat hepatocytes. No statistically significant increases in cell proliferation were noted at any concentration of IPU tested in CAR KO rat hepatocytes, but a statistically significant decrease was seen following treatment with 500µM IPU to 43.1% of control. There were no statistically significant changes in cell proliferation following treatment with IPU at any concentration in human hepatocytes.

The expected effects were observed for both positive control compounds indicating that the experimental system responded as expected.

1.3 Conclusion

Responsiveness of the test systems (wild-type rat, CAR-knockout rat and human hepatocyte cultures) to enzyme induction and cell proliferation was confirmed by response to concurrent positive controls in the assay. Responses were in the range of the laboratory's historical controls.

Treatment of cultured male WT Sprague Dawley rat hepatocytes with IPU resulted in increased CYP2B/3A activities and increased cell proliferation.

Treatment of cultured male CAR KO rat hepatocytes with IPU resulted in increased CYP2B/3A activities, but no increase in cell proliferation.

Treatment of cultured male human hepatocytes with IPU resulted in no increase in CYP2B activity, a very slight increased CYP3A activity, but no cell proliferation.

	Rat Hepatocytes		Human Hepatocytes
	Wild-type Rat	CAR Knockout Rat	
CYP2B/3A activities	↑	↑	(↑)
Cell Proliferation	↑	-	-

These data suggest that Isoproturon technical (IPU) increases the incidence of hepatocellular tumours in rats *via* a receptor-mediated rodent-specific mode of action involving the sustained activation of the hepatic constitutive androstane receptor (CAR), leading to hyperplasia and finally tumours (Elcombe *et al.*, 2014). The increased cell proliferation is the essential key event in this mode of action and in its absence hepatocarcinogenesis will not occur. Hence, the lack of IPU-stimulated hepatocellular proliferation in human hepatocytes suggests that IPU poses no liver tumour hazard to humans.

2.0 INTRODUCTION

This study investigated the ability of Isoproturon tech (IPU) to induce cytochrome P450 (CYP) activity (CYP2B/3A isoforms) and cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) in hepatocyte cultures. This study compared isolated male wild type (WT) Sprague Dawley rat hepatocyte cultures, male CAR KO rat hepatocyte cultures and male human hepatocyte cultures. Phenobarbital sodium salt (PB) and epidermal growth factor (EGF) were included as positive controls.

3.0 MATERIALS AND METHODS

3.1 Test Substances

Isoproturon was supplied by Agan Chemical Manufacturers Ltd.

Test substance name:	Isoproturon (IPU)
Chemical name:	3-(4-isopropylphenyl)-1,1-dimethylurea
Description:	white/off-white powder
Batch number:	D8837
Production date:	09 March 2014
Expiry date:	09 March 2017
Purity:	98.5% w/w
Molecular weight:	206.3
Storage conditions:	Room temperature (20-25°C) in the dark and protected from moisture

A certificate of analysis for IPU was obtained from the sponsor, accepted by CXR and is presented in Appendix 1.

The reference item phenobarbital sodium salt (PB, P-5178) was supplied by Sigma.

Product name:	Phenobarbital sodium salt
Product number:	P5178
Description:	white powder
Batch number:	SLBJ3684V
Quality release date:	10 December 2013
Retest date:	July 2015
Purity:	99.9%
Molecular weight:	254.22
Storage conditions:	Room temperature (20-25°C) in the dark and protected from moisture

A certificate of analysis for PB is presented in Appendix 1.

The reference item epidermal growth factor (EGF, E-9644) was supplied by Sigma.

Product name:	Epidermal growth factor
Product number:	E9644
Description:	white powder
Lot number:	011M4009V
Quality release date:	23 December 2010
Retest date:	December 2012 (when not reconstituted – see product information sheet in Appendix 1)
Purity:	99%
Bioactivity:	EC50: 0.258ng/ml
Storage conditions:	After reconstitution, for extended storage, working aliquots stored at approximately -70°C

A certificate of analysis for EGF is presented in Appendix 1.

3.2 Animals and Preparation of Hepatocyte Cultures

Male Sprague Dawley WT and CAR KO rats (product number TGRSWM4(SD), product number TGRS8190HM4 (CAR KO) approximately 300 g, age 10 weeks at hepatocyte isolation) were obtained from Sage Laboratories, Boyertown, PA 19512. The rats were allowed *ad libitum* access to water for human consumption and RM1 pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) *ad libitum* and were acclimatised for at least 5 days prior to study. Rats were terminally anaesthetised, using *i.p.* administered Euthatal™ at 150 mg/kg bodyweight, and hepatocytes isolated by *in situ* perfusion according to the method of Mitchell *et al*. (1984) using Krebs Ringer Phosphate and Krebs Ringer Hydrogen Carbonate buffers and 200mg/L collagenase A (Roche, 0.286U/mg). Viabilities of the hepatocyte preparations, determined by trypan blue exclusion, were in excess of 80% for all preparations. Hepatocytes from two independent perfusions for each strain were pooled. Primary monolayer cultures of hepatocytes were prepared in 25 cm^2 flasks, 96- and 6-well plastic tissue culture plates in Leibowitz Complete L15 (CL15) medium (Mitchell *et al*. 1984; Elcombe and Mitchell 1986). This medium incorporates foetal calf serum (8.3%), tryptose phosphate broth (8.3%), penicillin/streptomycin (41 IU/ml / 41µg/ml), insulin (1µM), hydrocortisone (10µM) and L-glutamine (240µg/ml). In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

3.3 Human Hepatocyte Cultures

Primary male human hepatocytes, plateable cryopreserved, were sourced from Invitrogen, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR. Viabilities of the hepatocyte preparation, determined by trypan blue exclusion, were in excess of 70%. Hepatocytes from one donor were used.

3.4 Treatment of Hepatocyte Cultures

Rat hepatocytes were cultured in Leibowitz CL15 medium for 4 h (to allow for adhesion) before the medium was removed and replaced with medium containing either IPU at one of 4 concentrations (0.5, 5, 50 and 500 µM), PB at 500 µM or vehicle (0.1% [v/v] dimethyl sulfoxide [DMSO]) alone. The concentrations of IPU used were selected with reference to a preliminary range finding study (Elcombe 2014, CXR1378), where 500 mM IPU was the limit of solubility in DMSO. 500 µM was therefore selected as a suitable top concentration for the current study. The total duration of exposure to IPU, PB or DMSO, was 96 h, with daily medium changes. There were 3 replicates for each concentration in 25 cm^2 flasks for PROD and BQ activities, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (proliferation) analysis and 6 replicates for each concentration in 96-well plates for ATP measurements.

Primary monolayer cultures of human hepatocytes were cultured on collagen coated 25 cm^2 flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz HCL15 (Mitchell *et al* 1984) as the medium. In all 96-well plate cultures the outside wells were not used, but filled with sterile phosphate buffered saline.

Human hepatocytes were resuscitated in Cryopreserved Hepatocyte Recovery Medium (CHRM®) then cultured in Cryopreserved Hepatocyte Plating Medium (CHPM®) for 6 h to allow adherence. The medium was then removed and replaced with Leibowitz HCL15 medium containing either IPU at one of 4 concentrations (0.5, 5, 50 and 500 µM), PB at 500 µM) or vehicle (0.1% [v/v] dimethyl sulfoxide [DMSO]) alone, as per the rat hepatocyte cultures. The total duration of exposure to IPU, PB or DMSO, was 96 h, with daily medium changes.

In addition to hepatocytes exposed to IPU or phenobarbital sodium salt, 5 replicates in 6-well plates were exposed to EGF (25 ng/mL) as a positive control for induction of replicative DNA synthesis (S-phase).

After 96 h in culture, hepatocytes were either fixed using methanol at approximately -20°C (for assessment of replicative DNA synthesis as described in section 3.5.2) or harvested (for assessment of PROD and BQ activities as described in sections 3.5.3 and 3.5.4, respectively) by scraping them into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH7.4), sonicating the mixture and storing it at approximately –70°C until analysis. Protein in hepatocytes scraped into SET buffer was determined by the method of Lowry *et al*. (1951) using bovine serum albumin standards.

3.5 Assays

3.5.1 Adenosine 5'-triphosphate (ATP)

The bioluminescent determination of ATP released from viable somatic cells was carried out using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay) according to manufacturers' instructions.

3.5.2 Replicative DNA synthesis (S-phase)

The number of hepatocytes undergoing replicative DNA synthesis (S-phase of the cell cycle) was determined immunocytochemically following the incorporation of 5-bromo-2-deoxyuridine (BrdU, 10 µM) into hepatocyte nuclei over the last 72 h of culture. Immunostaining was performed after fixation at 96 h. Data are expressed as a labelling index (% of total hepatocytes that have incorporated BrdU). Epidermal growth factor (EGF; 25 ng/mL, n=5) was included as a positive control. Approximately 1100 hepatocytes were manually counted per replicate for each concentration.

3.5.3 Pentoxyresorufin-O-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, in the presence of NADPH, as described by Burke *et al*. (1985).

3.5.4 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of CYP3A in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, in the presence of NADPH, as described by GENTEST HTS technical bulletin.

3.6 Data Evaluation

Statistical comparisons between treated and control groups were undertaken for all numerical data sets. The statistical analyses performed are documented with the results.

4.0 RESULTS

4.1 Cytotoxicity assessment

Treatment with 500 µM IPU (the highest tested concentration) resulted in no cytotoxicity in WT or CAR KO rat hepatocytes. Treatment with 500 µM IPU resulted in cytotoxicity in human hepatocytes, with intracellular ATP levels being statistically significantly reduced to 46.4% of control. PB did not induce cytotoxicity.

4.2 Pentoxyresorufin-*O*-depentylation (PROD)

Treatment with 500 µM IPU resulted in statistically significant increases in PROD activity in WT and CAR KO rat hepatocytes to 1.5- and 2.7-fold control, respectively. There were no statistically significant increases in PROD activity following treatment with IPU at any concentration in human hepatocytes.

Treatment with 0.5, 5 and 50 µM IPU resulted in statistically significant decreases in PROD activity in WT rat hepatocytes to 46.7, 56.4 and 59.9% of control respectively. In human hepatocytes treatment with 0.5 and 5 µM IPU resulted in statistically significant decreases in PROD activity to 72.3 and 74.5% of control respectively.

Treatment with 500 µM PB resulted in statistically significant increases in PROD activity to 2.2-fold control in WT rat hepatocytes only.

4.3 Benzyloxyquinoline-*O*-debenzylation (BQ)

Treatment with 0.5 µM IPU resulted in a statistically significant increase in BQ activity in WT rat hepatocytes to 1.4-fold control, whilst treatment with 500 µM IPU resulted in a statistically significant increase in BQ activity in CAR KO rat hepatocytes to 3.5-fold control and treatment with 50 µM IPU resulted in a statistically significant increase in BQ activity in human hepatocytes to 1.8-fold control.

Treatment with 500 µM PB resulted in statistically significant increases in BQ activity in WT rat and human hepatocytes to 2.0- and 4.1-fold control, respectively.

4.4 Replicative DNA synthesis (S-phase)

Treatment with EGF (25 ng/mL) resulted in statistically significant increases in replicative DNA synthesis in WT, CAR KO and human hepatocytes to 4.2- 3.6- and 9.4-fold control respectively, indicating that the hepatocytes could proliferate following exposure to proliferative stimuli and therefore demonstrating their suitability for use in investigations involving assessing induction of proliferation.

Treatment of WT rat hepatocytes with 0.5, 5, 50 and 500 µM IPU resulted in statistically significant increases in replicative DNA synthesis to 1.4-, 3.8-, 4.7- and 3.5-fold control, respectively. Treatment of CAR KO rat hepatocytes and human hepatocytes did not increase the labelling index.

Treatment with PB resulted in an increase in replicative DNA synthesis of 1.8-fold control in WT rat hepatocytes only.

5.0 DISCUSSION AND CONCLUSIONS

Treatment of cultured male WT Sprague Dawley rat hepatocytes with IPU resulted in increased cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and increased CYP2B/3A activities (measured as PROD and BQ activity).

Treatment of cultured male CAR KO rat hepatocytes with IPU resulted in no increase in cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) but increased CYP2B/3A activities (measured as PROD and BQ activity).

Treatment of cultured male human hepatocytes with IPU resulted in no increase in cell proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]), no increase in CYP2B activity (measured as PROD activity) but a very slightly increased CYP3A activity (measured as BQ activity).

Data Summary.

RAT	**PROD % control**		**BQ % control**		**S-phase % control**	
	WT	KO	WT	KO	WT	KO
DMSO	100	100	100	100	100	100
PB 500	**224****	121	**200*****	108	**179*****	99
IPU 0.5	47**	65	**145****	92	**144****	85
IPU 5	56*	80	118	96	**376*****	96
IPU 50	60*	110	87	88	**466*****	95
IPU500	**148***	**266****	115	**353*****	**350*****	43***
EGF	-	-	-	-	**418*****	**356*****
HUMAN	**PROD % control**		**BQ % control**		**S-phase % control**	
DMSO	100		100		100	
PB 500	101		**411*****		87	
IPU 0.5	72*		108		92	
IPU 5	75*		116		107	
IPU 50	84		**176*****		86	
IPU500	79		95		50	
EGF	-		-		**939*****	

*statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$. Grey boxes are increases.

These data suggest that Isoproturon technical (IPU) increases the incidence of hepatocellular tumours in rats *via* a receptor-mediated rodent-specific mode of action involving the sustained activation of the hepatic constitutive androstane receptor (CAR), leading to hyperplasia and finally tumours (Elcombe *et al.*, 2014). The increased cell proliferation is the essential key event in this mode of action and in its absence hepatocarcinogenesis will not occur. Hence, the lack of IPU-stimulated hepatocellular proliferation in human hepatocytes suggests that IPU poses no liver tumour hazard to humans.

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TABLES SECTION

TABLE 1 Biochemical Parameters in Cultured WT Male Rat Hepatocytes

Treatment	ATP content (luminescence units)[a]	S-phase labelling index (%) [b]	PROD (pmol resorufin/min/mg) [c]	BQ (nmol 7-OH quinoline/min/mg) [c]
Vehicle control (0.1% [v/v] DMSO)	100672 ± 5810[d] (100.0 ± 5.8)	6.84 ± 0.20 (100.0 ± 2.9)	0.5743 ± 0.1073 (100.0 ± 18.7)	0.0286 ± 0.0032 (100.0 ± 11.3)
PB 500 µM	99552 ± 3712 (98.9 ± 3.7)	12.21 ± 0.68*** (178.5 ± 9.9)	1.2837 ± 0.1260** (223.5 ± 21.9)	0.0571 ± 0.0017*** (199.6 ± 6.0)
Isoproturon 0.5 µM	95047 ± 6042 (94.4 ± 6.0)	9.82 ± 1.73** (143.5 ± 25.3)	0.2683 ± 0.0238** (46.7 ± 4.1)	0.0414 ± 0.0021** (144.6 ± 7.4)
Isoproturon 5 µM	102648 ± 4220 (102.0 ± 4.2)	25.73 ± 5.39*** (376.2 ± 78.8)	0.3240 ± 0.0986* (56.4 ± 17.2)	0.0337 ± 0.0030 (117.7 ± 10.5)
Isoproturon 50 µM	110442 ± 1847*** (109.7 ± 1.8)	31.91 ± 1.98*** (466.4 ± 28.9)	0.3438 ± 0.0298* (59.9 ± 5.2)	0.0249 ± 0.0029 (87.0 ± 10.2)
Isoproturon 500 µM	99135 ± 2964 (98.5 ± 2.9)	23.97 ± 3.47*** (350.4 ± 50.7)	0.8512 ± 0.1226* (148.2 ± 21.4)	0.0328 ± 0.0232 (114.6 ± 81.2)
EGF 25 ng/mL	-	28.60 ± 2.30*** (418.1 ± 33.6)	-	-

Values are Mean ± SD. Values in parenthesis are mean % control ± SD. [a] n = 6 per group, [b] n = 5 per group, [c] n = 3 per group, [d] n = 18 per group.

A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 2 Biochemical Parameters in Cultured CAR KO Male Rat Hepatocytes

Treatment	ATP content (luminescence units)[a]	S-phase labelling index (%) [b]	PROD (pmol resorufin/min/mg) [c]	BQ (nmol 7-OH quinoline/min/mg) [c]
Vehicle control (0.1% [v/v] DMSO)	95687 ± 7442[d] (100.0 ± 7.8)	7.99 ± 1.27 (100.0 ± 15.9)	0.2451 ± 0.0253 (100.0 ± 10.3)	0.0250 ± 0.0025 (100.0 ± 10.0)
PB 500 µM	105500 ± 4431*** (110.3 ± 4.6)	7.91 ± 1.30 (99.0 ± 16.2)	0.2959 ± 0.0946 (120.7 ± 38.6)	0.0268 ± 0.0013 (107.5 ± 5.3)
Isoproturon 0.5 µM	93490 ± 9768 (97.7 ± 10.2)	6.79 ± 1.69 (85.0 ± 21.2)	0.1597 ± 0.0736 (65.2 ± 30.0)	0.0231 ± 0.0038 (92.4 ± 15.3)
Isoproturon 5 µM	102466 ± 4735* (107.1 ± 4.9)	7.68 ± 2.21 (96.1 ± 27.7)	0.1959 ± 0.0375 (79.9 ± 15.3)	0.0241 ± 0.0016 (96.3 ± 6.4)
Isoproturon 50 µM	109497 ± 4065*** (114.4 ± 4.2)	7.62 ± 0.64 (95.3 ± 8.0)	0.2687 ± 0.0611 (109.6 ± 24.9)	0.0219 ± 0.0017 (87.8 ± 6.7)
Isoproturon 500 µM	105341 ± 3887*** (110.1 ± 4.1)	3.44 ± 0.51*** (43.1 ± 6.3)	0.6522 ± 0.1920** (266.1 ± 78.4)	0.0882 ± 0.0092*** (353.1 ± 36.8)
EGF 25 ng/mL	-	28.47 ± 1.55*** (356.4 ± 19.4)	-	-

Values are Mean ± SD. Values in parenthesis are mean % control ± SD. [a] n = 6 per group, [b] n = 5 per group, [c] n = 3 per group, [d] n = 18 per group.

A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

TABLE 3 Biochemical Parameters in Cultured Male Human Hepatocytes

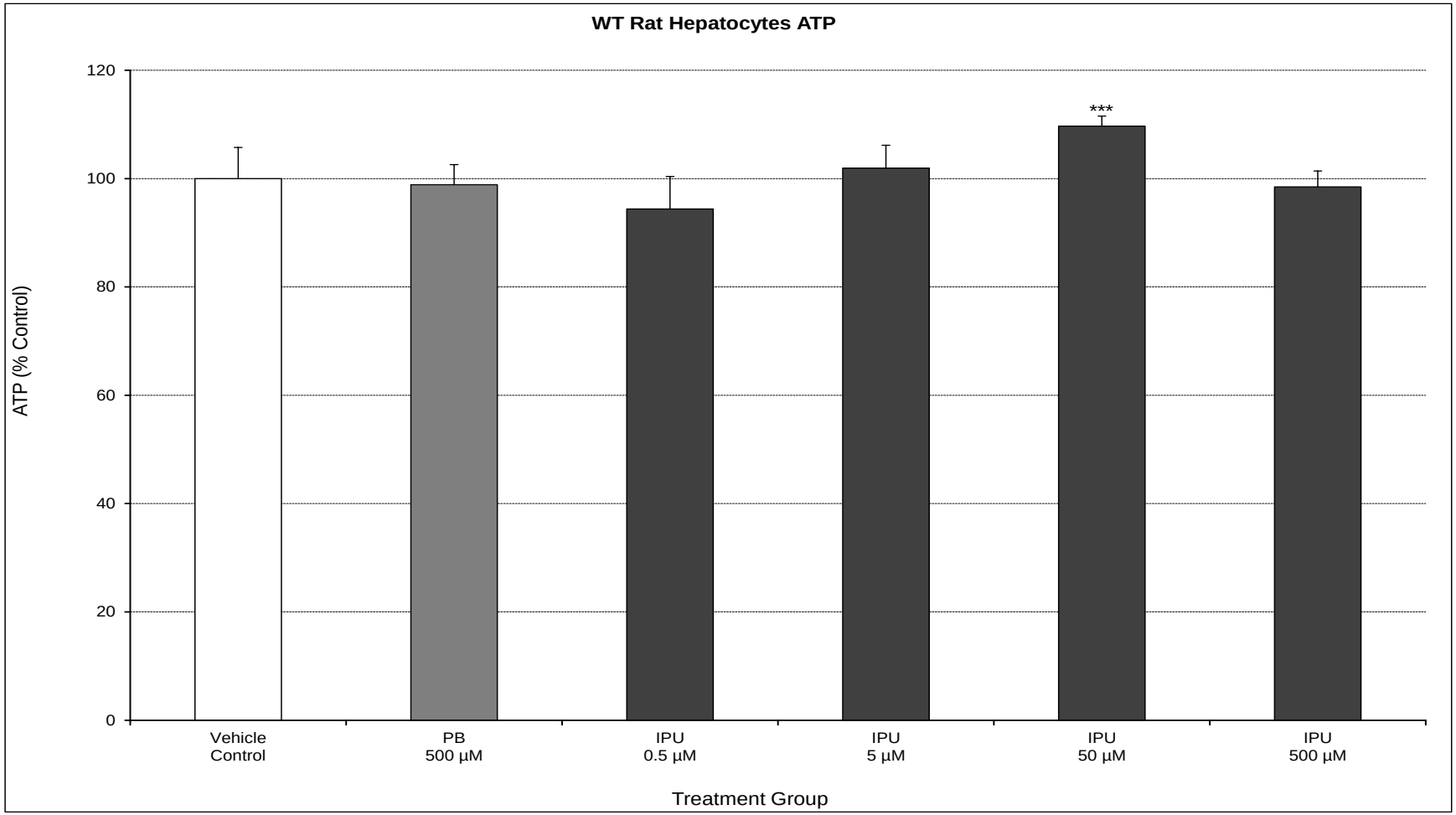
Treatment	ATP content (luminescence units)[a]	S-phase labelling index (%) [b]	PROD (pmol resorufin/min/mg) [c]	BQ (nmol 7-OH quinoline/min/mg) [c]
Vehicle control (0.1% [v/v] DMSO)	93054 ± 11514 (100.0 ± 12.4)	0.32 ± 0.14 (100.0 ± 43.9)	0.1762 ± 0.0372 (100.0 ± 21.1)	0.0457 ± 0.0051 (100.0 ± 11.1)
PB 500 µM	88374 ± 5131 (95.0 ± 5.5)	0.27 ± 0.08 (86.6 ± 23.9)	0.1770 ± 0.0484 (100.5 ± 27.5)	0.1874 ± 0.0199*** (410.5 ± 43.7)
Isoproturon 0.5 µM	91691 ± 5569 (98.5 ± 6.0)	0.29 ± 0.08 (91.6 ± 25.4)	0.1273 ± 0.0117* (72.3 ± 6.7)	0.0492 ± 0.0027 (107.8 ± 5.9)
Isoproturon 5 µM	97686 ± 4492 (105.0 ± 4.8)	0.34 ± 0.07 (107.2 ± 21.2)	0.1312 ± 0.0067* (74.5 ± 3.8)	0.0531 ± 0.0069 (116.3 ± 15.1)
Isoproturon 50 µM	83087 ± 9887 (89.3 ± 10.6)	0.27 ± 0.11 (85.5 ± 33.1)	0.1483 ± 0.0239 (84.2 ± 13.6)	0.0804 ± 0.0092*** (176.1 ± 20.1)
Isoproturon 500 µM	43155 ± 4890*** (46.4 ± 5.3)	0.16 ± 0.10 (49.6 ± 32.0)	0.1391 ± 0.0187 (78.9 ± 10.6)	0.0432 ± 0.0044 (94.6 ± 9.6)
EGF 25 ng/mL	-	2.98 ± 0.29*** (938.7 ± 90.1)	-	-

Values are Mean ± SD. Values in parenthesis are mean % control ± SD. [a] n = 6 per group, [b] n = 5 per group, [c] n = 3 per group, [d] n = 18 per group.

A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

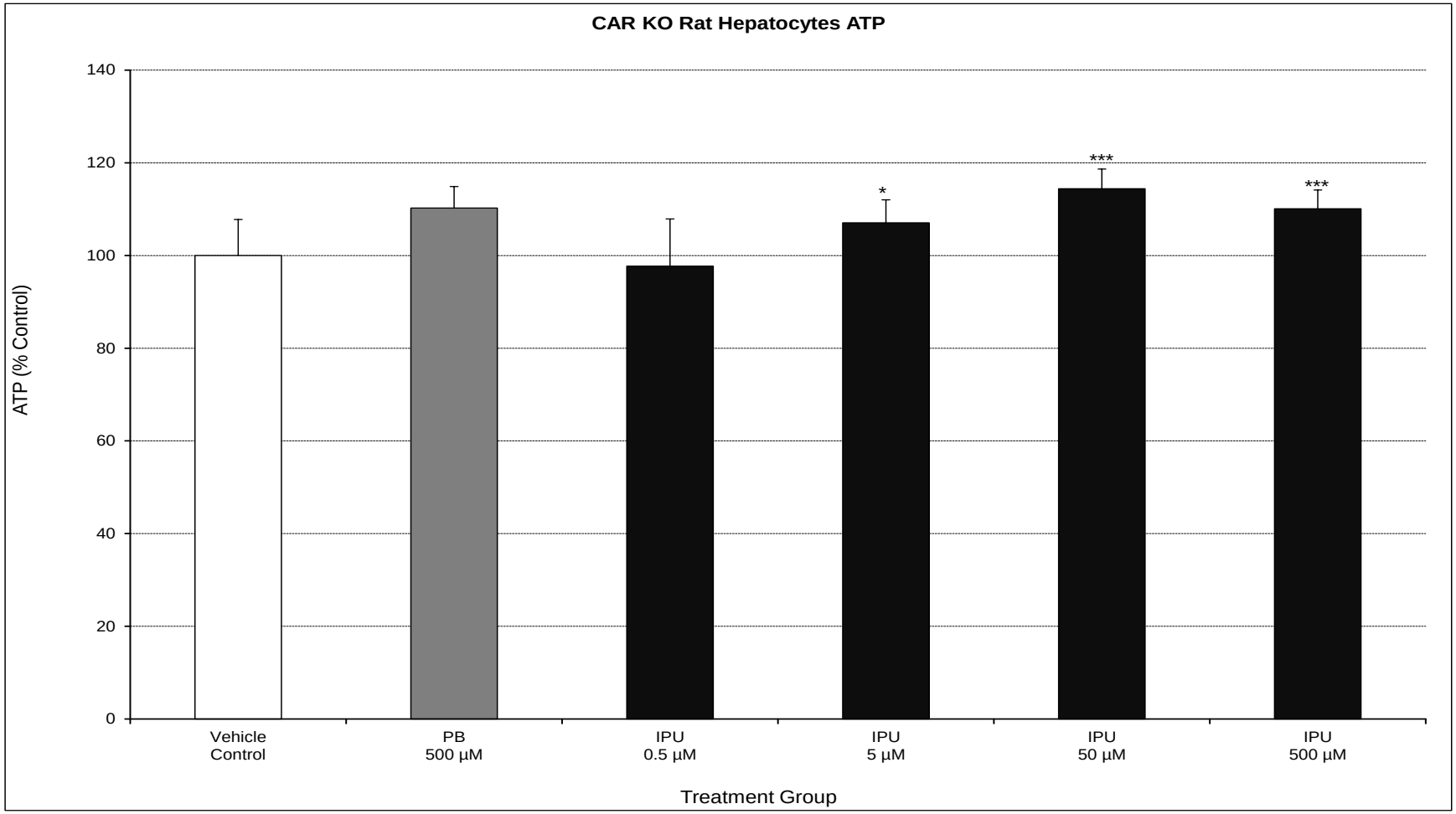
FIGURES SECTION

FIGURE 1 Adenosine 5'-Triphosphate (ATP) WT Male Rat Hepatocytes



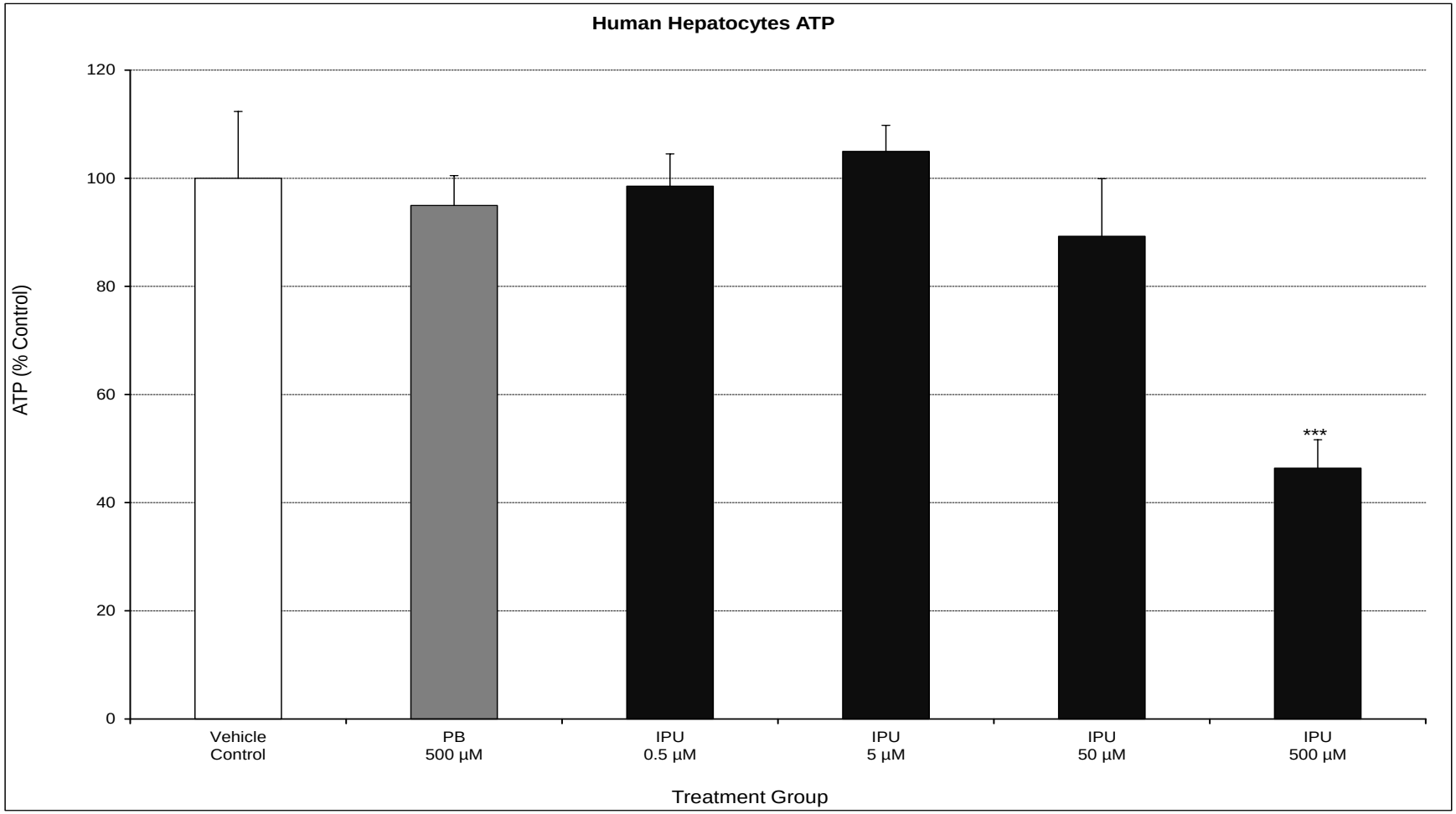
Graph presents data as mean % of control ± standard deviation (n=6). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 2 Adenosine 5'-Triphosphate (ATP) CAR KO Male Rat Hepatocytes



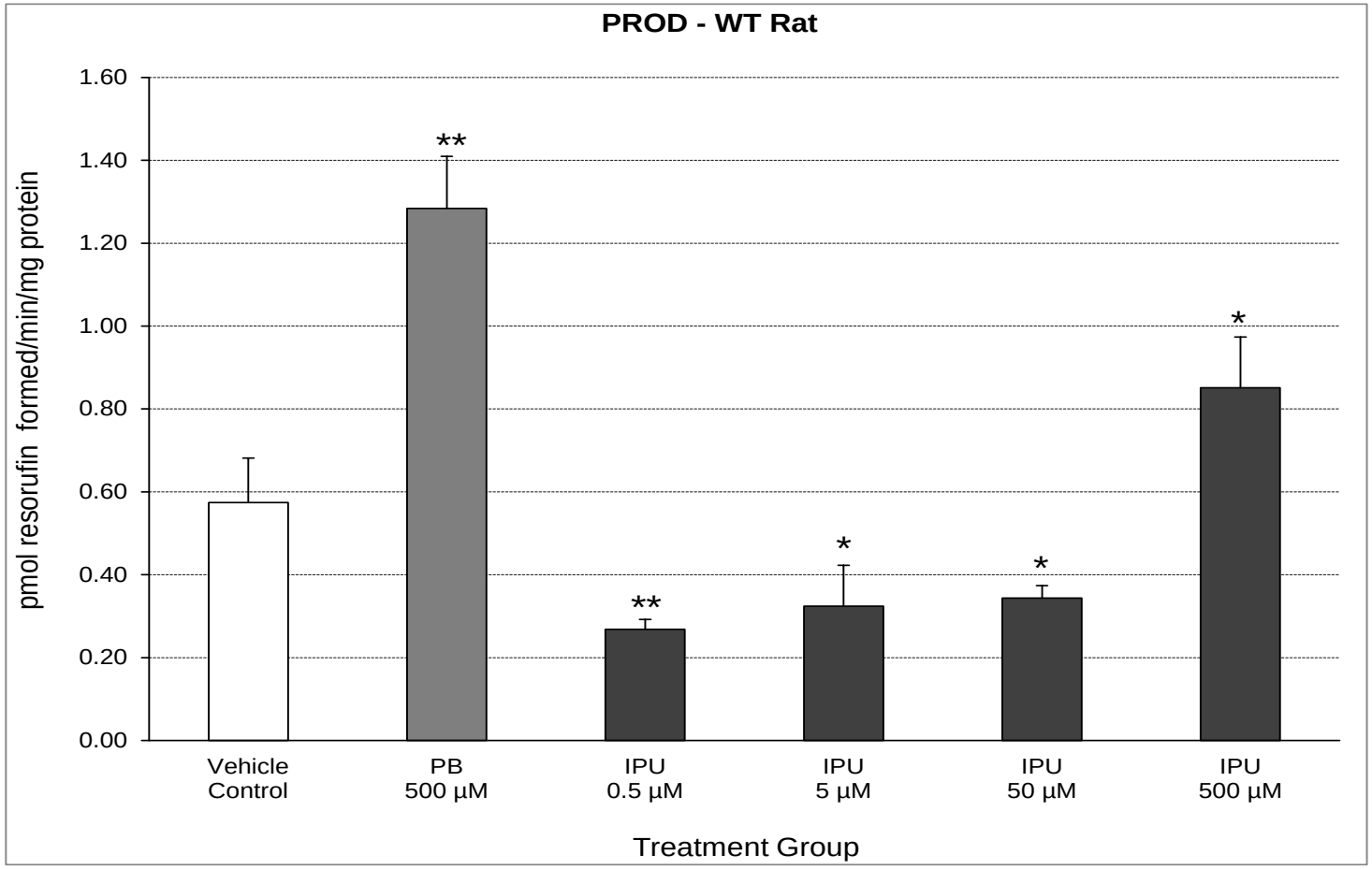
Graph presents data as mean % of control ± standard deviation (n=6). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 3 Adenosine 5'-Triphosphate (ATP) Male Human Hepatocytes



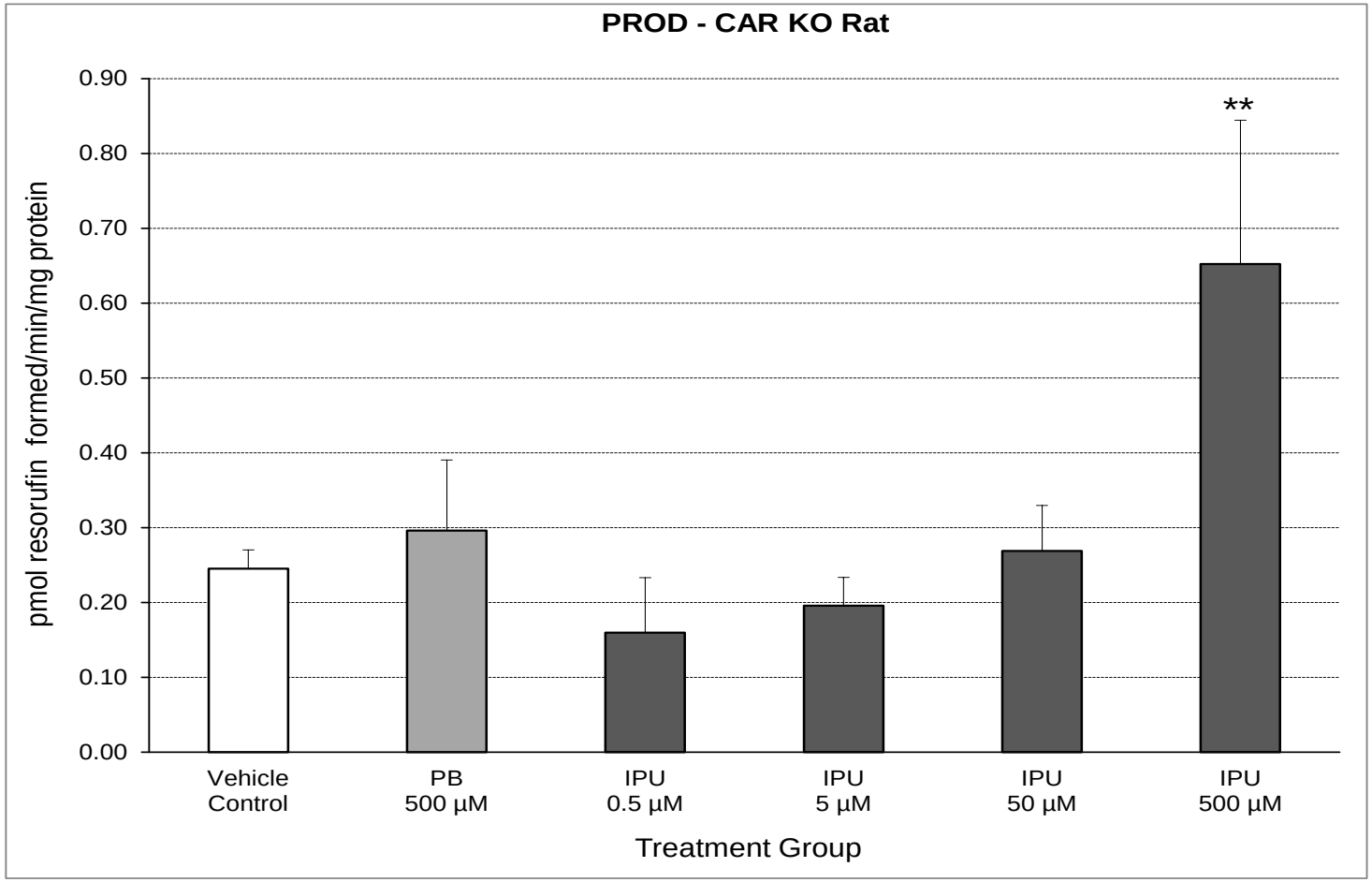
Graph presents data as mean % of control ± standard deviation (n=6). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 4 Pentoxyresorufin-*O*-Depentylation (PROD) WT Male Rat Hepatocytes



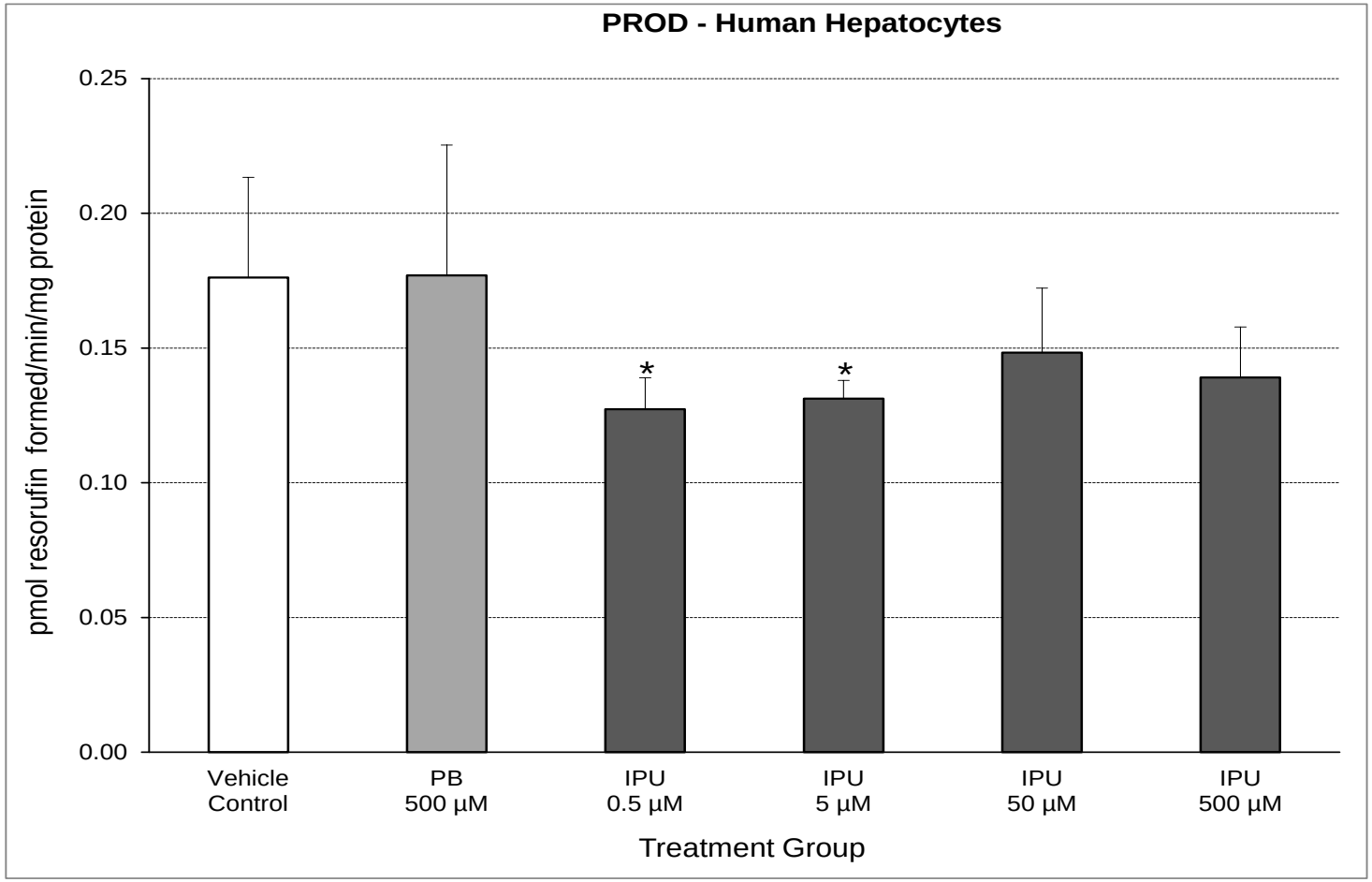
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 5 Pentoxyresorufin-*O*-Depentylation (PROD) CAR KO Male Rat Hepatocytes



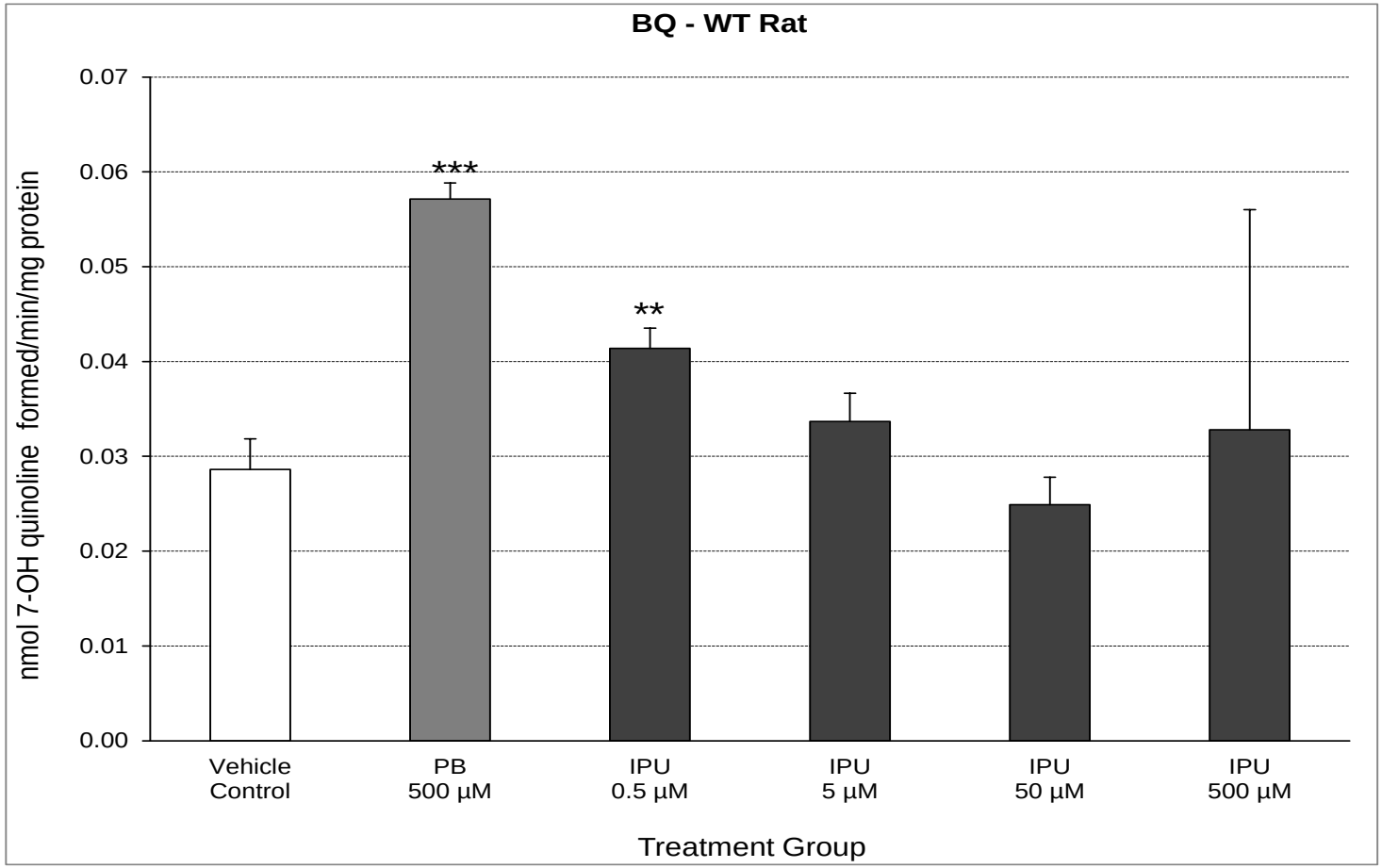
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 6 Pentoxyresorufin-*O*-Depentylation (PROD) Male Human Hepatocytes



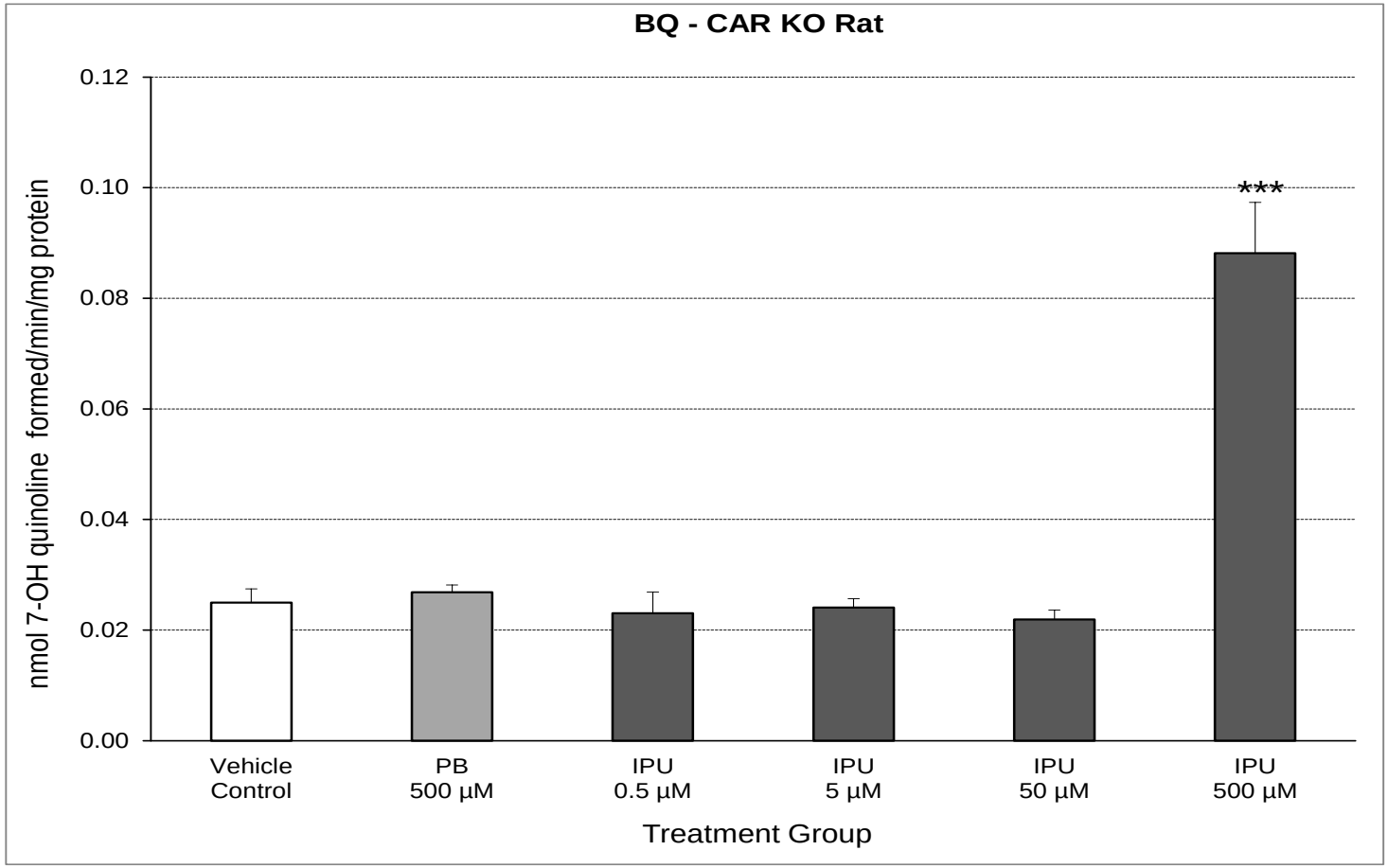
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 7 Benzyloxyquinoline-*O*-Debenzylation (BQ) WT Male Rat Hepatocytes



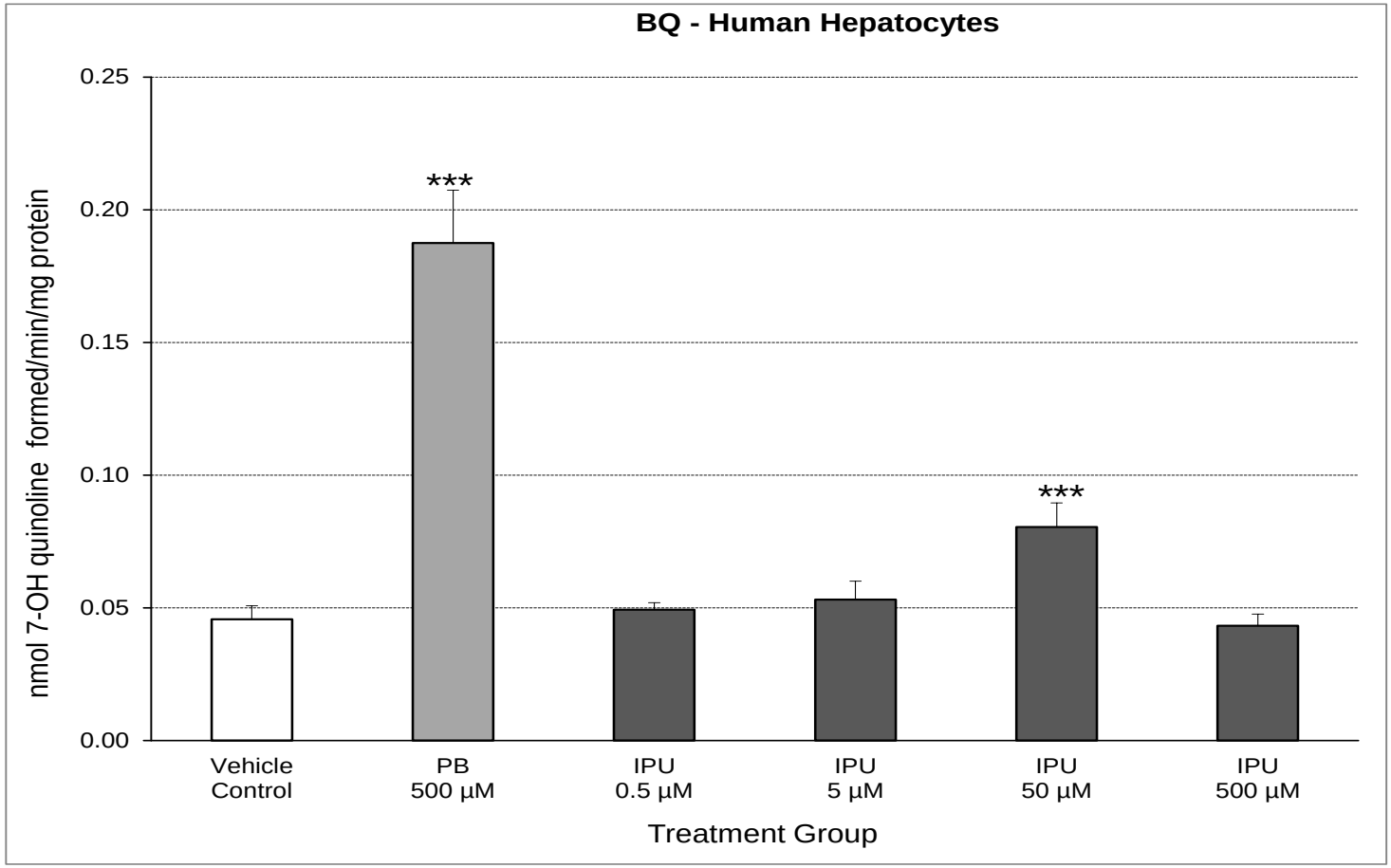
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 8 Benzyloxyquinoline-*O*-Debenzylation (BQ) CAR KO Male Rat Hepatocytes



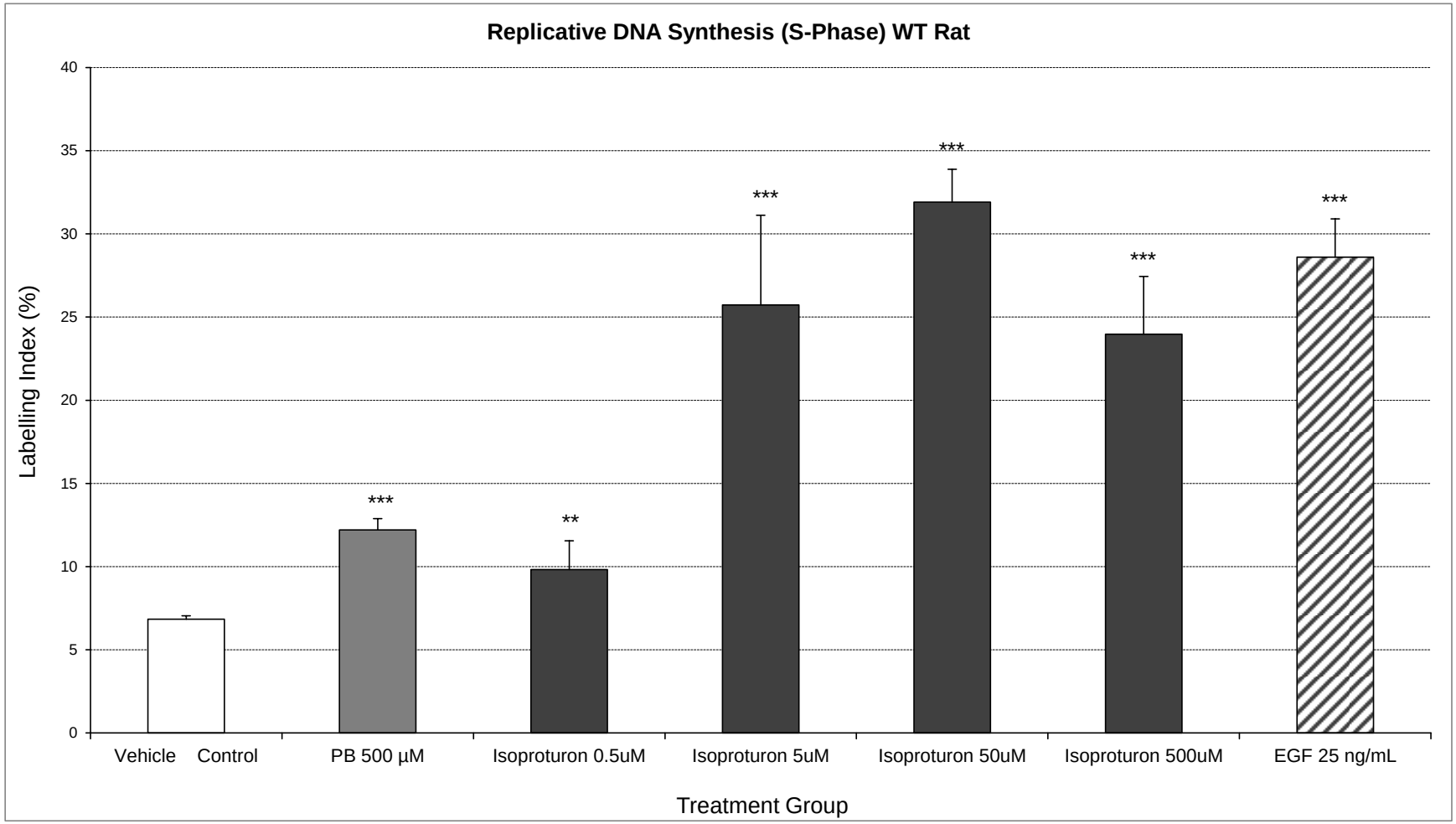
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 9 Benzyloxyquinoline-*O*-Debenzylation (BQ) Male Human Hepatocytes



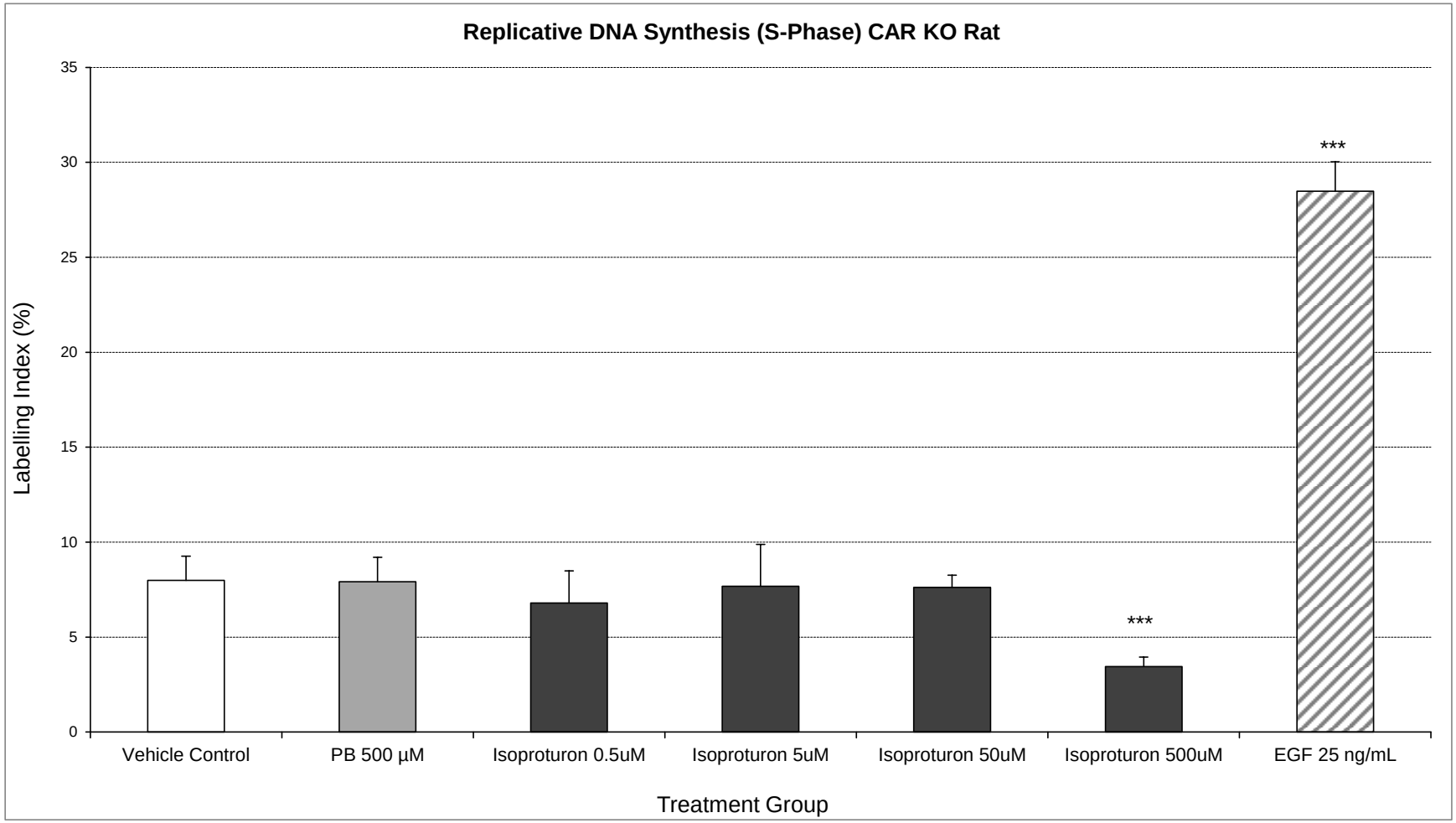
Graph presents data as mean ± standard deviation (n=3). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 10 Replicative DNA Synthesis (S-Phase) WT Male Rat Hepatocytes



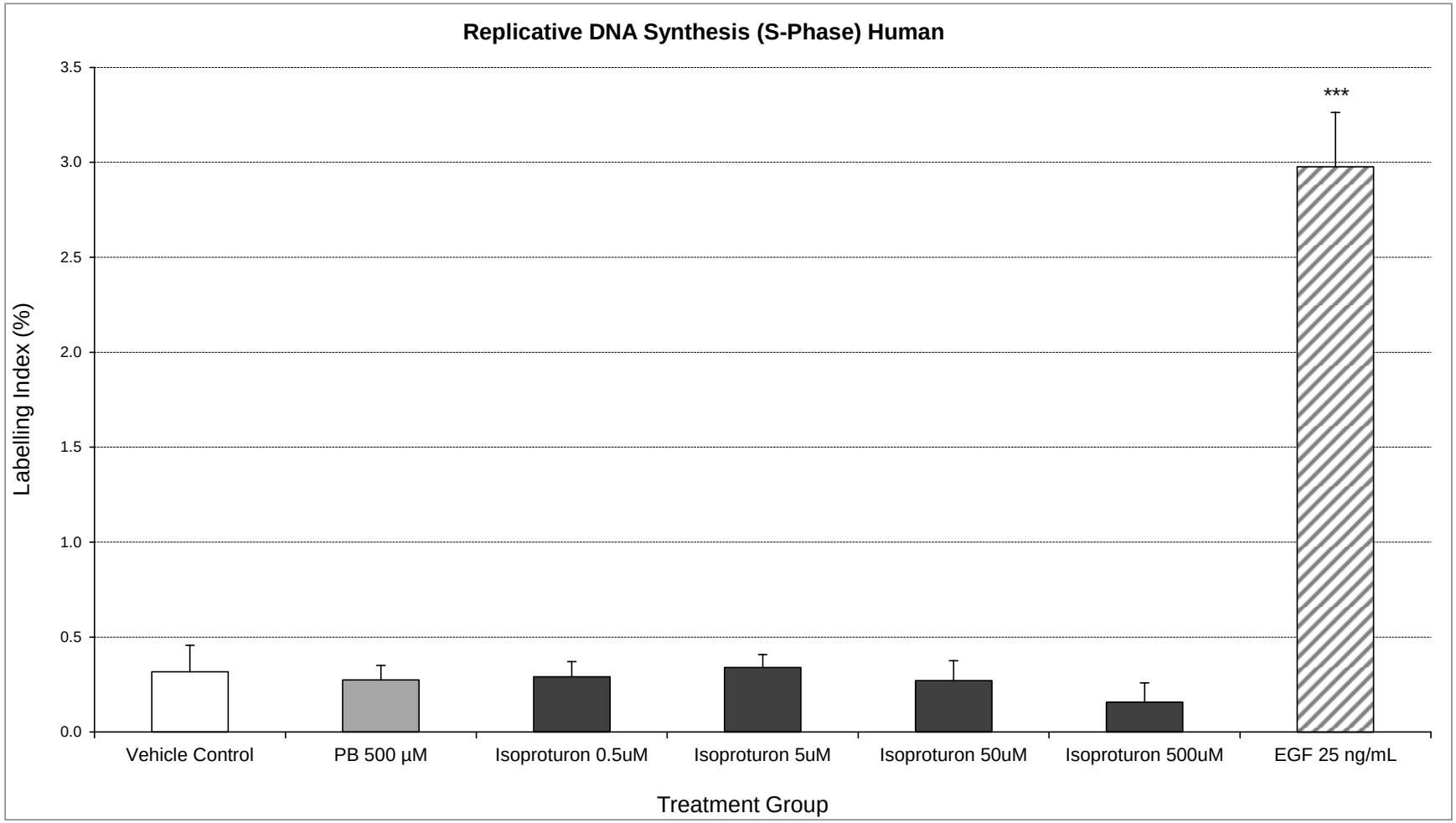
Graph presents data as mean ± standard deviation (n=5). A Student's t-test (2-sided) was performed on the results; *statistically different from control $p<0.05$; **$p<0.01$; ***$p<0.001$.

FIGURE 11 Replicative DNA Synthesis (S-Phase) CAR KO Male Rat Hepatocytes



Graph presents data as mean ± standard deviation (n=5). A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; ***p<0.001.

FIGURE 12 Replicative DNA Synthesis (S-Phase) Male Human Hepatocytes



Graph presents data as mean ± standard deviation (n=5). A Student's t-test (2-sided) was performed on the results; *statistically different from control p<0.05; **p<0.01; ***p<0.001.

APPENDICES SECTION

APPENDIX 1 Certificate of Analysis

AGAN CHEMICAL MANUFACTURERS LTD.

CERTIFICATE OF ANALYSIS

ISOPROTURON TECH.

Lot no.: 8837

Assay .....98.5 % (w/w) isoproturon.

This above material is good for three years after date of manufacture when stored in original packaging under normal storage conditions.
Production date: March 2014
Expiry date: March 2017

Ze'ev Freifeld, MSc
Quality Assurance Manager

09 March 2014

Northern Industrial Zone, P.O.B. 262, Ashdod 77102, Israel
Phone: 8-8515211 Fax: 8-8522806 E-mail: info@agan.co.il Website: www.agan.co.il

APPENDIX 1 Certificate of Analysis (Continued)

SIGMA-ALDRICH

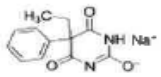
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Phenobarbital sodium salt

Product Number:	**P5178**
Batch Number:	**SLBJ3684V**
Brand:	SIGMA
CAS Number:	57-30-7
MDL Number:	MFCD00036211
Formula Weight:	254.22 g/mol
Quality Release Date:	10 DEC 2013
Recommended Retest Date:	JUL 2015



Test	Specification	Result
Appearance (Color)	White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless	Colorless
Solubility (Turbidity) 50 mg/mL, H2O	Clear	Clear
Infrared Spectrum	Conforms to Structure	Conforms
Loss on Drying	≤ 7.0 %	6.8 %
Assay Dry basis	99.0 - 101.1 %	99.9 %

Rodney Burbach, Manager
Analytical Services
St. Louis, Missouri US

Sigma-Aldrich warrants, that at the time of the quality release or subsequent retest date this product conformed to the information contained in this publication. The current Specification sheet may be available at Sigma-Aldrich.com. For further inquiries, please contact Technical Service. Purchaser must determine the suitability of the product for its particular use. See reverse side of invoice or packing slip for additional terms and conditions of sale.

Version Number: 1 Page 1 of 1

APPENDIX 1 Certificate of Analysis (Continued)

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Epidermal Growth Factor human, recombinant, expressed in *Escherichia coli*, lyophilized powder, suitable for cell culture
Product Number	E9644
Product Brand	SIGMA
CAS Number	62253-63-8

TEST	SPECIFICATION	LOT 011M4009V RESULTS
APPEARANCE	WHITE POWDER	CONFORMS
BIOACTIVITY (CELL PROLIFERATION, BALB/3T3 CELLS)	EC50: 0.08-0.8 NG/ML	EC50: 0.258 NG/ML
PURITY BY SDS-PAGE	GREATER THAN 97%	99%
ENDOTOXIN ASSAY	NOT MORE THAN 1 EU/UG EGF	CONFORMS
NOTE	LYOPHILIZED FROM A 0.2 UM-FILTERED SOLUTION OF PHOSPHATE BUFFERED SALINE, PH 7.5 +/-5	
QC RELEASE DATE		23 DEC 2010
RECOMMENDED RETEST	2 YEARS	DEC 2012

Rodney Burbach, Manager
Quality Control
St. Louis, Missouri USA

http://www.sigmaaldrich.com/catalog/CertOfAnalysisPage.do?symbol=E9644&LotN... 26/09/2011

APPENDIX 1 Certificate of Analysis (Continued)

SIGMA

3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: sigma-techserv@siai.com
http://www.sigma-aldrich.com

ProductInformation

EPIDERMAL GROWTH FACTOR (EGF)
Human, Recombinant
Expressed in *E. coli*

Product No. **E9644**

Description

Epidermal Growth Factor (EGF) is a small mitogenic polypeptide which is present in many mammalian species and is distributed throughout a wide number of tissues and body fluids.[1] Human EGF is identical to β-urogastrone, a polypeptide which was recognized and isolated on the basis of its ability to inhibit gastric acid secretion.[2] EGF is a member of a growth factor family which is characterized by the presence of 6 conserved cysteine motifs that form three disulfide bonds. The location of 3 intrachain disulfide bonds in recombinant human EGF is identical to that of mouse EGF.[3] EGF is structurally homologous to human transforming growth factor-α (TGF-α), and both exert their actions through EGF receptors.[4] EGF is homologous to a sequence contained in a 19 kD protein of vaccinia virus,[5] which appears to utilize the EGF receptor to gain entry into cells.[6] EGF is mitogenic for a variety of epidermal and epithelial cells, including fibroblasts, glial cells, mammary epithelial cells, vascular and corneal endothelial cells, bovine granulosa, rabbit chondrocytes, HeLa cells and SV40-3T3 cells.[1]

Performance Characteristics

The biological activity of recombinant, human EGF is measured by its ability to stimulate the mouse fibroblast cell line, BALB/3T3. The EC_{50} is defined as the effective concentration of growth factor that elicits a 50% increase in cell growth in a cell based bioassay.

Product Information

Expressed in *E. coli*
Molecular Weight: 6 kD
Purity: ≥ 97% as determined by SDS-PAGE
EC_{50}: 0.08 - 0.8 ng/ml
Package Size: 200 μg/vial

Formulation: Lyophilized from a 0.2 μm-filtered solution of phosphate buffered saline, pH 7.4
Sterility: 0.2 μm-filtered, aseptic fill
Endotoxin: ≤0.1 ng/μg EGF

Reconstitution and Use

Reconstitute the contents of the vial using 0.2 μm-filtered 10 mM acetic acid containing 0.1% HSA or BSA to a concentration of not less than 10 μg/ml.

Storage

Prior to reconstitution, store at -20°C. After reconstitution, store at 2-8°C for a maximum of one month. For extended storage, freeze in working aliquots at -70°C or -20°C. Repeated freezing and thawing is not recommended.

References

1. Carpenter, G., et al., Annu. Rev. Biochem., **48**, 193 (1979).
2. Gregory, H., Nature, **257**, 325 (1975).
3. George-Nascimento, C., Biochemistry, **27**, 797 (1988).
4. Todaro, G., et al., Proc. Natl. Acad. Sci. USA, **77**, 5258 (1980).
5. Blomquist, M., et al., Proc. Natl. Acad. Sci. USA, **81**, 736 (1984).
6. Eppstein, D., et al., Nature, **318**, 663 (1985).

Pcs2/99

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Sigma is a Member of the Sigma-Aldrich Family
Providing Biochemicals and Reagents for Life Science Research.

APPENDIX 1 Certificate of Analysis (Continued)

GIBCO®

Quality Certificate

Primary Hepatocytes: Cryopreserved

Species: Human

Number of Donors: 1

Catalog Number: HMCPISA

Lot Number: Hu4246

Shipping/Storage Temperature: Below -135 °C

☐ **Product Information Sheet**

Sample Information: Store using Vapor Phase Liquid Nitrogen

☑ **Certificate of Analysis**

Volume: 1.5-2.0 mL Vial

FOR RESEARCH USE ONLY. CAUTION: Not intended for human or animal diagnostic or therapeutic uses. Users should treat all human and animal cells as potential pathogens. Wear protective clothing and eyewear. Practice appropriate disposal techniques for potentially pathogenic or biohazardous materials.

Contact the in-house Safety Officer for information on safe handing of human cell products, universal precautions, and for information on what to do in case of exposure. For recommended post exposure procedures, call the Centers for Disease Control (CDC) Information line at +1 800 232 4636.

DONOR INFORMATION

Donor ID	Gender	Race	Age (yrs)	BMI	Smoker	Alcohol Use	Drug Use	Medications	Cause of Death
Hu4246	*Male*	*Caucasian*	*46*	*23*	*No*	*No*	*No*	*Flagyl, Levothyroxine, Rocephin, Zithromax, Coreg, Proventil, Atrovent, Dopamine, Morphine, Invanz, Crystalloids, FiberSource, D5NS, Insulin (administered in Hospital)*	*Anoxia-Second to Cardiovascular*

SEROLOGY (PCR detection)

Hepatitis B:	*Non-Reactive*	HTLV 1 & 2:	*Not Available*
Hepatitis C:	*Non-Reactive*	CMV:	*Reactive*
HIV 1 & 2:	*Non-Reactive*		

OTHER MEDICAL HISTORY:

History of Duchenne Muscular Dystrophy and ventilator dependant for several years.

For technical questions about this product, contact Invitrogen's Hepatic Biology Support Team.
Email: hepaticproducts@invitrogen.com Phone: (USA) +1 866 952 3559 (Europe) +44(0)141 814 5900

Page 1 of 4

Form Human QC Rev 1f, 19MAY11

APPENDIX 1 Certificate of Analysis (Continued)

GIBCO®

Quality Certificate
(Continued)

POST-THAW VIABILITY

Average Viability: 78%
Viable Cells/Vial: 5.9×10^6

METABOLIC ACTIVITY

The cell suspension per 37°C incubation is 0.5×10^6 cells/mL.
Metabolic activities are determined by HPLC or LC-MS/MS analysis and recorded as pmol/10^6 cells/min.

Enzyme	Substrate	Concentration (µM)	Incubation Time (Min)	Marker Metabolite	Metabolic Activity
CYP1A2	Phenacetin	100	15	Acetaminophen	N/A
CYP2B6	Bupropion	500	15	Hydroxybupropion	N/A
CYP2C8	Paclitaxel	20	45	6α-Hydroxypaclitaxel	N/A
CYP2C9	Diclofenac	25	15	4´-Hydroxydiclofenac	N/A
CYP2C19	(S)-Mephenytoin	250	30	4´-Hydroxymephenytoin	N/A
CYP2D6	Dextromethorphan	15	15	Dextrorphan	N/A
CYP2E1	Chlorzoxazone	250	15	6-Hydroxychlorzoxazone	N/A
CYP3A	Testosterone	200	15	6β-Hydroxytestosterone	N/A
CYP3A	Midazolam	10	10	1'-Hydroxymidazolam	N/A
FMO	Benzydamine	250	30	Benzydamine-N-oxide	N/A
Phase I	7-Ethoxycoumarin	100	30	(ECOD) 7-Hydroxycoumarin (7-HC)	N/A
Phase II	7-Hydroxycoumarin	100	30	7-Hydroxycoumarin Glucuronide (7-HCG)	N/A
Phase II	7-Hydroxycoumarin	100	30	7-Hydroxycoumarin Sulfate (7-HCS)	N/A

ENZYME INDUCTION

The number of plated cells per 37°C incubation is 0.375×10^6 cells/well. Enzyme activities are determined after 72 hours in culture by LC-MS/MS analysis (vehicle/induced activity). Relative mRNA contents are determined after 48 hours by qRT-PCR analysis (TaqMan®).
Results are recorded as pmol/10^6 cells/min.

Enzyme	Inducer *	Substrate	Conc. (µM)	Incubation Time (Min)	Marker Metabolite	Vehicle Activity	Induced Activity	Fold Induction	mRNA Relative-Fold
CYP1A2	3-MC (2 µM)	Phenacetin	100	15	Acetaminophen	4.04	136	33.7	58.8
CYP2B6	PB (1000 µM)	Bupropion	500	20	Hydroxybupropion	1.32	12.7	9.60	11.9
CYP3A	RIF (10 µM)	Testosterone	200	14	6β-Hydroxytestosterone	69.5	657	9.40	8.67

* 3-MC = 3-Methylcholanthrene; PB = Phenobarbital; RIF = Rifampicin

For technical questions about this product, contact Invitrogen's Hepatic Biology Support Team.
Email: hepaticproducts@invitrogen.com Phone: (USA) +1 866 952 3559 (Europe) +44(0)141 814 5900

Page 2 of 4 Form Human QC Rev 1e, 08JUN10

APPENDIX 1 Certificate of Analysis (Continued)

GIBCO®

Quality Certificate
(Continued)

GENOTYPING RESULTS

Invitrogen screens donor tissues for thirteen different SNPs within four drug-metabolizing genes. These include the following: CYP2C9*2, CYP2C9*3, CYP2C9*6, CYP2C19*2, CYP2C19*3, CYP2C19*6, CYP2D6*3, CYP2D6*4, CYP2D6*6, CYP2D6*9, CYP3A5*3, CYP3A5*6, and CYP3A5*8. All SNPS were identified by qRT-PCR with Taqman® primer/probe sets.

DONOR	DONOR ID	CYP2C9			CYP2C19			CYP2D6				CYP3A5		
		*2	*3	*6	*2	*3	*6	*3	*4	*6	*9	*3	*6	*8
1	Hu4246	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

PLATED METABOLISM

Cryopreserved Human Hepatocytes were thawed in CHRM™ and plated. The concentrations and incubation times for prototypical CYP substrates are included in the chart below. Incubations were conducted in duplicate in serum-free Williams Medium E culture medium and reactions allowed to proceed in a humidified incubator at 37°C, 95% relative humidity, and 5% CO_2 on an orbital shaker. Reactions were stopped with the addition of ice-cold Acetonitrile. Well contents were stored at -70°C prior to analysis. The disappearance of parent was monitored by LC-MS/MS analysis and intrinsic clearance (CL_{int}) values determined by linear regression.

SUBSTRATE	Concentration (µM)	Incubation Times (Hours)	Intrinsic Clearance (CL_{int}) values (µL/min/10^6 cells)
Dextromethorphan	1	0,1,2,4,6,8	N/A
Midazolam	0.5	0,1,2,4,6,8	N/A
Tolbutamide	1	0,4,6,8,18,24	N/A

TRANSPORTER RESULTS

Cryopreserved hepatocytes were thawed in CHRM™ and plated. The Transporter assay was performed on Day 5. Rates of substrate uptake were determined by the use of buffer with calcium. Hepatocyte cultures were incubated in triplicate with radio-labeled Taurocholate, Digoxin and Estradiol 17β glucuronide (E2-17G). Substrate concentrations and incubation times are listed in the table below. To account for non-specific binding of the radio-labeled substrate, a negative control plate absent of cells was included. Cells were lysed following incubations and samples analyzed by use of a liquid scintillation counter.

SUBSTRATE	CONDITIONS		Uptake Rate + Calcium (pmol/min/mg protein)
	Concentration (µM)	Incubation Time (min)	
Taurocholate	1	10	N/A
Digoxin	1	10	N/A
Estradiol 17β Glucuronide (E2-17G)	1	10	N/A

For technical questions about this product, contact Invitrogen's Hepatic Biology Support Team.
Email: hepaticproducts@invitrogen.com Phone: (USA) +1 866 952 3559 (Europe) +44(0)141 814 5900

Page 3 of 4 Form Human QC Rev 1e, 08JUN10

APPENDIX 1 Certificate of Analysis (Continued)

GIBCO®

Quality Certificate
(Continued)

The Liver Cells from Lot Hu4246 were derived from tissue obtained from accredited institutions. Consent was obtained by these institutions from the donor or the donor's legal next of kin, for use of the tissue and its derivatives for research purposes.

Matthew Riggber 31 May 2011
Prepared By Date

Quality Systems Department 31 May 2011
Date

For technical questions about this product, contact Invitrogen's Hepatic Biology Support Team.
Email: hepaticproducts@invitrogen.com Phone: (USA) +1 866 952 3559 (Europe) +44(0)141 814 5900

Page 4 of 4 Form Human QC Rev 1e, 08JUN10

APPENDIX 2 Individual Sample Data

ATP 96 Hours, WT Rat Heps									
Treatment	L.U.	Mean	±	SD	% Control	±	SD	T-Value	p
Vehicle Control	103287	100672	±	5810	100.0	±	5.8		
	97225								
	102549								
	96007								
	98572								
	104861								
	109267								
	106134								
	106735								
	107942								
	106034								
	104820								
	99937								
	94503								
	95405								
	93566								
	95965								
	89287								
500 µM PB	103369	99552	±	3712	98.9	±	3.7	0.548	
	102489								
	101662								
	96161								
	99533								
	94098								
0.5 µM Isoproturon	95781	95047	±	6042	94.4	±	6.0	1.994	
	98448								
	103342								
	95948								
	90825								
	85937								
5 µM Isoproturon	101651	102648	±	4220	102.0	±	4.2	-0.898	
	107269								
	98215								
	106437								
	104917								
	97401								
50 µM Isoproturon	112476	110442	±	1847	109.7	±	1.8	-6.249	***
	108751								
	109155								
	110119								
	113011								
	109137								
500 µM Isoproturon	104159	99135	±	2964	98.5	±	2.9	0.841	
	98889								
	98125								
	100609								
	97436								
	95593								

APPENDIX 2 Individual Sample Data (Continued)

ATP 96 Hours, CAR KO Rat Heps									
Treatment	L.U.	Mean	±	SD	% Control	±	SD	T-Value	p
Vehicle Control	98371	95687	±	7442	100.0	±	7.8		
	97655								
	86483								
	90810								
	87698								
	90237								
	110584								
	104373								
	104034								
	86307								
	104975								
	102858								
	94603								
	94606								
	89943								
	93923								
	89217								
500 µM PB	112569	105500	±	4431	110.3	±	4.6	-3.840	***
	107188								
	101595								
	104390								
	100323								
	106933								
0.5 µM Isoproturon	98137	93490	±	9768	97.7	±	10.2	0.502	
	101699								
	97614								
	95658								
	93481								
	74349								
5 µM Isoproturon	111310	102466	±	4735	107.1	±	4.9	-2.563	*
	98686								
	103546								
	99454								
	99405								
	102394								
50 µM Isoproturon	116324	109497	±	4065	114.4	±	4.2	-5.632	***
	109091								
	106054								
	105743								
	107692								
	112077								
500 µM Isoproturon	111434	105341	±	3887	110.1	±	4.1	-4.017	***
	105282								
	104685								
	101275								
	101478								
	107894								

APPENDIX 2 Individual Sample Data (Continued)

ATP 96 Hours, Human Heps									
Treatment	L.U.	Mean	±	SD	% Control	±	SD	T-Value	p
Vehicle Control	92340	93054	±	11514	100.0	±	12.4		
	93460								
	94335								
	88859								
	118039								
	86372								
	84706								
	75596								
	87449								
	85259								
	83718								
	88234								
	98135								
	87422								
	90905								
	93126								
	120173								
	106851								
500 µM PB	89058	88374	±	5131	95.0	±	5.5	1.365	
	83849								
	88791								
	87495								
	97613								
	83435								
0.5 µM Isoproturon	96220	91691	±	5569	98.5	±	6.0	0.385	
	92870								
	87034								
	96874								
	94282								
	82864								
5 µM Isoproturon	102533	97686	±	4492	105.0	±	4.8	-1.414	
	90945								
	98800								
	101546								
	93792								
	98499								
50 µM Isoproturon	89712	83087	±	9887	89.3	±	10.6	2.049	
	82840								
	86195								
	89960								
	63636								
	86177								
500 µM Isoproturon	45363	43155	±	4890	46.4	±	5.3	14.811	***
	50487								
	43238								
	42096								
	35474								
	42272								

APPENDIX 2 Individual Sample Data (Continued)

Pentyloxyresorufin - O depentylation (PRODs) male WT Rat Heps

Flask No	FU/min sample	FU std	pmols /1FU	pmols res formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	pmols res formed / min / mg prot	mean ± SD	% of control	T-VALUE (vs control)	
1	0.0250	8.1	12.3457	0.3086	2.300	250	0.57493	0.5368	0.5743 ± 0.1073	100.0 ± 18.7		
2	0.0253	6.2	16.1290	0.4081	2.348	250	0.58693	0.6953	Control 0 µM			
3	0.0216	7.3	13.6986	0.2959	2.412	250	0.60292	0.4908				
4	0.0674	7.8	12.8205	0.8641	2.588	250	0.64691	1.3357	1.2837 ± 0.1260	223.5 ± 21.9	**-7.425**	**
5	0.0743	8.1	12.3457	0.9173	2.668	250	0.66690	1.3755	Phenobarbital 500 µM			
6	0.0522	7.1	14.0845	0.7352	2.580	250	0.64491	1.1400				
7	0.0121	8.6	11.6279	0.1407	2.268	250	0.56694	0.2482	0.2683 ± 0.0238	46.7 ± 4.1	**4.823**	**
8	0.0128	7.5	13.3333	0.1707	2.604	250	0.65090	0.2622	Isoproturon 0.5 µM			
9	0.0151	8.1	12.3457	0.1864	2.532	250	0.63291	0.2945				
10	0.0208	7.7	12.9870	0.2701	2.468	250	0.61692	0.4379	0.3240 ± 0.0986	56.4 ± 17.2	**2.975**	*
11	0.0136	7.9	12.6582	0.1722	2.572	250	0.64291	0.2678	Isoproturon 5 µM			
12	0.0134	8.4	11.9048	0.1595	2.396	250	0.59893	0.2664				
13	0.0169	7.0	14.2857	0.2414	2.572	250	0.64291	0.3755	0.3438 ± 0.0298	59.9 ± 5.2	**3.586**	*
14	0.0141	7.2	13.8889	0.1958	2.476	250	0.61892	0.3164	Isoproturon 50 µM			
15	0.0141	6.8	14.7059	0.2074	2.444	250	0.61092	0.3394				
16	0.0409	9.4	10.6383	0.4351	2.404	250	0.60092	0.7241	0.8512 ± 0.1226	148.2 ± 21.4	**-2.944**	*
17	0.0536	9.3	10.7527	0.5763	2.380	250	0.59493	0.9688	Isoproturon 500 µM			
18	0.0441	9.1	10.9890	0.4846	2.252	250	0.56294	0.8609				

Pentyloxyresorufin - O depentylation (PRODs) male CAR KO Rat Heps

Flask No	FU/min sample	FU std	pmols /1FU	pmols res formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	pmols res formed / min / mg prot	mean ± SD	% of control	T-VALUE (vs control)	
1	0.0191	8.6	11.6279	0.2221	3.479	250	0.86981	0.2553	0.2451 ± 0.0253	100.0 ± 10.3		
2	0.0162	7.8	12.8205	0.2077	3.497	250	0.87416	0.2376	Control 0 µM			
3	0.0192	7.9	12.6582	0.2430	3.488	250	0.87198	0.2787				
4	0.0168	10.0	10.0000	0.1680	2.940	250	0.73505	0.2286				
5	0.0162	9.8	10.2041	0.1653	3.175	250	0.79374	0.2083				
6	0.0179	8.4	11.9048	0.2131	3.253	250	0.81330	0.2620				
7	0.0132	6.9	14.4928	0.1913	3.288	250	0.82199	0.2327	0.2959 ± 0.0946	120.7 ± 38.6	**-0.914**	
8	0.0172	7.4	13.5135	0.2324	3.714	250	0.92849	0.2503	Phenobarbital 500 µM			
9	0.0230	7.3	13.6986	0.3151	3.114	250	0.77852	0.4047				
10	0.0057	8.5	11.7647	0.0671	3.549	250	0.88720	0.0756	0.1597 ± 0.0736	65.2 ± 30.0	**1.952**	
11	0.0168	9.1	10.9890	0.1846	3.870	250	0.96762	0.1908	Isoproturon 0.5 µM			
12	0.0160	7.9	12.6582	0.2025	3.810	250	0.95240	0.2127				
13	0.0117	7.8	12.8205	0.1500	3.557	250	0.88937	0.1687	0.1959 ± 0.0375	79.9 ± 15.3	**2.051**	
14	0.0138	7.5	13.3333	0.1840	4.079	250	1.01978	0.1804	Isoproturon 5 µM			
15	0.0162	7.0	14.2857	0.2314	3.879	250	0.96979	0.2386				
16	0.0145	7.3	13.6986	0.1986	3.905	250	0.97631	0.2034	0.2687 ± 0.0611	109.6 ± 24.9	**-0.644**	
17	0.0178	6.7	14.9254	0.2657	3.818	250	0.95458	0.2783	Isoproturon 50 µM			
18	0.0221	6.5	15.3846	0.3400	4.192	250	1.04804	0.3244				
19	0.0225	5.0	20.0000	0.4500	3.818	250	0.95458	0.4714	0.6522 ± 0.1920	266.1 ± 78.4	**-3.656**	**
20	0.0337	5.2	19.2308	0.6481	4.105	250	1.02630	0.6315	Isoproturon 500 µM			
21	0.0445	5.1	19.6078	0.8725	4.088	250	1.02195	0.8538				

APPENDIX 2 Individual Sample Data (Continued)

Pentyloxyresorufin - O depentylation (PRODs) male human Heps

Flask No	FU/min sample	FU std	pmols /1FU	pmols res formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	pmols res formed / min / mg prot	mean ± SD	% of control	T-VALUE (vs control)
1	0.0060	26.3	3.8023	0.0228	0.837	250	0.20922	0.1090	0.1762 ± 0.0372	100.0 ± 21.1	
2	0.0120	27.7	3.6101	0.0433	0.837	250	0.20922	0.2071	Control 0 µM		
3	0.0133	29.4	3.4014	0.0452	0.850	250	0.21252	0.2129			
4	0.0103	26.4	3.7879	0.0390	0.883	250	0.22079	0.1767			
5	0.0111	26.1	3.8314	0.0425	0.929	250	0.23235	0.1830			
6	0.0105	26.3	3.8023	0.0399	0.949	250	0.23731	0.1682			
7	0.0069	24.3	4.1152	0.0284	0.797	250	0.19930	0.1425	0.1770 ± 0.0484	100.5 ± 27.5	**-0.025**
8	0.0080	24.3	4.1152	0.0329	0.843	250	0.21087	0.1561	Phenobarbital 500 µM		
9	0.0112	24.6	4.0650	0.0455	0.784	250	0.19600	0.2323			
10	0.0081	28.4	3.5211	0.0285	0.830	250	0.20757	0.1374	0.1273 ± 0.0117	72.3 ± 6.7	**2.939** *
11	0.0081	27.8	3.5971	0.0291	0.896	250	0.22409	0.1300	Isoproturon 0.5 µM		
12	0.0070	27.1	3.6900	0.0258	0.903	250	0.22574	0.1144			
13	0.0083	27.6	3.6232	0.0301	0.867	250	0.21665	0.1388	0.1312 ± 0.0067	74.5 ± 3.8	**2.867** *
14	0.0073	27.7	3.6101	0.0264	0.837	250	0.20922	0.1260	Isoproturon 5 µM		
15	0.0080	27.2	3.6765	0.0294	0.913	250	0.22822	0.1289			
16	0.0091	26.3	3.8023	0.0346	0.827	250	0.20674	0.1674	0.1483 ± 0.0239	84.2 ± 13.6	**1.356**
17	0.0074	27.5	3.6364	0.0269	0.886	250	0.22161	0.1214	Isoproturon 50 µM		
18	0.0101	29.3	3.4130	0.0345	0.883	250	0.22079	0.1561			
19	0.0068	30.0	3.3333	0.0227	0.771	250	0.19269	0.1176	0.1391 ± 0.0187	78.9 ± 10.6	**1.990**
20	0.0082	27.7	3.6101	0.0296	0.777	250	0.19435	0.1523	Isoproturon 500 µM		
21	0.0078	28.6	3.4965	0.0273	0.741	250	0.18526	0.1472			

Benzyloxyquinoline debenzylation male WT Rat Hep

Flask No	FU/min sample	FU std	nmols / 1 FU	nmols 7-OH formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	nmols 7-OH formed / min/mg prot	mean ± SD	% of control	T-VALUE (VS CONTROL)
1	0.0355	11.3	0.4425	0.0157	2.300	250	0.57493	0.0273	0.0286 ± 0.0032	100.0 ± 11.3	
2	0.0311	10.1	0.4950	0.0154	2.348	250	0.58693	0.0262	Control 0 µM		
3	0.0405	10.4	0.4808	0.0195	2.412	250	0.60292	0.0323			
4	0.0766	10.1	0.4950	0.0379	2.588	250	0.64691	0.0586	0.0571 ± 0.0017	199.6 ± 6.0	**-13.509** ***
5	0.0715	9.7	0.5155	0.0369	2.668	250	0.66690	0.0553	Phenobarbital 500 µM		
6	0.0749	10.1	0.4950	0.0371	2.580	250	0.64491	0.0575			
7	0.0509	10.4	0.4808	0.0245	2.268	250	0.56694	0.0432	0.0414 ± 0.0021	144.6 ± 7.4	**-5.721** **
8	0.0559	11.0	0.4545	0.0254	2.604	250	0.65090	0.0390	Isoproturon 0.5 µM		
9	0.0563	10.6	0.4717	0.0266	2.532	250	0.63291	0.0420			
10	0.0424	10.7	0.4673	0.0198	2.468	250	0.61692	0.0321	0.0337 ± 0.0030	117.7 ± 10.5	**-1.990**
11	0.0421	10.3	0.4854	0.0204	2.572	250	0.64291	0.0318	Isoproturon 5 µM		
12	0.0507	11.4	0.4386	0.0222	2.396	250	0.59893	0.0371			
13	0.0365	10.6	0.4717	0.0172	2.572	250	0.64291	0.0268	0.0249 ± 0.0029	87.0 ± 10.2	**1.482**
14	0.0288	10.8	0.4630	0.0133	2.476	250	0.61892	0.0215	Isoproturon 50 µM		
15	0.0335	10.4	0.4808	0.0161	2.444	250	0.61092	0.0264			
16	0.0788	11.0	0.4545	0.0358	2.404	250	0.60092	0.0596	0.0328 ± 0.0232	114.6 ± 81.2	**-0.309**
17	0.0259	11.4	0.4386	0.0114	2.380	250	0.59493	0.0191	Isoproturon 500 µM		
18	0.0288	13.0	0.3846	0.0111	2.252	250	0.56294	0.0197			

APPENDIX 2 Individual Sample Data (Continued)

Benzyloxyquinoline debenzylation male CAR KO Rat Heps **St**

Flask No	FU/min sample	FU std	nmols / 1 FU	nmols 7-OH formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	nmols 7-OH formed / min/mg prot	mean ± SD	% of control	T-VALUE (VS CONTROL)
1	0.0251	6.3	0.7937	0.0199	3.479	250	0.86981	0.0229	0.0250 ± 0.0025	100.0 ± 10.0	
2	0.0263	6.3	0.7937	0.0209	3.497	250	0.87416	0.0239	Control 0 µM		
3	0.0309	6.3	0.7937	0.0245	3.488	250	0.87198	0.0281			
4	0.0224	6.6	0.7576	0.0170	2.940	250	0.73505	0.0231			
5	0.0248	6.6	0.7576	0.0188	3.175	250	0.79374	0.0237			
6	0.0298	6.5	0.7692	0.0229	3.253	250	0.81330	0.0282			
7	0.0288	6.2	0.8065	0.0232	3.288	250	0.82199	0.0283	0.0268 ± 0.0013	107.5 ± 5.3	**-1.469**
8	0.0276	5.8	0.8621	0.0238	3.714	250	0.92849	0.0256	Phenobarbital 500 µM		
9	0.0278	6.7	0.7463	0.0207	3.114	250	0.77852	0.0266			
10	0.0225	6.6	0.7576	0.0170	3.549	250	0.88720	0.0192	0.0231 ± 0.0038	92.4 ± 15.3	**0.786**
11	0.0282	6.3	0.7937	0.0224	3.870	250	0.96762	0.0231	Isoproturon 0.5 µM		
12	0.0312	6.1	0.8197	0.0256	3.810	250	0.95240	0.0269			
13	0.0268	6.4	0.7813	0.0209	3.557	250	0.88937	0.0235	0.0241 ± 0.0016	96.3 ± 6.4	**0.664**
14	0.0327	6.2	0.8065	0.0264	4.079	250	1.01978	0.0259	Isoproturon 5 µM		
15	0.0274	6.2	0.8065	0.0221	3.879	250	0.96979	0.0228			
16	0.0263	6.2	0.8065	0.0212	3.905	250	0.97631	0.0217	0.0219 ± 0.0017	87.8 ± 6.7	**2.175**
17	0.0245	6.3	0.7937	0.0194	3.818	250	0.95458	0.0204	Isoproturon 50 µM		
18	0.0288	5.8	0.8621	0.0248	4.192	250	1.04804	0.0237			
19	0.1108	6.1	0.8197	0.0908	3.818	250	0.95458	0.0951	0.0882 ± 0.0092	353.1 ± 36.8	**-11.704** ***
20	0.1072	5.7	0.8772	0.0940	4.105	250	1.02630	0.0916	Isoproturon 500 µM		
21	0.0906	5.7	0.8772	0.0795	4.088	250	1.02195	0.0778			

Benzyloxyquinoline debenzylation male human heps

Flask No	FU/min sample	FU std	nmols / 1 FU	nmols 7-OH formed /min /ml	Protein mg/ml	volume (ul) mics used	protein mg/ml in cuvette	nmols 7-OH formed / min/mg prot	mean ± SD	% of control	T-VALUE (VS CONTROL)
1	0.0133	8.1	0.6173	0.0082	0.837	250	0.20922	0.0392	0.0457 ± 0.0051	100.0 ± 11.1	
2	0.0142	8.6	0.5814	0.0083	0.837	250	0.20922	0.0395	Control 0 µM		
3	0.0168	8.3	0.6024	0.0101	0.850	250	0.21252	0.0476			
4	0.0187	8.4	0.5952	0.0111	0.883	250	0.22079	0.0504			
5	0.0183	8.4	0.5952	0.0109	0.929	250	0.23235	0.0469			
6	0.0203	8.5	0.5882	0.0119	0.949	250	0.23731	0.0503			
7	0.0541	8.0	0.6250	0.0338	0.797	250	0.19930	0.1697	0.1874 ± 0.0199	410.5 ± 43.7	**-12.122** ***
8	0.0758	8.6	0.5814	0.0441	0.843	250	0.21087	0.2090	Phenobarbital 500 µM		
9	0.0612	8.5	0.5882	0.0360	0.784	250	0.19600	0.1837			
10	0.0167	8.6	0.5814	0.0097	0.830	250	0.20757	0.0468	0.0492 ± 0.0027	107.8 ± 5.9	**-1.376**
11	0.0194	8.3	0.6024	0.0117	0.896	250	0.22409	0.0522	Isoproturon 0.5 µM		
12	0.0185	8.4	0.5952	0.0110	0.903	250	0.22574	0.0488			
13	0.0203	8.4	0.5952	0.0121	0.867	250	0.21665	0.0558	0.0531 ± 0.0069	116.3 ± 15.1	**-1.658**
14	0.0161	8.5	0.5882	0.0095	0.837	250	0.20922	0.0453	Isoproturon 5 µM		
15	0.0218	8.2	0.6098	0.0133	0.913	250	0.22822	0.0582			
16	0.0247	8.4	0.5952	0.0147	0.827	250	0.20674	0.0711	0.0804 ± 0.0092	176.1 ± 20.1	**-6.115** ***
17	0.0325	8.2	0.6098	0.0198	0.886	250	0.22161	0.0894	Isoproturon 50 µM		
18	0.0299	8.4	0.5952	0.0178	0.883	250	0.22079	0.0806			
19	0.0122	7.9	0.6329	0.0077	0.771	250	0.19269	0.0401	0.0432 ± 0.0044	94.6 ± 9.6	**0.755**
20	0.0148	7.9	0.6329	0.0094	0.777	250	0.19435	0.0482	Isoproturon 500 µM		
21	0.0130	8.5	0.5882	0.0076	0.741	250	0.18526	0.0413			

APPENDIX 2 Individual Sample Data (Continued)

RAT HEPATOCYTES - S-Phase by BrdU incorporation (wt male)								
	Total	S-Phase						
DMSO control		cells	**INDEX**	**Mean**	**± SD**	**% control**	**± SD**	
	1347	90	6.6815					
	1358	93	6.8483	6.84	0.20	100.0	2.9	
	1368	93	6.7982					
	1366	98	7.1742					
	1298	87	6.7026					
PB 500uM								
	1401	181	12.9193					
	1382	168	12.1563	12.21	0.68	178.5	9.9	***
	1455	180	12.3711					
	1458	162	11.1111					
	1432	179	12.5000	t-value	**-17.0638**			
Isoproturon 0.5uM								
	1348	103	7.6409					
	1382	130	9.4067	9.82	1.73	143.5	25.3	**
	1429	138	9.6571					
	1364	170	12.4633					
	1431	142	9.9231	t-value	**-3.8275**			
Isoproturon 5uM								
	1475	356	24.1356					
	1461	297	20.3285	25.73	5.39	376.2	78.8	***
	1469	358	24.3703					
	1480	515	34.7973					
	1486	372	25.0336	t-value	**-7.8302**			
Isoproturon 50uM								
	1551	516	33.2689					
	1522	517	33.9685	31.91	1.98	466.4	28.9	***
	1440	417	28.9583					
	1447	467	32.2737					
	1465	455	31.0580	t-value	**-28.1894**			
Isoproturon 500uM								
	937	279	29.7759					
	932	225	24.1416	23.97	3.47	350.4	50.7	***
	964	215	22.3029					
	1039	238	22.9066					
	1259	261	20.7307	t-value	**-11.02**			
EGF								
	1613	458	28.3943					
	1601	470	29.3567	28.60	2.30	418.1	33.6	***
	1588	416	26.1965	**EGF**	+ve Control			
	1649	529	32.0800					
	1572	424	26.9720	t-value	**-21.0793**			

APPENDIX 2 Individual Sample Data (Continued)

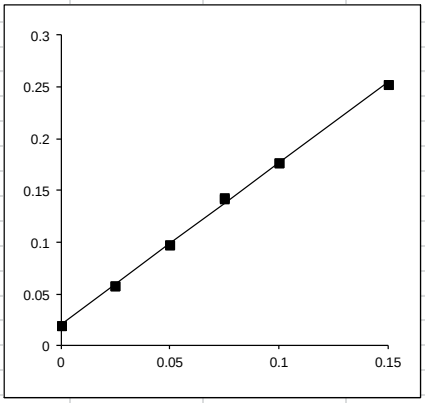
KO RAT HEPATOCYTES - S-Phase by BrdU incorporation (male)								
	Total	S-Phase						
DMSO control		cells	**INDEX**	**Mean**	**± SD**	**% control**	**± SD**	
	1544	95	6.1528					
	1633	121	7.4097	7.99	1.27	100.0	15.9	
	1647	157	9.5325					
	1575	134	8.5079					
	1546	129	8.3441					
PB 500uM								
	1633	157	9.6142					
	1626	125	7.6876	7.91	1.30	99.0	16.2	
	1607	108	6.7206					
	1555	104	6.6881					
	1618	143	8.8381	t-value	**0.0981**			
Isoproturon 0.5uM								
	1570	110	7.0064					
	1639	153	9.3350	6.79	1.69	85.0	21.2	
	1551	89	5.7382					
	1544	75	4.8575					
	1507	106	7.0338	t-value	**1.2631**			
Isoproturon 5uM								
	1563	138	8.8292					
	1601	136	8.4947	7.68	2.21	96.1	27.7	
	1572	162	10.3053					
	1553	86	5.5377					
	1555	81	5.2090	t-value	**0.2753**			
Isoproturon 50uM								
	1607	129	8.0274					
	1607	137	8.5252	7.62	0.64	95.3	8.0	
	1622	120	7.3983					
	1568	110	7.0153					
	1559	111	7.1199	t-value	**0.5836**			
Isoproturon 500uM								
	1588	47	2.9597					
	1576	51	3.2360	3.44	0.51	43.1	6.3	
	1567	48	3.0632					
	1559	60	3.8486					
	1534	63	4.1069	t-value	**7.4191**			***
EGF								
	1642	472	28.7454					
	1623	423	26.0628	28.47	1.55	356.4	19.4	***
	1618	456	28.1829	**EGF**	+ve Control			
	1621	491	30.2899					
	1609	468	29.0864	t-value	**-22.8098**			

APPENDIX 2 Individual Sample Data (Continued)

HUMAN HEPATOCYTES - S-Phase by BrdU incorporation (male)								
	Total	S-Phase						
DMSO control		cells	INDEX	Mean	± SD	% control	± SD	
	1200	4	0.333333					
	1290	4	0.310078	0.32	0.14	100.00	43.89	
	1255	2	0.159363					
	1210	3	0.247934					
	1308	7	0.535168					
PB 500uM								
	1246	4	0.321027					
	1230	3	0.243902	0.27	0.08	86.63	23.89	
	1292	2	0.154799					
	1229	4	0.325468					
	1217	4	0.328677	t-value	0.5982			
Isoproturon 0.5uM								
	1200	3	0.25					
	1263	4	0.316706	0.29	0.08	91.62	25.45	
	1265	5	0.395257					
	1284	4	0.311526					
	1114	2	0.179533	t-value	0.37			
Isoproturon 5uM								
	1173	4	0.341006					
	1189	3	0.252313	0.34	0.07	107.24	21.21	
	1370	5	0.364964					
	1383	6	0.433839					
	1296	4	0.308642	t-value	-0.3323			
Isoproturon 50uM								
	1080	2	0.185185					
	1158	4	0.345423	0.27	0.11	85.52	33.14	
	1249	2	0.160128					
	1225	5	0.408163					
	1166	3	0.25729	t-value	0.5888			
Isoproturon 500uM								
	978	2	0.204499					
	893	1	0.111982	0.16	0.10	49.59	31.98	
	857	0	0					
	871	2	0.229621					
	832	2	0.240385	t-value	2.08			
EGF								
	1311	38	2.898551					
	1352	35	2.588757	2.98	0.29	938.73	90.06	***
	1299	38	2.925327	EGF	+ve Control			
	1189	37	3.111859					
	1368	46	3.362573	t-value	-18.7187			

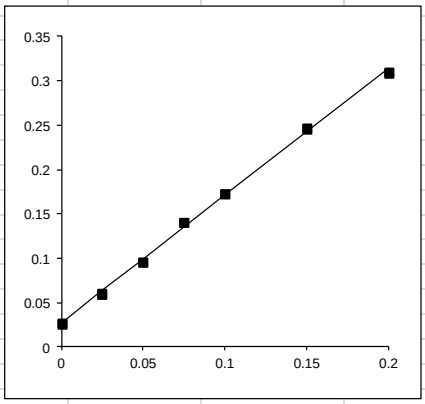
APPENDIX 2 Individual Sample Data (Continued)

Lowry		Male WT Rat Heps							
Correlation coeff		0.99950484	Diln.Factor	25					
Intercept		0.02020714							
Slope		1.56314286							
mg/ml st	O.D. std	Y Calc.				**Protein**			
0	0.020	0.020	**Flask No.**	**O.D.1**	**O.D.2**	**mg/ml**	**Mean**	**SD**	**CV**
0	0.019	0.020	1	0.164	0.164	2.300	0.164	0.000	0.0
0.025	0.058	0.059	2	0.168	0.166	2.348	0.167	0.001	0.8
0.025	0.058	0.059	3	0.172	0.170	2.412	0.171	0.001	0.8
0.05	0.097	0.098	4	0.182	0.182	2.588	0.182	0.000	0.0
0.05	0.097	0.098	5	0.187	0.187	2.668	0.187	0.000	0.0
0.075	0.142	0.137	6	0.182	0.181	2.580	0.182	0.001	0.4
0.075	0.143	0.137	7	0.162	0.162	2.268	0.162	0.000	0.0
0.1	0.177	0.177	8	0.184	0.182	2.604	0.183	0.001	0.8
0.1	0.177	0.177	9	0.178	0.179	2.532	0.179	0.001	0.4
0.15	0.253	0.255	10	0.176	0.173	2.468	0.175	0.002	1.2
0.15	0.252	0.255	11	0.179	0.183	2.572	0.181	0.003	1.6
			12	0.171	0.169	2.396	0.170	0.001	0.8
			13	0.181	0.181	2.572	0.181	0.000	0.0
			14	0.174	0.176	2.476	0.175	0.001	0.8
			15	0.173	0.173	2.444	0.173	0.000	0.0
			16	0.171	0.170	2.404	0.171	0.001	0.4
			17	0.170	0.168	2.380	0.169	0.001	0.8
			18	0.161	0.161	2.252	0.161	0.000	0.0



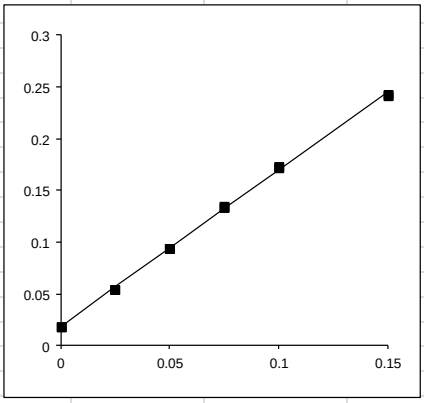
APPENDIX 2 Individual Sample Data (Continued)

Lowry		Male CAR KO Rat Heps							
Correlation coeff		0.99924428	Diln.Factor	25					
Intercept		0.02690419							
Slope		1.43778443							
mg/ml st	O.D. std	Y Calc.				**Protein**			
0	0.026	0.027	**Flask No.**	**O.D.1**	**O.D.2**	**mg/ml**	**Mean**	**SD**	**CV**
0	0.027	0.027	1	0.228	0.226	3.479	0.227	0.001	0.6
0.025	0.059	0.063	2	0.228	0.228	3.497	0.228	0.000	0.0
0.025	0.060	0.063	3	0.227	0.228	3.488	0.228	0.001	0.3
0.05	0.096	0.099	4	0.196	0.196	2.940	0.196	0.000	0.0
0.05	0.096	0.099	5	0.209	0.210	3.175	0.210	0.001	0.3
0.075	0.140	0.135	6	0.214	0.214	3.253	0.214	0.000	0.0
0.075	0.140	0.135	7	0.216	0.216	3.288	0.216	0.000	0.0
0.1	0.173	0.171	8	0.241	0.240	3.714	0.241	0.001	0.3
0.1	0.173	0.171	9	0.206	0.206	3.114	0.206	0.000	0.0
0.15	0.246	0.243	10	0.231	0.231	3.549	0.231	0.000	0.0
0.15	0.247	0.243	11	0.253	0.246	3.870	0.250	0.005	2.0
0.2	0.309	0.314	12	0.246	0.246	3.810	0.246	0.000	0.0
0.2	0.310	0.314	13	0.231	0.232	3.557	0.232	0.001	0.3
			14	0.261	0.262	4.079	0.262	0.001	0.3
			15	0.250	0.250	3.879	0.250	0.000	0.0
			16	0.251	0.252	3.905	0.252	0.001	0.3
			17	0.247	0.246	3.818	0.247	0.001	0.3
			18	0.268	0.268	4.192	0.268	0.000	0.0



APPENDIX 2 Individual Sample Data (Continued)

Lowry		Male hu4246 human Heps							
Correlation coeff		0.99957013	Diln.Factor	10					
Intercept		0.01839286							
Slope		1.51285714							
mg/ml st	O.D. std	Y Calc.				**Protein**			
0	0.018	0.018	**Flask No.**	**O.D.1**	**O.D.2**	**mg/ml**	**Mean**	**SD**	**CV**
0	0.018	0.018	1	0.145	0.145	0.837	0.145	0.000	0.0
0.025	0.054	0.056	2	0.145	0.145	0.837	0.145	0.000	0.0
0.025	0.054	0.056	3	0.147	0.147	0.850	0.147	0.000	0.0
0.05	0.094	0.094	4	0.152	0.152	0.883	0.152	0.000	0.0
0.05	0.094	0.094	5	0.159	0.159	0.929	0.159	0.000	0.0
0.075	0.135	0.132	6	0.162	0.162	0.949	0.162	0.000	0.0
0.075	0.134	0.132	7	0.139	0.139	0.797	0.139	0.000	0.0
0.1	0.172	0.170	8	0.146	0.146	0.843	0.146	0.000	0.0
0.1	0.173	0.170	9	0.137	0.137	0.784	0.137	0.000	0.0
0.15	0.242	0.245	10	0.144	0.144	0.830	0.144	0.000	0.0
0.15	0.243	0.245	11	0.154	0.154	0.896	0.154	0.000	0.0
			12	0.155	0.155	0.903	0.155	0.000	0.0
			13	0.150	0.149	0.867	0.150	0.001	0.5
			14	0.145	0.145	0.837	0.145	0.000	0.0
			15	0.156	0.157	0.913	0.157	0.001	0.5
			16	0.143	0.144	0.827	0.144	0.001	0.5
			17	0.152	0.153	0.886	0.153	0.001	0.5
			18	0.152	0.152	0.883	0.152	0.000	0.0



APPENDIX 3 Protocol

CXR Biosciences Ltd.
2, James Lindsay Place
DUNDEE DD1 5JJ

PROTOCOL

ISOPROTURON TECH – ENZYME AND CELL PROLIFERATTION INDUCTION IN CULTURED MALE WILD TYPE SPRAGUE DAWLEY RAT HEPATOCYTES, MALE CAR-/- RAT HEPATOCYTES ANDMALE HUMAN HEPATOCYTES

CXR STUDY NUMBER:	CXR1449
Sponsor Ref. NoS.	90017747/90017748/90017749
Sponsors:	Agan Chemical Manufacturers Ltd. PO Box 262, Northern Industrial Zone Ashod 77102 Israel
Sponsors Representative:	[redacted]
Study Director: Test Facility	[redacted] Medical School Resource Unit (MSRU), Dundee University, Dundee, DD1 9SY.
Proposed Start Date:	26 May 2014

Approved by:

[redacted]
Study Director

26 May 2014
Study Start Date

Accepted (on behalf of Agan Chemical Manufacturers Ltd.) by:

[redacted]
Signature

2nd June 2014
Date

Accepted (on behalf of CXR) by:

[redacted]
Director

26 May 2014
Date

CIRCULATION

Original: Study File/Archives
Copies to: Study Director
Sponsors Representative (electronic)

Project Licence Details:

This study will be conducted under the "Utility, metabolism and toxicity of compounds (UMT) project licence".

[redacted]

Project Licence Holder or Deputy

26 May 2014
Date

Agreed by Home Office Licensees:
I have read the protocol and understand that to the best of my knowledge, the conditions of my personal licence allow me to carry out the procedures contained within it.

[redacted]

26 May 2014

[redacted]

26 May 2014

1. INTRODUCTION

This study will investigate the proliferation and CYP P450 enzyme inducibility effects of Isoproturon (IPU) tech in isolated male wild type (WT) rat hepatocyte cultures, male CAR KO rat hepatocyte cultures and male human hepatocyte cultures..

2. ANIMALS (Scientific Procedures) ACT 1986 COMPLIANCE

The in-life procedures to be undertaken during the course of this Study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986. The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques, and the issue of project licences for specified programmes of work.

This Study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

Animal Order Number:	60/4440/848
Home office Licensees:	[redacted]

2.1 Licensee Responsibilities

The procedure to be performed in this study is PD2 as detailed in the UMT project licence (60/4440). The severity limit for this procedure is UNCLASSIFIED. [redacted] will complete the Home Office animal returns at the start of the study under the UMT project licence.

3. MATERIALS AND METHODS

3.1 Test and Reference Items

3.1.1 Isoproturon

Name of the Report:	Isoproturon
Chemical name:	3-(4-isopropylphenyl)-1,1-dimethylurea
Appearance:	white/off-white powder
Batch No.:	D8837
Date of Batch Production:	will be given in the report
Purity:	98.5%
Storage:	at room temperature (20-25°C) in the dark and protected from moisture
Expiry Date:	will be given in the report

The reference items phenobarbital sodium salt (PB, P-5178) and epidermal growth factor (EGF, E-9644) will be supplied by Sigma.

Details on the test and reference items and vehicle will be kept in the raw data and included in the study report.

The Test Items will be handled, and their identities verified, according to CXR Biosciences SOP 0010. This SOP describes routine procedures for the receipt, identification, labelling, handling, sampling and storage of Test and Reference Items. These procedures are implemented to ensure that:

- Test and Reference Items used are those Items specified in Study Protocols.
- Handling and storage of Test and Reference Items preserves their identity and integrity during use.

In order for CXR Biosciences to comply with the Health and Safety at Work etc. Act 1974, and the current Control of Substances Hazardous to Health Regulations, the Study Sponsor will be asked to complete a Test Item Data Sheet (TIDS) and provide a Certificate of Analysis for the items supplied. The Sponsor shall provide CXR Biosciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Sponsor to CXR Biosciences. The Sponsor shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Any items remaining after this study will be returned to the Study Sponsor.

3.2 Safety Precautions

The normal safety precautions as detailed in the relevant SOP's and COSHH assessments will apply, no additional precautions are considered necessary.

3.3 Test System

3.3.1. Animals

Rat (*Rattus norvegicus*)

Strain 1: Sprague Dawley (product number TGRSWM4(SD)

Strain 2: CAR KO (SD background, product number TGRS8190HM4)

Sex: male

Age/weight at time of hepatocyte isolation: 10 weeks /300g

Supplier: Sage Laboratories, USA

Acclimatization: On arrival and prior to the start of the study, all rats will be observed to ensure that they are physically normal and that they exhibit normal activity. Animals showing signs of ill health will be excluded from the study.

3.3.2 Animal Accommodation and Husbandry

In the animal room the environment will be controlled to provide conditions suitable for the Sprague Dawley strain of rat. The rats will be housed in groups of 4 on saw-dust in solid-bottom, polypropylene cages (Techniplast Series 119).The temperature will be maintained within a range of 19-23° C and relative humidity within a range of 40-70%. There will be a nominal 14-15 air changes per hour. Twelve-hour periods of light will be cycled with twelve-hour periods of darkness.

The rats will be allowed *ad libitum* access to water for human consumption and RM1 pelleted diet (Special Diet Services Ltd., Stepfield, Witham, Essex, UK) *ad libitum* and will be acclimatised for at least 5 days prior to study. The specification of the diet and the results of the regular communal analysis of the water are held by the Medical School Resource Unit.

4. EXPERIMENTAL PROCEDURES

4.1 Rat Hepatocyte Isolation

Rats will be terminally anaesthetised, using *i.p.* administered euthatal at 150 mg/kg bodyweight, and hepatocytes isolated by *in situ* perfusion according to Mitchell A.M. et al (1984) using Krebs Ringer Phosphate and Krebs Ringer Hydrogen Carbonate buffers and 200mg/L collagenase A (Roche). Viabilities of the preparations, determined by trypan blue exclusion, must be in excess of 80% or the preparations are not used for experimental work. Hepatocytes from two independent perfusions for each strain will be pooled.

4.2 Rat Hepatocyte Culture

Primary monolayer cultures of hepatocytes will be prepared in 25 cm^2 flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz Complete L15 (CL15) (Mitchell et al 1984; Elcombe and Mitchell 1986) as the medium. This medium incorporates foetal calf serum, tryptose phosphate broth, penicillin/streptomycin, insulin, hydrocortisone and L-glutamine. In all 96-well plate cultures the outside wells will not be used, but filled with sterile phosphate buffered saline. The hepatocytes will be cultured in humidified air at a temperature of 37°C; all materials and solutions coming in contact with the cells after isolation will be sterile and pre-warmed to 37°C.

Hepatocytes will be cultured in Leibowitz CL15 (Laboratory Method Sheet (LMS) TIC001) medium for 4h to allow adherence. The medium will then be changed and the hepatocytes exposed to the medium containing phenobarbital sodium salt at 500 µM as an assay control or to test item at 4 concentrations (0.5, 5, 50 and 500 µM) or a vehicle control (0.1% DMSO). The concentrations of the test item to be used were selected with reference to a preliminary range finding study. The media, with test item, will be

replenished daily for a further 3 days. There will be 3 replicates for each concentration in 25 cm^2 flasks for enzyme activity measurements, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements.

Cells will be investigated microscopically for appropriate densitiy and morphology of the cultures before incubation. Only cultures of appropriate density and morphology will be used. The test item will be formulated in DMSO, and administered such that the final DMSO concentration in all cultures will be 0.1%, 1 µL DMSO/mL medium. The pH of the media containing vehicle and highest test dose for each test item will be determined prior to incubation.

In addition to hepatocytes exposed to test items or phenobarbital sodium salt, 5 replicates in a 6-well plate will be exposed to EGF (25 ng/mL) as a positive control for induction of replicative DNA synthesis (S-phase).

4.3 Human Hepatocyte Culture

Primary male human hepatocytes, plateable cryopreserved, will be sourced from Invitrogen, 7 Kingsland Grange, Warrington, Cheshire, WA1 4SR. Viabilities of the hepatocyte preparation, determined by trypan blue exclusion, must be in excess of 70%. Hepatocytes from one donor will be used.

Primary monolayer cultures of hepatocytes will be prepared in collagen coated 25 cm^2 flasks, 96- and 6-well plastic tissue culture plates, using Leibowitz HCL15 (Mitchell *et al* (1984) Arch. Toxicol. **55,** 239-246) as the medium. In all 96-well plate cultures the outside wells will not be used, but filled with sterile phosphate buffered saline.

Hepatocytes will be resuscitated in Cryopreserved Hepatocyte Recovery Medium (CHRM®) then cultured in Cryopreserved Hepatocyte Plating Medium (CHPM) for 4-6 h to allow adherence. The medium will then be changed to Leibowitz HCL15 medium and the hepatocytes will be exposed to the medium containing phenobarbital sodium salt at 500 µM as an assay control or to the test item at 4 concentrations (0.5, 5, 50 and 500 µM) or a vehicle control (0.1% DMSO) as for the rat hepatocytes.

The media, with test/control substances, will be replenished daily for a further 3 days. There will be 3 replicates for each concentration in 25 cm^2 flasks for enzyme activity measurements, 5 replicates for each concentration in 6-well plates for replicative DNA synthesis (S-phase) analysis and 6 replicates for each concentration in 96-well plates for cell toxicity (ATP) measurements.

4.4 Assays

After 96 hours in culture, hepatocytes will be methanol fixed, or harvested by scraping them into SET buffer (0.25 M sucrose, 5 mM EDTA, 20 mM Tris-HCl, pH7.4), sonicating the mixture and storing it at –70°C until analysis. Protein will be determined by the method of Lowry *et al* (1951) using bovine serum albumin standards. (MET-SPEC001).

4.4.1 Adenosine 5'-Triphosphate (ATP)

Hepatocyte toxicity will be assessed following 96 hours of culture as indicated by ATP depletion. Cellular Adenosine 5'-Triphosphate (ATP) will be determined by luminometry according to LMS FLUOR003.

The bioluminescent determination of ATP released from viable somatic cells will be carried out using an assay kit supplied by Promega (CellTitre-Glo luminescent cell viability assay). A comprehensive explanation of this assay and its applications is to be found in the Promega Technical Bulletin No. TB288, which is available online at www.promega.com/tbs.

Results will be expressed as a percentage of the ATP released by control hepatocytes.

4.4.2 Replicative DNA Synthesis (S-Phase)

The number of hepatocytes undergoing replicative DNA synthesis (S-phase) in any given cell population can be determined by the incorporation of BrdU followed by immunostaining. S-phase will be determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. Immunostaining will be performed after fixation at 96 h. The number of hepatocytes undergoing DNA synthesis will be recorded as the labelling index [(no. of labelled hepatocytes /total no. of hepatocytes) x 100].

4.4.3 Pentoxyresorufin-O-depentylation (PROD)

The activity of CYP2B in cultured hepatocytes will be determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin, in the presence of NADPH, as described by Burke et al (1985), according to LMS Fluor-002.

4.4.4 Benzyloxyquinoline-O-debenzylation (BQ)

The activity of CYP3A1 in cultured hepatocytes will be determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline, in the presence of NADPH, as described by GENTEST HTS technical bulletin, according to LMS Fluor-005.

5. STATISTICS

Statistical comparisons between treated hepatocytes and their control group will be

undertaken for all numerical data sets using a 2-tailed (paired) Student's t-test.

6. PROTOCOL AMENDMENTS

Changes to this protocol will be documented as Protocol Amendments signed by the Study Director. Where appropriate and where time allows, Protocol Amendments will be agreed first with the Study Monitor. Deviations to the protocol will be recorded in the raw data and mentioned in the report.

7. DATA STORAGE AND ARCHIVING

The data will be held in a Study File according to CXR Biosciences SOPs 0003 and 0005. On completion of the study, the following items will be retained in the CXR Archive:

- Study protocol and possible amendments
- Study Report and possible amendments
- Study File (including Study Diary)
- Relevant correspondence

All items will be kept for ten years from the date of signing the Study Report unless, in the opinion of CXR Biosciences, the quality of the specimens no longer allows evaluation. At the end of the ten-year period, the Study Sponsor will be contacted and the items either sent to the Study Sponsor or retained by CXR Biosciences under a separate agreement.

8. REPORT

Data will be presented in tabular and graphical formats as appropriate.

On completion of the study a draft report fulfilling US EPA PR notice 2011-02 formalistic requirements will be issued to the Sponsor after scientific peer review within CXR. Once finalised the report will be issued to the Sponsor as follows:

One signed electronic copy and one originally signed unbound hardcopy.

Copies retained by CXR will be as follows:

One signed unbound copy and an electronic copy.

The Study Report will be prepared as described in CXR Biosciences SOP 0004.

9. QUALITY ASSURANCE

Study activities at the Test Facility (CXR Biosciences Ltd and MSRU) will be conducted according to relevant SOPs.

Deviations from this protocol and from company procedures that may occur and that do not affect the quality, integrity or achievement of the study objectives, will be documented in the study records and, where appropriate, detailed in the final report

No claim of GLP compliance will be made for this study.

10. REFERENCES

Burke *et al* (1985) Biochem. Pharmacol. **34**, 3337-3345
Elcombe C.R and Mitchell A.M. (1996). Environ. Health Perspect. **70**, 211-219.
Lowry *et al* (1951). J. Biol. Chem. **193**, 265-275
Mitchell A.M. et al (1984). Arch. Toxicol. **55,** 239-246.