Mechanism Of Action Of Liver Growth Induced By Non-Genotoxic Carcinogens (Peroxisome Proliferators)

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Abstract:

The peroxisome proliferator activated receptor α (PPAR) is a member of the steroid/hormone receptor. Peroxisome proliferators cause non-genotoxic hepatocarcinogenesis in rodents. It is important to clarify the mechanism of action of the peroxisome proliferators in order to provide an assessment of the hazard, of such compounds to humans. It is also known that the peroxisome proliferators begin their actions by inducing hepatic DNA synthesis. Peroxisome proliferators (ciprofibrate) were investigated. Previous work had indicated that two successive doses of ciprofibrate treatment separated by 24hr, 48hr led to two rounds of liver cell replication,

but it was not clear whether the same or different hepatocyte cells were involved in this growth response. To study this phenomenon, histochemical experimental work was undertaken to assess whether the same or different hepatocyte cells were stained during the two rounds of cell division following ciprofibrate treatment. The two histochemical stains used were EdU and BrdU, which are both base-pair analogues that stain nuclei undergoing DNA replication. It was hypothesized that if EdU was used to stain cells at 24 hr and then BrdU at 48 hr, that if the same cells were responding to ciprofibrate treatment then cells would be co-stained by both dyes, whereas if different cells were responding then there would be little or no double staining of hepatocyte cells – instead different cells would be stained. We found that different cells were stained by the two dyes, indicating that ciprofibrate treatment was targeting different cells.

Introduction:

Peroxisome proliferators (PPs) are a class of chemicals that have diverse effects in rats and mice including increased DNA synthesis and peroxisome proliferation. These chemicals act through ligand activation of nuclear membrane receptors termed 'peroxisome-proliferator-activated receptors' (PPARs), which themselves act as nuclear transcription factors. These chemicals cause an increase in the size and number of peroxisome and ultimately lead to hepatocarcinodensis in rodents. PPARα forms a heterodimer with the retinoid x receptor α (RXR) -after being induced by the peroxisome proliferators- which then binds to precise regions on the DNA termed peroxisome proliferator hormone response elements (PPREs) of the targeted genes. Previous work (Figure 1) had indicated that two successive doses of ciprofibrate treatment separated by 24hr led to two

rounds of liver cell replication, but it was not clear whether the same or different hepatocytes were involved in this growth response. To study this phenomenon, histochemical experimental work was undertaken to assess whether the same or different hepatocyte cells were stained during the two rounds of cell division following ciprofibrate treatment. The two histochemical stains used were EdU and BrdU, which are both base-pair analogues that stain nuclei undergoing DNA replication. It was hypothesized that if EdU was used to stain cells at 24 hr and then BrdU at 48 hr, that if the same cells were responding to ciprofibrate treatment then cells would be co-stained by both dyes, whereas if different cells were responding then there would be little or no double staining of hepatocyte cells.

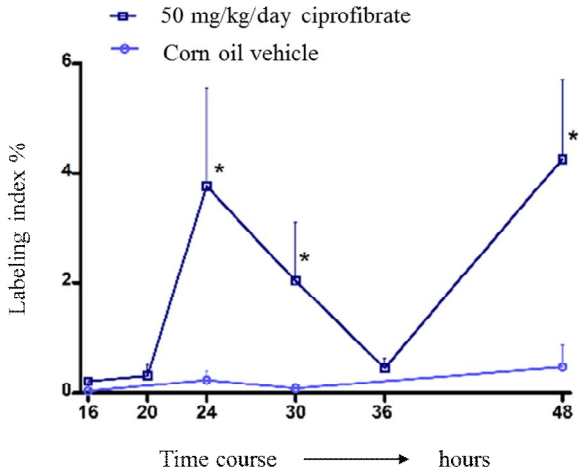


Figure 1; Labelling index of time courses of effect 50 mg/kg/day ciprofibrate. Induction of hepatic DNA synthesis in male F-344/NHsd rats aged 14-15 weeks. Biphasic pattern of DNA synthesis of cell division, one at 24 hr and the other at 48 hr. The rats were gavaged with either corn oil or with ciprofibrate at time Ø and at 24hr. The animals injected i.p with BrdU were then killed, and visualization of BrdU-stained hepatocyte nuclei undertaken. The same procedure was performed at 30, 36 and 48 hr (n=6). The data showed significant differences in percentage cells labelled between the test and the control treated for 24hr and 48 hr (P<0.0001) (Amer, 2011).

Animals and Gavage dosing schedule:

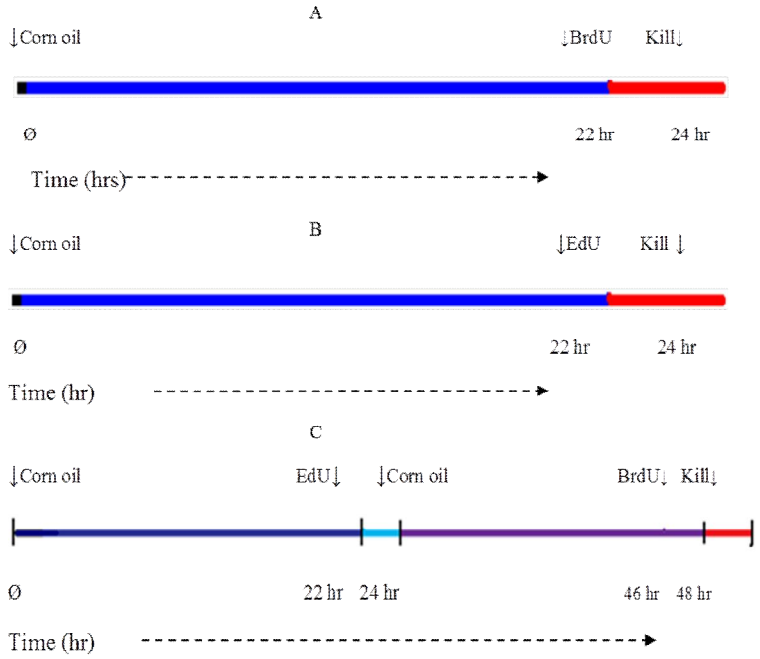
Male and female Fisher (F-344/NHsd) rats were purchased from Harlan UK limited (Bicester, UK). Two groups of rats were purchased, one group aged 8-9 weeks and other aged 14-15 weeks, which were all housed 6 rats per cage. The experiments were performed in accordance with protocols from Scientific Procedures (Act 1986). All animals were given human care handling and husbandry. They were housed in plastic cages in biologically clean rooms with filtered air. Temperature and relative humidity were held at 22 ± 2 ^{0}C and 50 ± 5% respectively and were maintained on a 12-hr light/dark cycle. Rats were maintained on a standard lab diet and purified water with addition of libitum.

It was necessary to optimize protocols for EdU staining. F-344/NHsd rats, aged 8-9 weeks, were injected with three different concentrations of EdU to determine the amount of the EdU required allowing measurement of the labelling index of hepatocytes. 2 mg/kg EdU was found to be an efficient amount for detecting replicative DNA synthesis. BrdU protocol was optimised, primary anti-BrdU antibodies were purchased from two sources, Sigma and Amersham. The primary antibody purchased from Amersham provided clear and reliable results at a concentration of 1:750 µl. The concentration of secondary anti-BrdU antibody (1:50 µl) used was the same as in previous work.

Experimental work was then undertaken to determine if the same, of different hepatocytce cells, were induced to divide during the two rounds of cell division following ciprofibrate treatment. Male F-344/NHsd rats aged 14-15 weeks, were treated with 50mg/kg/day ciprofibrate at time Ø and injected at 22 hrs either with EdU or BrdU, before being killed 2 hrs later. Male F-344/NHsd rats, aged 14-15 weeks were then gavaged with with

50mg/kg/day. Ciprofibrate at time Ø and injected with i.p, EdU at 22 hrs. They were gavaged again with ciprofibrate after 24 hrs and injected with i.p. BrdU at 46 hrs. At 48 hrs, the rats were killed. Liver sections were taken from both the treated animals and control animals and then stained with click-iT EdU and BrdU.

The control vehicle groups:



Figur2;Time course of procedures for treatment of rats in control vehicle groups. Six male F-344/NHsd rats aged 14-15 weeks were gavaged with 20ml/kg corn oil at time Ø and then injected i.p with 2 mg/kg EdU in 5 ml/kg PBS at 22 hr, and were then gavaged again with 20 ml/kg corn oil at 24 hr. At 26 hr the animals were injected with 100 mg/kg BrdU in 10mlkg prior to killing at 48 hr. The body weight was taken before every gavaging and injection treatment

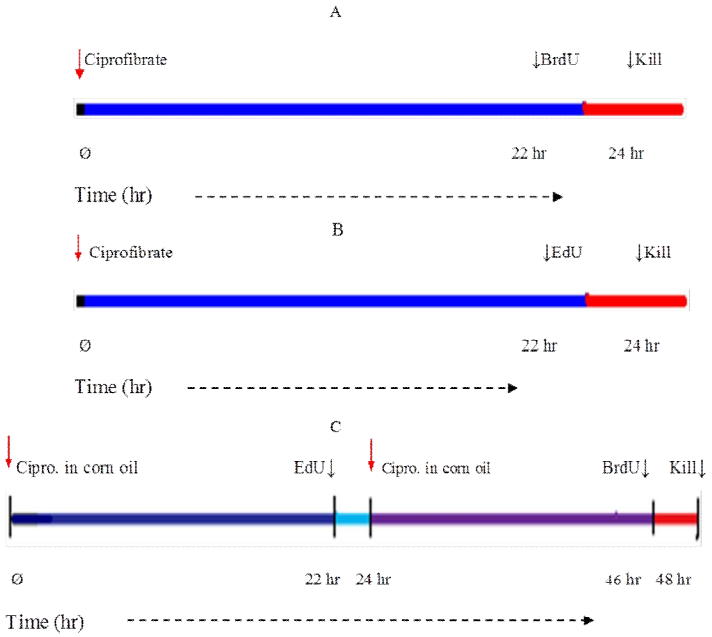


Figure3; Time course of effects of 50mg/kg/day ciprofibrate on hepatic DNA synthesis in male F-344/NHsd rats aged 14-15 weeks. 6 male F-344 rats, each were gavaged with 50 mg/kg/day ciprofibrate was dissolved in 20ml/kg corn oil at time Ø and then injected i.p with 2mg/kg EdU in 5 ml/kg PBS at 22 hrs and then gavaged again with 50 mg/kg/day ciprofibrate was dissolved in 20ml/kg corn oil at 24 hrs. At 46 hrs the animals were injected with 100mg/kg BrdU in 10ml/kg. The body weight was taken before every gavaging and injection treatment.

Results:

BrdU is a synthetic thymidine analogue that is incorporated into DNA during the S phase of the cell cycle. The BrdU immunohistochemistry technique has widespread use (Muskhelishvili, Latendresse et al. 2003). However, it was still necessary to check that the protocol worked properly. Two male F-344/NHsd rats aged 7-8 weeks were injected i.p with 100 mg/kg BrdU 2 hrs before killing. Immunohistochemistry for the BrdU paraffin section technique was used to stain the liver and colon sections as follows: stain with 1:1000 µl, 1:750 µl

and 1:500 µl of primary anti-BrdU body with the diluent buffer. The same concentration of secondary antibody (1:50 µl) was used for all the different concentrations of primary antibodies. The results showed that using 1:750 µl of primary antibody with 1:50 µl of secondary antibody gave the best labelling index (Figure 4). The protocol was able to restrict incorporated BrdU to the cell nuclei with a good signal-to-noise ratio.

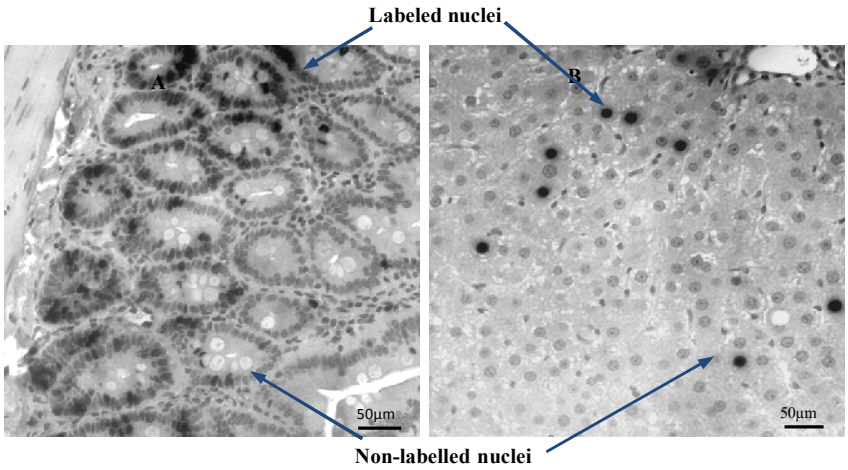


Figure4; Assessment of BrdU immunohistochemistry protocol: A; colon section and B; liver section. BrdU labelling was stained with immunohistochemistry protocol with primary and secondary (Bio-Rad) anti-BrdU antibodies using Amersham cell proliferation kit as described in section (2) materials and methods. Liver and colon sections were harvested from an animal which was injected i.p with 100mg/kg BrdU. The concentration of primary anti-BrdU was 1:750, and the concentration of secondary anti-BrdU was 1:50. The slides were examined under light microscope at magnification (400X). The micrographs were taken with LucaEM Camera DL-604. Scale bar = 50µm.

Investigation of EdU doses to monitor hepatic DNA synthesis:

Three different concentrations of EdU were checked, based on its ability to detect hepatic DNA synthesis, with the aim of determining the lowest dose to reduce the severity of impact on rat cells. Eight male F-344 rats at age 7-8 weeks were brought from Harlan. The rats were divided into

4 groups and each two of them were injected intraperitoneal (i.p) 2 hours prior kill with 100 mg/kg BrdU in PBS or 2, 5 or 25 mg/kg EdU in PBS respectively. 2 hours after injection the rats were sacrificed and animals body weight (data not shown), and liver weight to body weight ratio % was determined. Liver tissues were put in formaldehyde fixative with a part of colon included as a positive control for immunohistochemistry. The results showed that all three concentrations of EdU yielded acceptable results, with a significantly higher liver weight to body weight ratio detected when using 25 mg/kg EdU concentration (Figure 4). The average liver weight to body weight ratio (%) in the rats dosed with BrdU was is 4.3 ± 0.04 and the average of the three dosing EdU concentrations were 4.27 ± 0.06, 4.3 ± 0.12 and 4.65 ± 0.3 respectively.

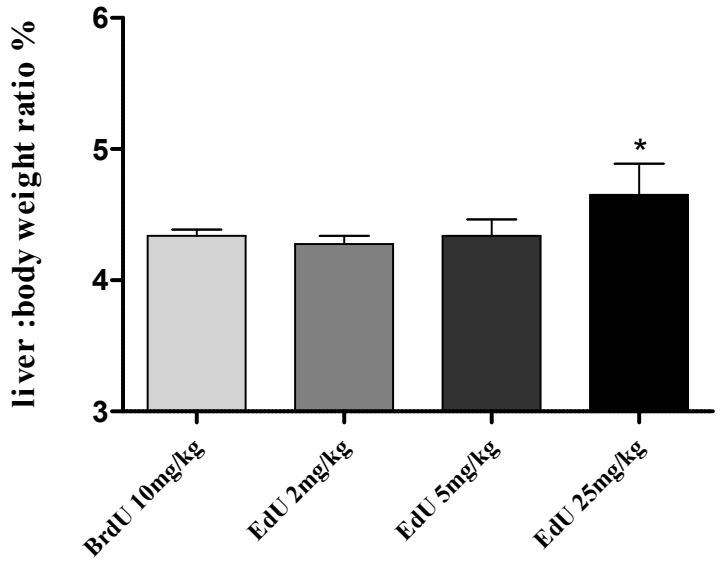


Figure 5; Liver weight to body weight ratio (%) of three different EdU concentrations.

Male F-344NHsd rats aged 7-8 weeks, were injected with either BrdU or EdU and killed after 2 hours. All data are expressed as mean ± SD, statistically not significant difference from BrdU to EdU groups. (n=2) except EdU 25mg/kg concentration which exhibited a significantly different liver weight to body weight ratio (starred) compared with all concentrations of EdU and BrdU. Statistics performed one way ANOVA test using Tukey's multiple comparisons test (P<0.05).

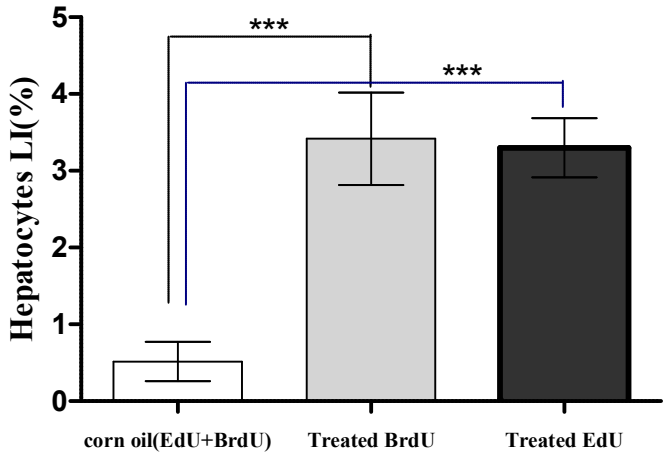


Figure 6; Time course of effect of ciprofibrate in F-344/NHsd rats, comparing between treated groups of EdU and BrdU. The diagram shows the comparison of labelling indices of treated groups which injected with EdU or BrdU against control. Groups of six male F-344 rats aged 14-15 weeks were dosed with 50 mg/kg/day ciprofibrate or corn oil (vehicle) at time Ø ad then injected either i.p with 100 mg/kg BrdU at (22h) or 2 mg/kg EdU two hours prior to kill(24h). Control and treated groups were statistically different from each other. All the data were expressed as mean ± SD, (n=6) whereas control group showed mean 0.51 ± 0.25, mean of EdU and BrdU groups were 3.4 ± 0.6 and 3.29 ±0.38 respectively. Significant analysis was determined by Dunnett's Multiple Comparison Test. * Significant difference between control and both treated groups, P < 0.05, One way ANOVA.**

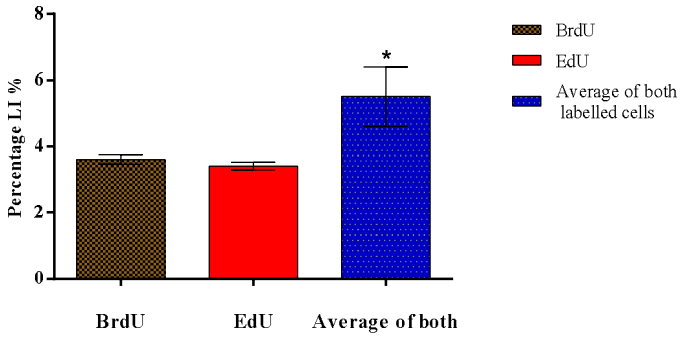


Figure 7; Percentages of labelled hepatocytes of F-344 liver rats induced with 50% ciprofibrate. Three groups of animals were gavaged with ciprofibrate and then injected either BrdU or EdU. The third group is EdU and BrdU (dual-labelled) at 24 h and 48h. Dual-labelled was performed for paraffin liver section of 48h (n=6). Statistically the difference not significant between EdU with BrdU Also statistically different from single labelled to dual labelled. Statistics was done with one-way analysis of variance (Tukey's multiple comparison test), P<0.05. All the data was expressed as mean ± SE, (n=6).

A t-test showed no significant differences in the labelling index of hepatocytes between BrdU and EdU staining in both the treated and a control group.

Subsequent analysis showed that the cells undergoing division at 24 hrs were not related to those that undergoing division at 48 hrs.

A key aim of the present study was to try and find out if ciprofibrate causes the same cells to divide repetitively, or whether different cells divide at different treatment times. To investigate these questions, two groups of six male -344/NHsd rats were used. The animals were gavaged with either corn oil (control vehicle group) or ciprofibrate (treated group) at time Ø. Both groups were then injected i.p with 2 mg/kg EdU at 22 hr. At 24 hr the animals were gavaged again with either corn oil or Ciprofibrate. At 46 hr the animals were injected i.p with 100 mg/kg BrdU. At 48 hr the animals were killed. After fixation the samples underwent tissue processing, where sections were sliced to a thickness of 4.5µm. Then successive tissue slices were stained i.e. these were slices directly in contact with each other, and were expected to pass through the same hepatocytes. The first of these slices was stained with click-iT EdU and second one was stained with BrdU. Staining was performed separately to allow for the different staining chemical procedures, which meant that double staining of the same tissue slice was not feasible (results not shown). The two micrographs were then superimposed together using the program Photoshop CS4 (Adobe). This experiment was performed with liver sections taken from both control and treated rats. Gut sections were used as a positive control.

The results revealed the key observation that the cells which divide at 24 hr are different from those which divide at 48 hr in both control and treated liver rat tissues (Figures 8). This result was consistently obtained in at least three independent experimental groups of animals.

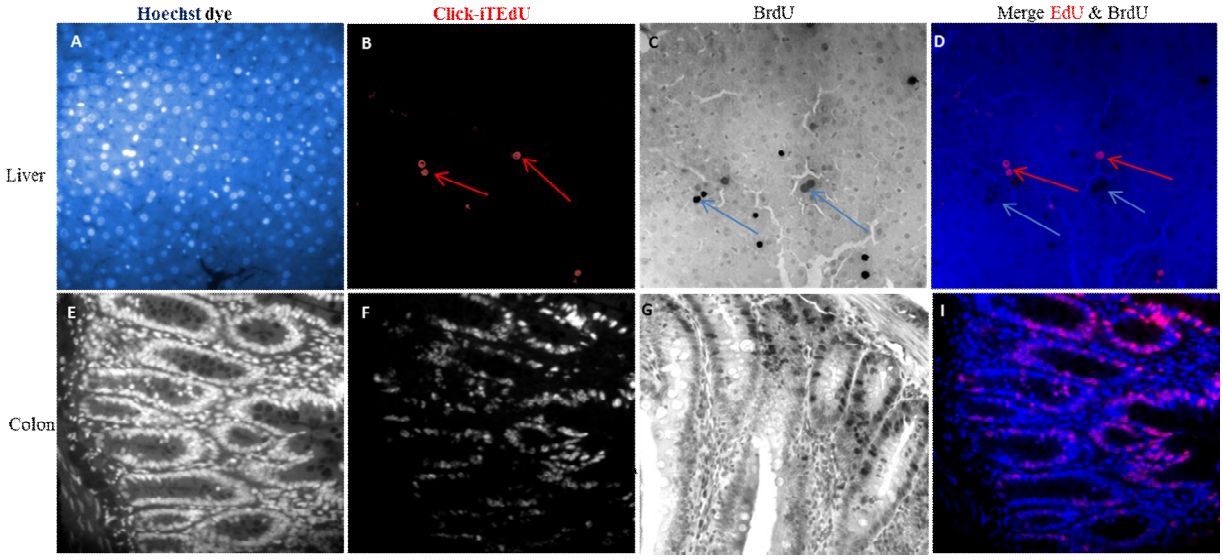


Figure8 Using EdU and BrdU staining to investigate whether the two peaks of cells at 24 hrs and 48 hrs are related after treated with ciprofibrate. The figure shows slides prepared from male F-344/NHsd rats aged 14-15. A) displays nuclei detected with Hoechst dye and A) same micrograph after coloured with the Photoshop programme. B) shows the replicative cells were detected with click-iT EdU. C) Replicative cells were detected with BrdU stain. D) Shows EdU and BrdU stains together after merge with the Photoshop programme. The replicative cells detected with BrdU stain appeared in black spots, whereas the click-iT EdU protocol detected the replicative cells as red spots. The animal was killed at 48 hrs. E, F, G and H sections harvested from colon used as positive control. The programme Photoshop was used to merge the two micrographs together. The micrographs were taken with a Luca^{EM} Camera DL-604 using an exposure time of 100 ms and a medium setting (2) for binning at magnification (400X). Red arrows indicate to detected replicative DNA synthesis by EdU marker, blue arrows indicate to replicative DNA synthesis detected with BrdU marker.

Discussion:

A main objective is determine whether particular liver cell populations respond to repeated treatment by the peroxisome proliferator (ciprofibrate), or whether independent cell populations respond to treatment. In order to achieve these objective various experimental works was undertaken as discussed below.

Rationale and method validation

Optimisation of Hoechst dye concentration

A histochemical approach was utilised in order to determine whether the two peaks of cell division observed by Amer (2011) following repeated ciprofibrate treatment (one at 24 hr and the other at 48 hr, following treatment at Ø and 24 hr) were related. The two histochemical and immunohistochemical stains used were EdU and BrdU, which are both base-pair analogues that stain nuclei undergoing DNA replication as a result of incorporation into DNA during novel DNA synthesis (Cappella, Gasparri et al. 2008). It was hypothesized that if EdU was administered to animals at 24hr which had been pretreated with ciprofibrate at Ø hr, then this stain would be incorporated into nuclei of dividing hepatocyte cells at 24hr. If animals were then treated with a second dose of ciprofibrate and then allowed to live for a further day, then administration of BrdU at 48 hr would be expected to lead to incorporation of the latter stain into nuclei of dividing hepatocyte cells at that stage. Thus, if the same cells are responding to ciprofibrate treatment then cells would be co-stained by both dyes, whereas if different cells were responding then there would be little or no double staining of hepatocyte cells.

In order to achieve a good background to noise ratio staining by Hoechst dye was optimised before being used to reveal the total number of nuclei. Meanwhile EdU was used to detect specifically cell nuclei undergoing replicative DNA synthesis. Many studies have investigated the relative fluorescence intensity of Hoechst dye, e.g Lalande and Miller (1979) examined concentrations from 1µg to 20µg/ml. A reduction of Hoechst 33258 dye (Bisbenzimide) concentration to below 1µg/ml has

been linked to decreased background fluorescence (McGowan, Kurtis et al. 2002).

Hoechst dye at a concentration of 2µg/ml was found to accurately and clearly detect nuclei in liver cells prepared from adult rats (Lamas, Chassoux et al. 2003).

In this study, 5 different concentrations were examined, from 0.1µg/ml to 50µg/ml, to reveal the effect of these concentrations on fluorescent signal. A Hoechst dye stock of 5µg/ml was prepared using distilled water then PBS was used to make the five concentrations. Stock solution was stored at 4 ^{0}C and protected from light. The experiments show that using 1.0 µg/ml dye concentration gave the best results as background fluorescent was reduced. In order to obtain good background to noise ratio, the washing solution (PBS) was optimised as well. The results show that a concentration of 0.2M NaCl was better than 1M NaCl. In addition, the time of washing was optimised concluding that 2 X 5 min gave the best results. It was also found that Hoechst dye gave much better results in the presence of moisture so a small chamber was designed to meet this condition (Figure 3.7).

Assessment of immunohistochemistry protocol:

BrdU and IHC techniques are widely used as an experimental procedure to label DNA synthesis within replicative nuclei and have been used in a variety of studies of cell biology including investigations of cell proliferation of liver growth *in vitro* and *vivo* (McGinley, Knott et al. 2000; Ueda, Saito et al. 2005). BrdU is incorporated into DNA during DNA synthesis of cell proliferation during the S-phase of the cell cycle. In this study BrdU Immunohistochemistry for the BrdU paraffin section technique

was used to stain liver and colon sections, the latter included as these undergo much cell division so provide a suitable control for cell and nuclei staining. Primary anti-BrdU antibodies purchased from Amersham and Sigma were assessed at three concentrations 1:1000 µl, 1:750 µl and 1:500 µl. The same concentration of secondary antibody 1:50 µl was used for all the different concentrations of primary antibodies. Primary anti-BrdU antibodies were applied to positive sections and incubated for 45 min. Negative control section were held in 0.2M PBS during the primary incubation. The secondary antibody (Bio-Rad laboratories) was applied to all sections (positive and negative) and incubated for 30 min. Using 1:750 µl of primary antibody with 1:50 µl of secondary antibody gave the best labeling index. These optimisations were concordant to several former studies, where in most of them the secondary antibody used was the same as the above concentration, but different results with the primary antibody were obtained depending on the source of purchase (Connolly and Bogdanffy 1993; Constan, Sprankle et al. 1999; Ezaki, Yoshida et al. 2009; Ross, Plummer et al. 2010).

Optimisation of the dose of the EdU:

EdU was used as stain to detect DNA synthesis in proliferating cells during cell division. EdU is incorporated into DNA of nuclei of proliferating cells and can be subsequently detected by fluorescence resulting from an antibody bound copper azide (Salic and Mitchison 2008). In particular, the click-iT EdU reaction relies on incorporation during DNA replication – with the terminal alkyne group of EdU containing a thymidine analogue which replaces methyl groups in DNA. Then the terminal alkyne group can be detected via reaction with a

fluorescent copper azide. EdU staining has been utilised in a variety of different eukaryotic cells and has been incorporated strongly into the DNA of proliferating mammalian cells. For example, Salic *et al* (2007) injected EdU into an adult mouse i.p with 100µg of EdU in PBS. This resulted in staining of cells of the small intestine, with red nuclei observed of cells that had been in S-phase (Salic and Mitchison 2008). In this project three concentrations of EdU were tested to establish an optimal dose of EdU. A group of six animals was injected with 2, 5, and 25 mg/kg EdU (n=2 per dose). All of the EdU concentrations gave acceptable staining, therefore the lowest concentration 2 mg/kg was used. Indeed, using the this small amount lowest concentration of EdU showed no signs of EdU toxicity, which confirms results of Cappella in his *vivo* study in adult mice (Warren, Puskarczyk et al. 2009).

Using base analogue stains EdU and BrdU:

It was necessary to make sure that the hepatic DNA synthesis which was detected by both EdU and BrdU with immunohistochemistry was at the same level i.e. to ensure that these stains detected the same cells, and same number of cells, rather than providing artifactual results. Comparisons of BrdU and EdU histochemistry showed no statistically significant differences. Thus, click-iT EdU incorporated into DNA synthesis of cells in the same manner as BrdU, revealing that all cells showed similar labelling indices with both stains (Nwe and Brechbiel 2009). The EdU showed high efficient fluorescence without the need for denaturation, unlike BrdU which required all slides to be denaturated (Warren, Puskarczyk et al. 2009). A study by Cappella showed that labelling with EdU gave slightly increased staining compared to BrdU

(Cappella, Gasparri et al. 2008). The finding is in disagreement with this project, because there were no statistically significant differences between EdU and BrdU stains. Indeed, on the contrary, the labelling with BrdU showed a slightly increased, although this was not statistically significant.

Use of both EdU and BrdU to distinguish if the two peaks of DNA replication and cell division are related:

This project was initiated to test the underlying hypothesis that certain hepatocytes might have an increased ability to divide, due for instance to increased expression of specific genes, which might therefore make these hepatocytes, more susceptible to carcinogens. If so these cells would be more likely to undergo division at two different times following toxin treatment. To determine whether ciprofibrate causes the same cells to divide repetitively, or whether different cells divide at different times, two different stains were used. Liver and gut tissues of 4.5 μm thickness were prepared. The first and second slices of tissue were taken, the first slice was stained with click-iT EdU and second one was stained with BrdU separately. The two micrographs were then superimposed together using the programme Photoshop. This experiment was performed with liver sections taken from both control and treated rats. The results revealed the key observation that the cells which divide at 24 hr are different from those which divide at 48 hr in both control and treated liver rat tissues as displayed in Figures (8). In other words, ciprofibrate does not cause the same cells to divide repetitively but instead different cells divided at different times. This suggests that that there is no specific type of hepatocytes which have a particular physiological character, such as increased expression of certain genes, that makes them more likely to

divide at the different times and be susceptible to carcinogens. This finding is consistent with that of Gournay, 2002 who suggested that only mature hepatocytes have the ability divide. (Gournay, Auvigne et al. 2002).

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