

BENOXAPROFEN MODIFIED LIVER PROTEINS IN MICE AND RATS AFTER A SINGLE TREATMENT

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Earlier studies have proposed that the formation of protein adduct may have a role in the mechanism of hepatotoxicity of non-steroidal anti-inflammatory drugs (NSAIDs). The objective of the present study was to investigate the pattern of protein adducts induced by a hepatotoxic NSAID, benoxaprofen [BNP, Operan (UK); Oraflex (USA)] in mice and rats after single dose. To investigate this further, specific polyclonal antibody has been raised in rabbits against BNP-modified keyhole limpet haemocyanin. Antibody titres from different bleeds were monitored by Enzyme-Linked Immunosorbent Assay (ELISA) and the specificity and sensitivity of the antibody were characterised further by SDS-PAGE and immunoblotting. Mice and rats were given single doses of BNP; their liver were excised, and subcellular fractions were prepared by differential centrifugation. The subcellular fractions were subjected to SDS-PAGE and probed for BNP-adducts by immunoblotting. In addition, liver sections were examined histologically and adducts localisation by immunohistochemistry. Various protein adducts ranging from 60 – 200 kDa were detected with a single major adduct of 110 kDa, observed in the 600 x g fraction that were expressed in a dose dependent manner. In mice, these adducts were found to be localised in the bile canalicular membrane regions of the liver. However, in rats, they were found in the nuclei of the hepatocytes. Hepatotoxicity was not observed in both mice and rats although formation of protein adducts were observed. These data suggested that hepatotoxicity is not simply triggered by adduct formation *per se*. Additional events are required before toxicity becomes apparent.

Keywords: Benoxaprofen, Hepatotoxicity, Protein Adducts

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) have a wide range of adverse drug reactions. Their principal side-effects are in the guts and kidneys (Murray and Brater 1993). Adverse reactions affecting the liver (Zimmerman 1998), skin (Girschick 1999) and blood (Miescher 1986) have also been reported. The mechanism(s) of NSAID-induced hepatotoxicity remain obscure. Clinical evidence suggest that it may be immune-mediated as some patients have rashes, fever, and/or eosinophilia, features indicating a hypersensitivity reaction (Pirmohamed *et al.* 1992). The absence of these biomarkers in some patients has raised the possibility of active metabolites that may be responsible (Boelsterli 1995).

Benoxaprofen (BNP), an arylpropanoic acid derivative, was introduced to the UK market in April 1980. It was considered to be advantageous

especially to the elderly in treating rheumatoid arthritis (Cashin *et al.* 1977). It has a distinctive pharmacological profile as an anti-inflammatory agent. Its weak inhibition of cyclooxygenase (COX) explains the lower incidence of gastrointestinal tract disturbance compared to other NSAIDs (Ridolfo *et al.* 1980). The long plasma half-life of the drug in human allows the drug to be administered once daily, which makes it more convenient for elderly patients (Chatfield and Green 1978). Almost all reports indicate that BNP was very effective in arthritic patients (Jones *et al.* 1980; Berry *et al.* 1980). Later, however, a number of adverse reactions were seen, including photosensitivity reactions, which accounted for approximately 70% of all the adverse effects reported (Halsey and Cardoe 1982). As the number of patients treated increased, a number of unexpected cases of jaundice and hepatotoxicity were seen especially in the elderly, despite the lack of hepatic adverse effects in laboratory animals (Taggart and Alderdice 1982). Histological findings revealed severe canalicular and hepatocellular cholestasis (Fisher and McArthur 1982). The drug was withdrawn from the market in 1982 following the deaths of a number of elderly patients with impaired renal function from cholestatic jaundice (Taggart and Alderdice 1982).

In recent years, various studies have suggested that protein adduct following NSAIDs administration is involved in the aetiology of its hepatic adverse effect (Paulus 1982; Boelsterli *et al.* 1995; Zimmerman 1998). Thus, specific antibodies to NSAIDs modified proteins have been used to probe the mechanism of formation, intracellular and intrahepatic localisation of these adducts. In this study, we report on the development of a rabbit polyclonal antibody to BNP-modified keyhole limpet haemocyanin (KLH), and its use to investigate the formation of BNP-modified protein adducts in the liver of mice and rats treated with BNP.

METHODS

Production of Antisera

BNP-modified protein conjugates was synthesised by covalently coupled BNP to KLH, or rabbit serum albumin (RSA) by the two-step carbodiimide conjugation method of Wade *et al.* (1998). Female New Zealand white rabbits (2.5–3.0 kg) were immunised subcutaneously at more than 6 sites over the back of each rabbit with BNP-KLH conjugate in Freund's

complete conjugate adjuvant. Thereafter, booster injections of 1 ml of conjugate containing 50% (v/v) Freund's incomplete adjuvant were given after two weeks and as previously described. Tests bleed (20 ml) were obtained a fortnight later from the marginal ear vein. Serum was produced from the blood and antibody level was monitored by ELISA. Finally, when a satisfactory antibody titre was achieved (Wade *et al.* 1998), the rabbits were anaesthetized and exsanguinated by cardiac puncture. Clear serum was produced from the collected blood and stored in 2 ml aliquots at -20°C . The antibody titre was determined by ELISA and the assay was carried out as described by Kenna *et al.* (1984), in 96 well polystyrene microtiter plates (Immulon 4, Dynatech Laboratories Inc.).

Treatment of Animals

All animals used were acclimatised for 7 days before use. They had free access to food and water. Groups of 3 female CD1 mice (20–25 g) were dosed intraperitoneally with 5, 10, 15, 100 or 200 mg/kg of BNP, dissolved in 10% (v/v) DMSO/tricaprylin. The control group was given 10% (v/v) DMSO/tricaprylin. The volume of all doses given was 250 μl per mouse. After 6 hours, animals were sacrificed by cervical dislocation and their livers were immediately removed.

Male Sprague-Dawley rats (200–250 g) were also divided into groups of three and dosed with 30 and 200 mg/kg of BNP, dissolved in 10% (v/v) DMSO/tricaprylin given at 500 μl per rat, intraperitoneally. The control group received the dose vehicle given in the same manner. After 6 hours, the animals were sacrificed by cervical dislocation and their livers were immediately removed. The median lobe was removed, fixed in 10% formal saline and processed for histological analysis according to routine procedures.

Subcellular Fractionation of Liver

Liver subcellular fractions from mice and rats were prepared essentially according to the protocols described by Wade *et al.* (1998).

Sodium-Dodecyl-Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

SDS-PAGE was performed according to the method of Laemmli (1970) using a Mini-PROTEAN® II Electrophoresis Cell apparatus from BioRad (UK) with 3% stacking gel/8% resolving gel, using a discontinuous buffer system. Competitive antibody binding inhibition studies was carried out in order to determine the specificity of the antibody in recognizing BNP as the antigen. Essentially, before incubation with the nitrocellulose membrane, the primary antibody was incubated with various concentrations of inhibitors in wash buffer at 4°C overnight. Subsequent developments were carried out as previously described (Wade *et al.* 1998).

Histological Analysis

Freshly isolated liver samples were fixed, blocked in paraffin wax, sectioned and mounted on poly-(L-lysine) coated glass slides. Sections were stained with H & E and analysed under the light microscope.

Immunohistochemical Analysis

Sections from the median lobe of the liver were taken and fixed into formal saline [10% (v/v) formalin, 1.5 mM NaCl]. These were embedded into paraffin wax, sectioned and mounted onto microscope slide. All of the subsequent steps were carried out at room temperature and as described by Hargus *et al.* (1995).

RESULTS AND DISCUSSION

Monitoring the Antibody Titre Against BNP-KLH Conjugate by ELISA

The antibody response was monitored by ELISA using BNP-modified RSA or unmodified RSA as coating antigens. The level of antibody was monitored over time from all test bleeds using BNP-modified RSA conjugates to screen off the antibodies directed against the carrier protein, KLH (Pohl 1993). Each bleed tested was compared with the corresponding pre-immune sera, verifying the level of recognition on BNP-modified RSA. The antibody response found to have increased with time and number of

booster injections. The second bleed sera showed the best antibody response compared to all test bleeds. Significant response was only seen towards the respective BNP-RSA conjugate, and not toward unconjugated RSA or PBS (phosphate buffer saline). A very low level of immunoreactivity of the pre-immune sera was detected, showing that the rabbits were not already generating antibodies against BNP-modified proteins before immunisation. The pre-immune sera showed very low recognition to BNP-modified RSA conjugate, unmodified RSA and PBS (Fig. 1).

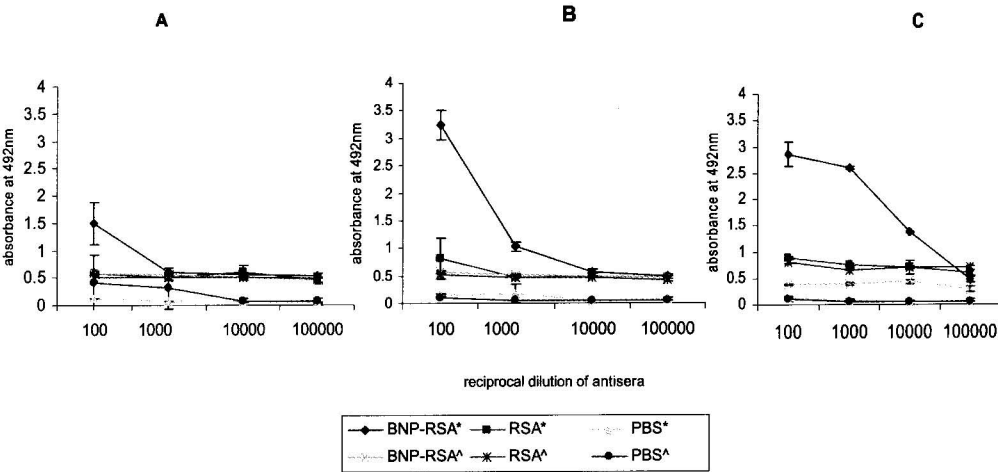


Fig. 1: Monitoring antibody titre of different bleeds obtained from rabbits immunised with BNP-KLH conjugate. Serial dilutions of *anti (BNP-KLH) or ^pre-immune sera were added to the 96 well ELISA plates, and pre-coated with BNP-RSA conjugate, unmodified RSA or PBS. The binding of primary antisera was detected by secondary antisera conjugated with HRP, and the reaction was developed by the addition of *o*-phenylenediamine and the absorbance was measured at 492 nm. (A) first, (B) second and (C) third bleed.

Screening of the Potency of Different Bleeds of Antisera Against BNP-KLH Conjugate by Western Blotting

There was an increase in antibody activity following each booster injection. Final bleed serum (bleed 3) showed the best recognition of protein adducts generated *in vivo* in mice given BNP. Protein adducts ranging from 60 – 200

kDa were detected in the whole liver homogenate of BNP-dosed mice with 110 kDa as the major protein adduct. No protein adducts were detected in the liver of the control mice receiving 10% (v/v) DMSO/tricaprylin. No protein adduct was detected when using pre-immune serum. This indicates the absence of antibodies towards BNP-modified proteins in the pre-immune serum (Fig. 2).

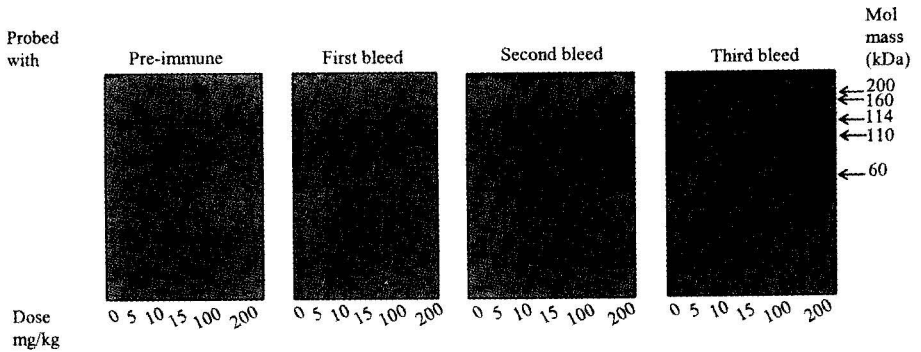


Fig. 2: The detection of BNP-protein adducts by anti (BNP-KLH) antisera obtained from different bleeds. Whole homogenate (50 mg/lane) was prepared from liver of mice treated with a single dose of BNP (0–200 mg/kg). Proteins were separated by SDS-PAGE and electroblotted to the nitrocellulose membrane. Anti (BNP-KLH) antisera (1 : 10,000) of different bleeds were used to detect BNP-protein adducts. Bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG secondary antiserum (1 : 10,000) and developed with Pierce SuperSignal substrate reagents. The adducts were visualised by exposing the membrane to HyperFilm®.

When different bleed sera were used to detect BNP protein adducts *in vivo*, the final bleed showed the highest titre. The discrepancies in antibody titre and specificity between ELISA and immunoblotting may be due to the nature of antigen being used. In ELISA, synthetically modified RSA was used, while proteins that were modified *in vivo* were detected in immunoblotting. The proteins modified *in vivo* may contain more epitopes recognized by anti BNP-KLH polyclonal antisera than synthetic conjugates.

Determination of Cross-Reactivity Between Different Antibodies in the Detection of BNP-Modified Protein Adducts.

To further characterise the specificity of the adducts detection, the nitrocellulose membrane was probed with antisera raised against NSAID-modified KLH conjugates. No specific protein adducts were detected by any of the antisera tested. Diclofenac-KLH antibody, which detected the diclofenac-modified 110 kDa protein in the bile canalicular plasma membrane in both mice and rats (Wade *et al.* 1998) also failed to detect the adducts formed following BNP treatment (results not shown). The results showed that the protein adducts were only detected by (BNP-KLH) antiserum (Fig. 3).

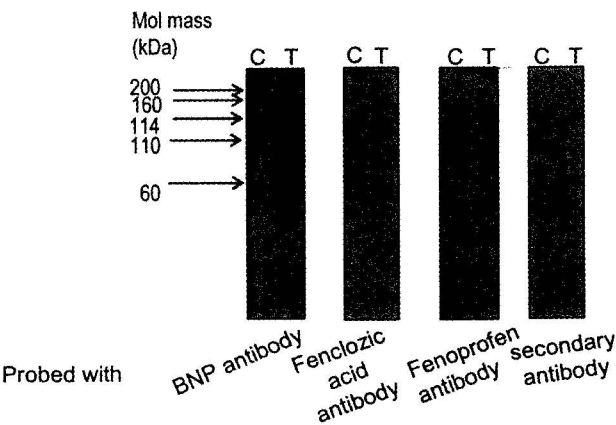


Fig. 3: Cross-reactivity study of anti (BNP-KLH) antiserum. The nitrocellulose membrane was probed with antisera raised against fenclozic acid and fenoprofen-modified KLH. Cross recognition of the secondary antibody used was also investigated. Bound antisera were visualized using Pierce SuperSignal substrate reagents. C: 600 × *g_{av}* fraction from control mice, T : 600 × *g_{av}* fraction from BNP-treated mice at 200 mg/kg.

Intracellular Localisation of BNP-Modified Proteins in Liver of Female CD1 Mice Treated with A Single Dose of BNP

Subcellular fractionation of crude homogenate of mice liver treated with BNP showed that the 110 kDa adducts was abundantly detected in the 600 × *g_{av}* fraction. Adducts of apparent molecular weight of 60, 114, 160 and 200 kDa were also seen in the 600 × *g_{av}* fraction (Fig. 4). At 6 h post-dosing,

a very low level of adducts were detected in subcellular fractions from rats given 30 mg/kg dose. Following the 200 mg/kg dose, a variety of protein adducts were detected. The 110 and 200 kDa adducts were densely expressed in the $600 \times g_{av}$ fraction. Adducts of 55, 85 and 130 kDa were clearly detected in the microsomal fractions (Fig. 5). Free BNP inhibited the anti BNP-KLH antibody and blocked the detection of adducts in a concentration dependent manner in both mice and rats, confirming the specificity of adducts detection.

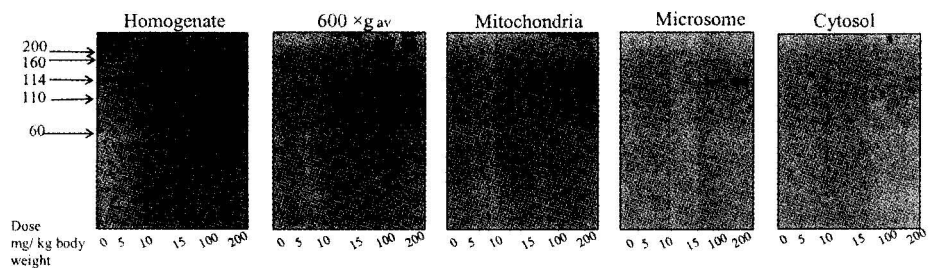


Fig. 4: Subcellular localisation of BNP-protein adducts in mouse liver. Whole liver homogenate, $600 \times g_{av}$ fraction, mitochondrial, microsomal and cytosolic fractions were prepared from livers of mice treated with BNP (0–200 mg/kg) and screened for BNP-modified protein using anti (BNP-KLH) antisera (1: 10,000). 50 mg/lane protein were resolved by SDS-PAGE and immunoblotted using specific BNP-modified KLH antisera. Bound antibodies were detected using HRP conjugated goat anti-rabbit antisera and visualised using Pierce SuperSignal substrate reagents.

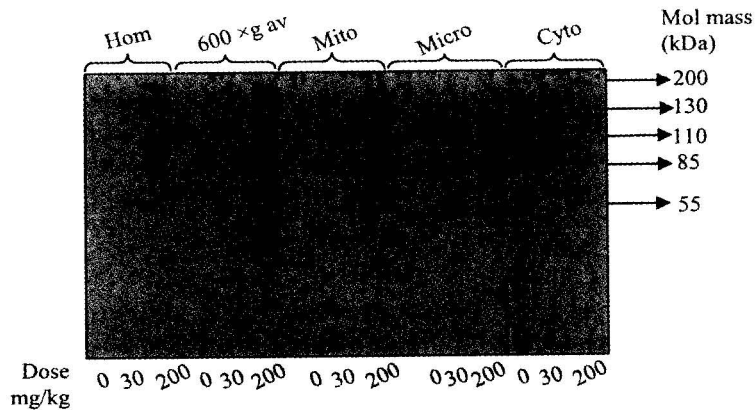


Fig. 5: Intracellular localisation of BNP-modified proteins in male Sprague-Dawley rat liver after 6 hours dosing. Subcellular fractions of rat liver were electrophoresed and electrophoretically transferred to nitrocellulose membrane. Anti (BNP-KLH) antiserum was used to detect BNP-modified proteins. The bound antibodies were

detected using HRP-conjugated goat anti-rabbit IgG secondary antibody and visualised using Pierce SuperSignal substrate reagents. Hom: homogenate, 600 × g_{av} fraction, Mito: mitochondria, Micro: microsomal, Cyto: cytosol.

In mice, at 100 nM BNP, partially inhibited the antibody and complete inhibition was seen at 1000 nM (Fig. 6). Meanwhile in rats, at 0.5 nM BNP, the recognition of BNP adducts was totally blocked (Fig. 7). The density of adducts expression in mice was found to be higher than in rats. This may suggest that proteins in mice are more susceptible to covalent modification by the reactive species compared to rats. Thus, a higher concentration of free BNP was required to block the antibody in detecting the BNP modified proteins.

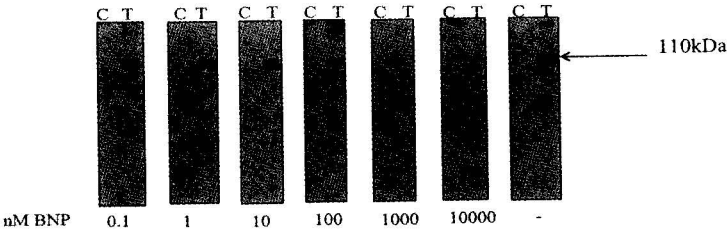


Fig. 6: Inhibition of the recognition of BNP-modified protein by anti (BNP-KLH) antibody in mice. Prior to immunoblotting, anti (BNP-KLH) antisera was pre-incubated with various concentration of BNP. Bound antibodies were detected using HRP-conjugated goat anti-rabbit IgG secondary antiserum (1 : 10000) and visualised with Pierce SuperSignal substrate reagents. Protein was loaded at 50 mg/lane, C : 600 × g_{av} fraction from control mice, T : 600 × g_{av} fraction from BNP-treated mice at 200 mg/kg.

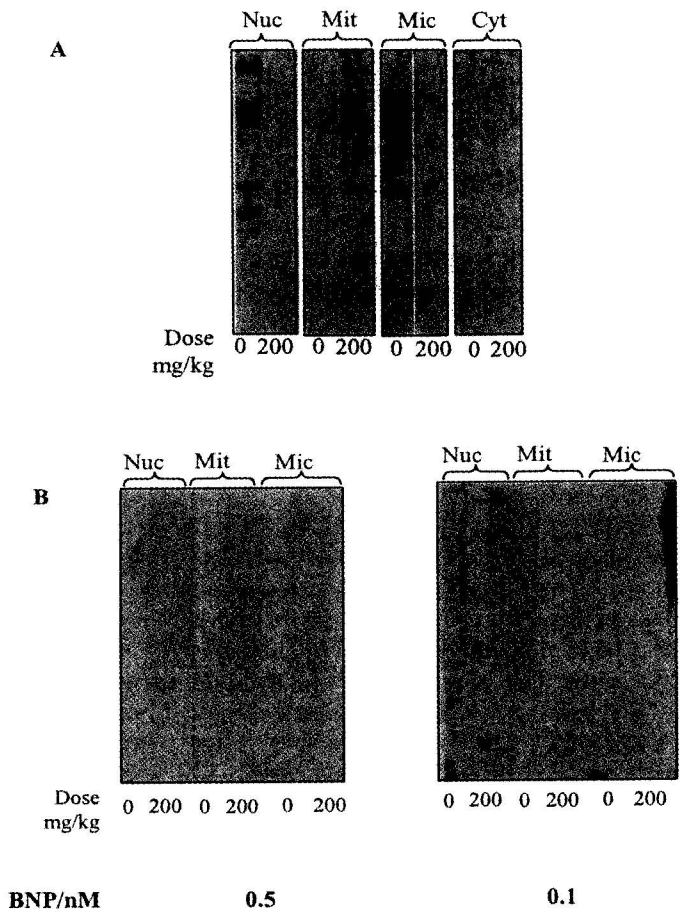


Fig. 7: Competitive inhibition studies on the recognition of BNP adducts by anti (BNP-KLH) antiserum in rats. The recognition of BNP adducts in a single dose of BNP-treated rats (A) was blocked by free BNP, (B) in a dose-dependent manner. At 0.5 nM BNP, the recognition of BNP adduct was totally blocked. Note: Nuc: $600 \times g_{av}$, Mit: mitochondria, Mic: microsome and Cyt: cytosol.

Histological and Immunohistochemical Analysis of the Liver Section of Female CD1 Mice and Male Sprague-Dawley Rats Given Single Doses

Histopathological examination of the liver sections did not reveal any significant hepatocyte abnormality in the mice and rats treated with BNP at all dose levels sacrificed after 6 hours (results not shown).

In the liver sections from BNP-treated mice probed with anti BNP-KLH antisera, clear dose-dependent staining of the bile canalicular membrane of the hepatocytes was seen. The staining was most intense in the centrilobular region with some staining was also seen in the bile duct (Fig. 8). No specific canalicular staining could be detected in the liver sections of control mice treated with 10% DMSO/tricaprylin when probed with anti BNP-KLH antisera. The staining was also absent when the liver sections from BNP-treated mice were probed with either pre-immune serum or the anti (diclofenac-KLH) antiserum. Incubation of the antiserum with free BNP before incubation with the liver section also blocked staining in the bile canalicular membrane (results not shown). In rats, liver sections probed with specific anti (BNP-KLH) polyclonal antiserum revealed cytoplasmic staining with some staining of the nuclei at higher dose (Fig. 9).

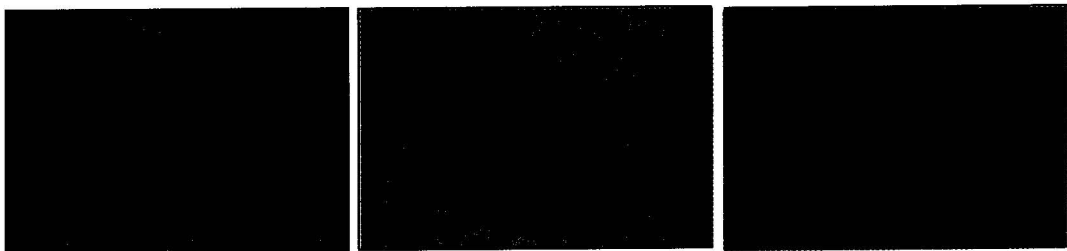
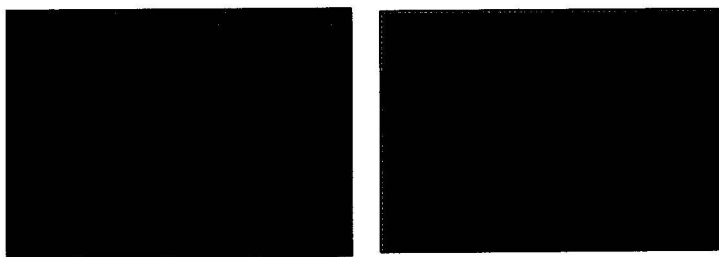


Fig. 8: Immunohistochemical analysis of liver section of mice treated with benoxaprofen and control. Liver sections from a mouse given a single dose of (A) 10% DMSO/tricaprylin, (B) 5 mg/kg, (C) 100 mg/kg and (D) 200 mg/kg BNP, showing a bile canalicular staining at the centrilobular area. The adduct staining was expressed in dose-dependent manner. Liver section of mice treated with 200 mg/kg BNP (E), probed with pre-immune sera, showed the absence of adduct.

All adducts were expressed in a dose-dependent manner and mainly discovered in the $600 \times g_{av}$ fraction although the adducts localisation in both species were found to be different. The localisation of BNP adducts to the bile canalicular plasma membrane of mice was consistent with previous studies on other NSAIDs carried out by several groups (Pumford *et al.* 1993; Wade *et al.* 1998).

The pattern of adducts of apparent molecular weight of 110 and 200 kDa is the same as that observed after the treatment of hepatotoxic NSAIDs such as diclofenac and sulindac (Pumford *et al.* 1993; Hargus *et al.* 1995; Wade *et al.* 1998). Hargus and co-workers in 1995 have further characterised the 110 kDa, which was identified as dipeptidyl peptidase IV (DPPIV). They have postulated that this enzyme might has a role in hepatotoxicity of NSAIDs, as the activity of DPPIV was reduced in rats given diclofenac (Hargus *et al.* 1995). Nevertheless, another study by Cadwell *et al.* (1998) found that the activity of this enzyme was also low in ibuprofen treated rats, which suggests that DPPIV is unlikely to have a central role in NSAID induced hepatotoxicity since the hepatotoxic potential of ibuprofen is very rare.

The result obtained from this study has also suggested that BNP and/or its metabolites is a less potent drug-inducing liver injury compared to diclofenac. Relevant to this suggestion is the difference between human doses: BNP was prescribed at 600 mg/day, while the dose of diclofenac is 75 mg twice a day. Acyl glucuronides have been implicated in toxicity of a number of NSAIDs, notably diclofenac. These glucuronides act as electrophiles attacking nucleophilic groups such as sulphydral, amino and hydroxyl groups. This may result in covalent binding to cellular proteins and thus may have considerable toxicological significance. NSAIDs are weak acids which can be divided into carboxylic and enolic acids. The presence of carboxylic acid group in the chemical structure of NSAIDs give rise to the formation of acyl glucuronide following conjugation with glucuronic acid.

The structure of the carboxylic drugs may allow the prediction of reactivity of the corresponding acyl glucuronides to form covalent adducts that may lead to hepatotoxicity (Benet *et al.* 1993). The degree of covalent binding to endogenous proteins will be a function of the concentration acyl glucuronide in plasma, its inherent reactivity and the degradation rate of each conjugate. There are evidences of the relationship between the structure of the NSAIDs and the extent of covalent binding with the endogenous proteins (Caldwell *et al.* 1998; Benet *et al.* 1993). In tolmetin,

ibufenac and diclofenac, the α -carbon to the carboxyl group is unsubstituted and these compounds found to exhibit the greatest degree of covalent binding (Benet *et al.* 1993). For ibuprofen, the α -carbon has a methyl substitution. These compounds have intermediate binding capacity to the endogenous proteins. The fully substituted α -carbon, such as the mefenamic acid exhibited the least binding capacity with proteins (Benet *et al.* 1993).

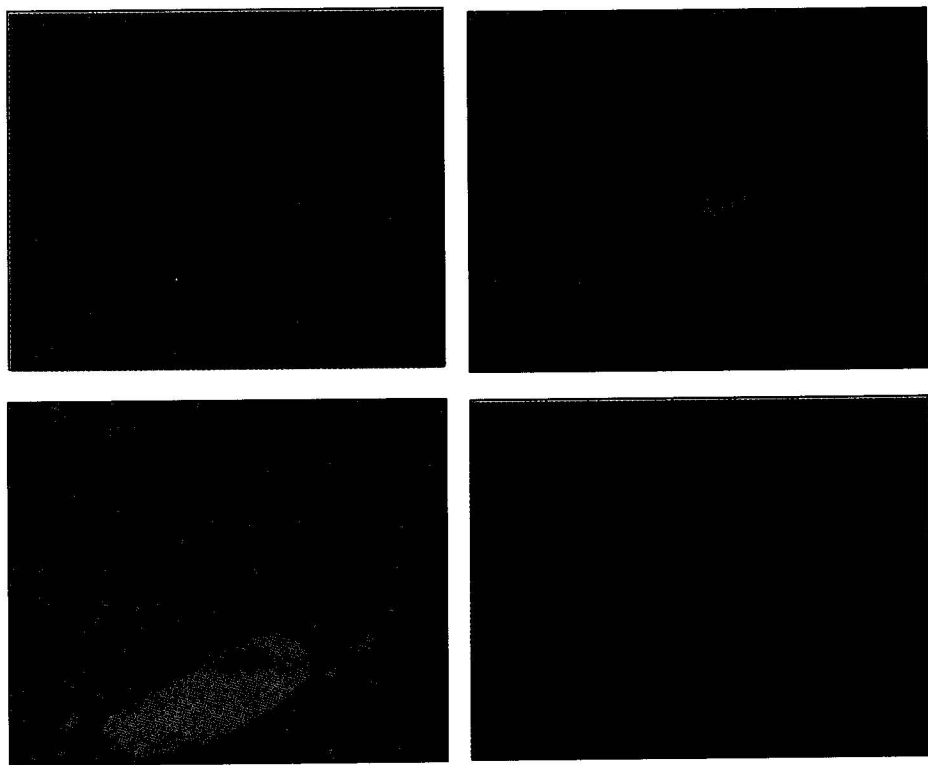


Fig. 9: Immunohistochemical analysis of liver section of rat treated with benoxaprofen and control. Liver sections after (A) DMSO/trycaprylin, (B) 30 mg/kg and (C) 200 mg/kg BNP, were probed with anti (BNP-KLH) antiserum (1:3000) and developed using a biotin/streptavidin-HRP amplification system. Significant brown staining was seen in the nuclei of the hepatocytes in treated rats, probed with specific BNP antibody, but when probed with secondary antibody alone, no staining was seen (D). Note: CV: Central vein.

Therefore, the substitution of the α -carbon in BNP glucuronide may provide steric hinderance and make it less likely to form covalent binding with proteins. The stability of the adduct towards hydrolysis also influence its reactivity and subsequently having toxicological significance (Spahn-Langguth and Benet 1992). This may explain the reason why hepatotoxicity of BNP was not evidence at this dose level, but with diclofenac, hepatotoxicity was observed.

CONCLUSION

The development of antibody obtained from this study may be used to detect the presence of BNP-modified proteins either in the subcellular fraction or in the paraffin-embedded section. The polyclonal antibody developed was specifically directed against BNP-modified proteins, particularly the 110 kDa molecular weight proteins. This protein was believed to be localised in the bile canalicular membrane of the mice and in rats, it was found to be in the cytosol. From the blocking study using free BNP, the antibody was found to show a high specificity in identifying proteins specifically modified by BNP but not other drug. Furthermore, no cross-reactivity action was seen with other drug or NSAID. Nevertheless, hepatocellular damage was not seen in both mice and rats, even at 200 mg/kg BNP after a single treatment, 6 h post-dosing. It may be probably worth to extend the study duration of more than 6 h in order for the toxicity to become apparent. Therefore, the modification of proteins by drug or its metabolite(s) does not necessarily induced toxicological event. Further reactions are required before toxicity becomes apparent.

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