

Canadian Journal of Applied Sciences; 2011; 1(3): 129-143, October, 2011
Intellectual Consortium of Drug Discovery & Technology Development Inc.
ISSN 1925-7430 Available online <http://www.canajas.com>

INFLUENCE OF HYPERGLYCEMIA ON OXIDATIVE STRESS AND DNAase-MEDIATED GENOMIC FRAGMENTATION COUPLED WITH APOPTOTIC AND NECROTIC CELL DEATHS IN KIDNEY

Mohammed Saleem¹, Muhammad Shoaib Akhtar^{2*} Sidharta D. Ray³, Bashir Ahmed⁴

¹Faculty of Pharmacy, G.C. University, Faisalabad, Pakistan, ²Faculty of Pharmacy, University of Sargodha, Sargodha, Pakistan, ³Long Island University Brooklyn, New York, USA, ⁴College of Pharmacy, University of Punjab, Lahore, Pakistan.

ABSTRACT

Diclofenac (DCLF) is a potent inhibitor of prostaglandin synthesis and an established antipyretic and analgesic agent. It also has a nephrotoxic profile caused by generation of reactive oxygen species and enhanced apoptotic DNA fragmentation. The specific goals of this investigation were to determine: (i) sensitivity of hyperglycemic mice to nephrotoxic doses of DCLF, (ii) whether hyperglycemia modulates genomic DNA-fragmenting potency of DCLF in kidney, (iii) whether hyperglycemia would increase DCF-induced oxidative stress in kidney and (iv) whether hyperglycemia can alter apoptotic and necrotic death of kidney cells. Male ICR CD-I mice were divided into normal and diabetic groups. Diabetes was induced by administering 100 mg/kg streptozotocin. Both normal and diabetic groups were divided into sub-groups on basis of nephrotoxic doses of DCLF i.e., 100 and 200 mg/kg i.p. The mice were sacrificed after 24-hrs and determined blood urea nitrogen (BUN), lipid peroxidation level and caspase-activated DNAase-mediated genomic DNA fragmentation. In addition, kidney tissues were examined histopathologically and checked for DNA damage. The results obtained showed that DCLF is a potent nephrotoxic agent as it increases BUN and powerfully induces oxidative stress depicted by significant increase in malandialdehyde (MDA) level which lead to massive DNA fragmentation coupled with apoptotic and necrotic cell deaths in both diabetic and non-diabetic groups *in vivo*. In addition, data have indicated that DCF causes greater nephrotoxicity in diabetics as compared to non-diabetics as shown by higher values of BUN, LPG and genomic DNA fragmentation.

Key words: Diclofenac, diabetes, non-steroidal anti-inflammatory drug, BUN.

***Address for Correspondence:** Muhammad Shoaib Akhtar, Professor of Pharmacology, Faculty of Pharmacy, University of Sargodha, Sargodha, Pakistan drmsakhtar@gmail.com

INTRODUCTION

Diabetes mellitus (DM) is an endocrine disorder characterized by inability of pancreas to secrete significant amount of insulin to maintain physiological levels of blood glucose (Huma et al., 2011). Long standing DM is associated with a complex array of disorders like neuropathy, cardiomyopathy, retinopathy, hepatomegaly and nephropathy. Among these, however, nephropathy is the most commonly encountered complication in patients with type I and type II DM. It has been reported that this complication is on the rise world wide (Jorns *et al.*, 2001). One possible explanation for this complication involves the hyperglycemia induced formation of advanced glycosylation end-products which generate reactive oxygen species, suggesting that hyperglycemia causes oxidative damage to cells through NF- κ B-dependent pathways (Mohamed *et al.*, 1999). Streptozotocin (STZ) is a powerful inducer of diabetes in experimental animals by destroying pancreatic β -cells. Diabetes mellitus could also induce polymorphic alterations of metabolic activities of cytochrome P-450-dependent monooxygenases in induced diabetic animals. The diabetic condition is known to increase (Ray *et al.*, 2001) or decrease (Rour *et al.*, 1986) activities of several drug metabolizing enzymes. For example, STZ-induced hyperglycemia is known to aggravate cardiotoxicity of doxorubicin (Al-Shabanah *et al.*, 2000), thioacetamide-induced hepatotoxicity (El-Hawari and Plaa, 1983) and increase cyclosporine-A-induced renal tumorigenicity (Carmo *et al.*, 2000). Increased toxicity in specific target organs is due to the inhibition of specific drug metabolizing enzymes, whereas decreased toxicity may be due to the polyuremia in the diabetics.

DCF is a potent inhibitor of prostaglandin synthesis and an antipyretic and analgesic agent. However, severe renal dysfunctions are occasional consequences of its toxicity. Although DCF is extensively used, mechanisms underlying its potential to cause multi-organ toxicity remain unknown. A majority of the previous publications primarily describe the forms of hepatic cytochrome P-450 that metabolize DCF, and its toxic metabolites (Tang *et al.*, 1999). However, basic biochemical mechanisms underlying DCF-induced nephrotoxicity remain poorly investigated, particularly in diabetics. Additionally, from a mechanistic standpoint, it is not known as to how diabetics respond to NSAID-induced nephrotoxicity. Previous studies have investigated the mechanisms underpinning the nephrotoxic potential of DCF in experimental animals (Hickey *et al.*, 2001). The nephrotoxic doses (100-300 mg/kg) of DCF induce apoptotic death of kidney cells in addition to necrosis (Hickey *et al.*, 2001). The present investigation was aimed at understanding the modulatory influence of diabetic condition on key events associated with DCF's apoptogenic potential including the induction of caspase-activated DNAase (CAD) which is considered a principal suspect in orderly fragmentation of genomic DNA. Additional focus was placed upon establishing a cause-and-effect relationship between oxidative stress, DNA fragmentation and DCF's apoptogenic potential.

MATERIALS AND METHODS

Chemicals used

Chemicals were analytical grade or higher and were used without purification and included streptozotocin, diclofenac (DCF) sodium, disodium EDTA, glacial acetic acid, perchloric acid, ethidium bromide, malonaldehyde, N-lauroylsarcosine, sulfuric acid, Lysis buffer, 0.5% triton X-I and trichloroacetic acid

Animals used

Adult male ICR (CD-1) mice weighing 35 g each were of Spargue-Dawely strain and were given access to lab chow (Purina Laboratory rodent chow and tap water *ad libitum*). The animals were allowed to acclimate in an environment of controlled temperature (22-25°C), humidity, and light/dark (12h/12h) cycle for one week prior to study. Food and water were withdrawn from all the experimental animals for one hour following drug treatments, and returned. All animal procedures received prior approval by the Institutional Laboratory Animal Care and Use Committee, and met all state and federal regulations.

Induction of diabetes

STZ was dissolved in citrate buffer (pH 4.7) and was injected ip to induce hyperglycemia. Five doses of STZ (100 mg/kg) were administered ip every other day for ten days to induce hyperglycemia. Blood glucose level >200mg/dl was considered as hyperglycemic. DCF was dissolved in normal saline (pH 7.4) and was administered ip. All animals were exposed to DCF for 24 hrs before they were sacrificed.

Grouping of animals

The animals were divided into following groups:

Group I (Control): All animals were injected normal saline ip. Group II: STZ-alone - Streptozotocin 100 mg/kg was given ip. Group III: DCF alone 100 mg/kg ip. Group IV: DCF alone 200 mg/kg ip. Group V: STZ +DCF 100 mg/kg ip and Group VI: STZ +DCF 200 mg/kg ip.

Lethality and nephrotoxicity studies

The numbers of deaths were counted in each cage at the end of 24 hours. Nephrotoxicity was determined by measuring BUN (blood urea nitrogen) levels and kidney histopathology. BUN levels with a UV /VIS Beckman DU-640 spectrophotometer using a Sigma kit based on the method of Talke and Scubert (1965). Enzyme activity is calculated from the decrease in NADH absorbance at 340 nm.

Histological studies

Sections of kidneys were collected at the time of sacrifice and preserved in 10% buffered formalin. Tissue specimens were sent to Pathology Associates (MD) for further processing, sectioning and PAS (periodic Acid Schiff) staining. Normal, apoptotic and necrotic cells were identified from 5mm PAS-stained kidney sections using a Carl-Zeiss bright field microscope (Axioskop 20 equipped with a camera). Previously published guidelines to characterize normal, apoptotic, and necrotic cells were followed (Ray *et al.*, 1996).

Oxidative stress and lipid peroxidation

The extent of oxidative stress was inferred from the degree of lipid peroxidation. The amount of lipid peroxidation was inferred from the levels of MDA (malondialdehyde) detected in kidney tissue. MDA levels from kidney homogenates were determined based on the methods of Bagchi *et al.* (1992) and Ray and Fariss, (1994). Kidney homogenates were reacted with TBA (thiobarbituric acid) to determine TBA-reactive substances. Absorbencies were extrapolated from a MDA standard curve and read at 532 nm.

Quantitative DNA fragmentation

DNA damage was quantified by using one kidney from each animal in all treatment groups. DNA fragmentation assay was based on method of Ray *et al.* (1996) and color reaction on method of Burton, 1956). To measure kidney DNA fragmentation, frozen kidneys (in liquid nitrogen) were weighed and homogenized in pre-chilled Lysis buffer (10 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were centrifuged at 27,000g for 20 min. at 4°C to separate intact chromatin in pellet from fragmented DNA in a supernatant. Pellets were suspended in 0.5N perchloric acid and supernatants were treated with concentrated perchloric acid to reach a final concentration of 0.5 N. All the samples were capped, boiled at 90°C for 15 min and centrifuged at 1500g for 10 min to co-precipitate protein along with other debris. The resulting supernatants were treated with diphenylamine reagent (1.5 g DPA + 1 ml 36N H₂SO₄ + 100 ml glacial acetic acid + 50 mM CH₃CHO) for 16-20 hrs at room temperature in darkness. Absorbencies were measured at 600nm with a Beckman DU 640 spectrophotometer. DNA fragmentation in the control sample was treated as 100% fragmentation based on the formula [(frag. DNA)/(frag. DNA + intact DNA)] (Ray *et al.*, 1996; Burton, 1956). DNA appearing in supernatant was divided by the total DNA to find percentage fragmentation. Treatment effects were reported as percent control fragmentation.

Quantitative DNA fragmentation assay

Prior to homogenization, portions from four kidneys were collected together in order to minimize statistical variation. Also, the collected kidneys were almost equally damaged based on average BUN levels and visual inspection. Kidneys were treated with chilled Tris- EDTA Sarkosyl buffer (0.1 M Tris HCl/10 mM EDTA/0.5% N-lauroylsarcosine, pH 7.8) and digested with proteinase-K at 50°C (50µg/ml) for 2 hrs. After digestion, DNA was extracted 3 times with phenol-chloroform and precipitated with absolute ethanol and 2.5 M sodium acetate (pH 4.0). After precipitation, DNA was washed 3 times with 66% ethanol, dissolved in 0.1 M Tris-HCl/20 mM EDTA buffer (pH 8) and digested for 15 min at 37°C with DNAase free RNAase-A (1 mg/ml). DNA was reprecipitated, dissolved in Tris-HCl/EDTA buffer and then quantified spectrophotometrically from absorbance at 260 nm and loaded onto an agarose gel. The status of integrity of DNA (15µg/lane) was determined by constant voltage mode electrophoresis (in a large submarine at 60 V, Hoeffler Instruments, San Francisco on a 1.2% agarose gel containing 0.4-µg/ml ethidium bromide. A HindIII digest of DNA served as molecular weight size standard on gel. The gel electrophoretogram was illuminated with 300 nm UV light and a photographic record was made (Ray *et al.*, 1986).

Statistical analysis

Data were analyzed for significance ($P < 0.05$) using ANOVA followed by the Fisher PLSD test (Stat View II for Macintosh, version 1.04, Abacus Concepts, California) or evaluated by linear regression analysis and correlation (Zar,1984). Simple statistical comparison of DNA fragmentation data was determined by Fischer PLSD test and converted to percentages.

RESULTS

Preliminary data showed that 100 mg/kg ip dose of STZ administered every other day was optimal for induction of hyperglycaemia and its doses exceeding 100 mg/kg, or their prolonged

administration in more than 5 doses were lethal. Its administration in excess of 10 days induced severe sickness and that is why these animals were not included in final studies. Consistent loss of weight (Fig. 1) was another stringent criterion which was taken into account while developing this protocol. During trial period, DCLF was administered ip or orally and normal saline was used as vehicle for its dissolution for injections whereas it was suspended in corn oil and sonified for oral administration. The degree of nephrotoxicity induced by DCLF was greater via ip route.

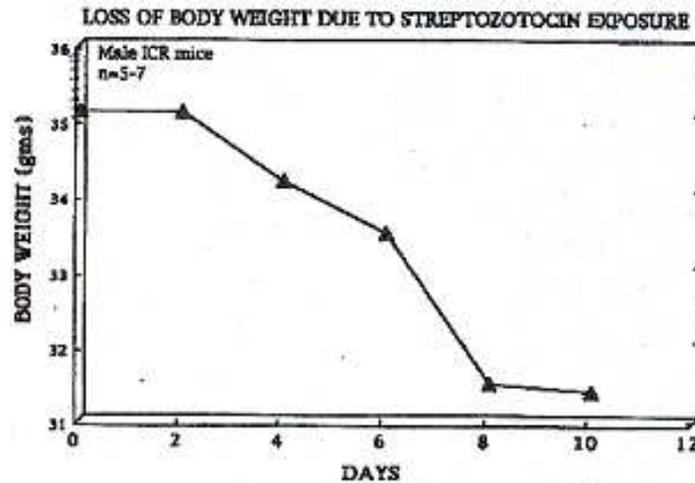


Fig. 1. Streptozotocin induced correlation number of doses for STZ and weight loss in male ICR mice

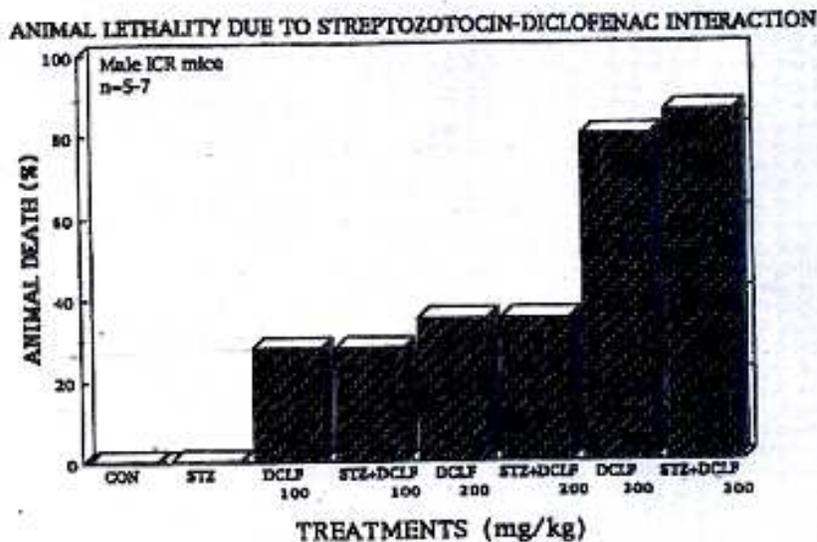


Fig. 2. Animal lethality due to streptozotocin-Diclofenac interaction in male ICR mice

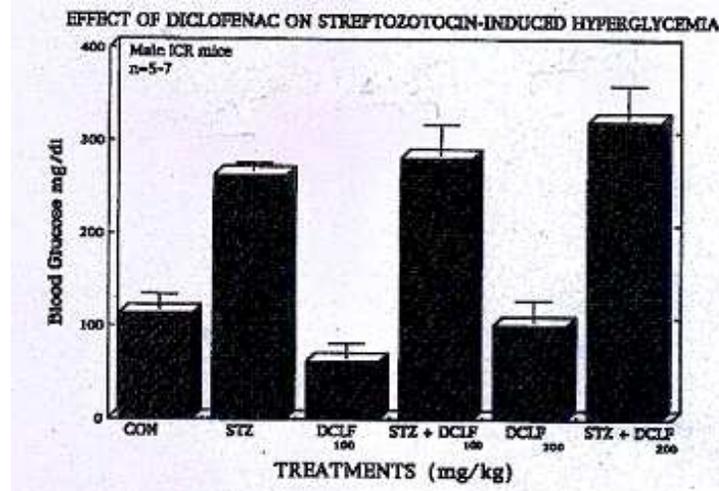


Fig. 3. Modulation of blood glucose level by DCLF in diabetic and no diabetic mice

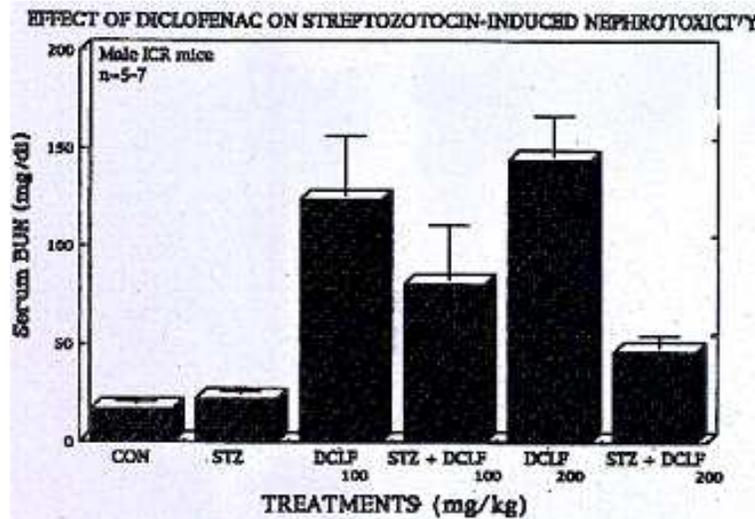


Fig. 4. Effect of Diclofenac on streptozotocin-induced nephrotoxicity

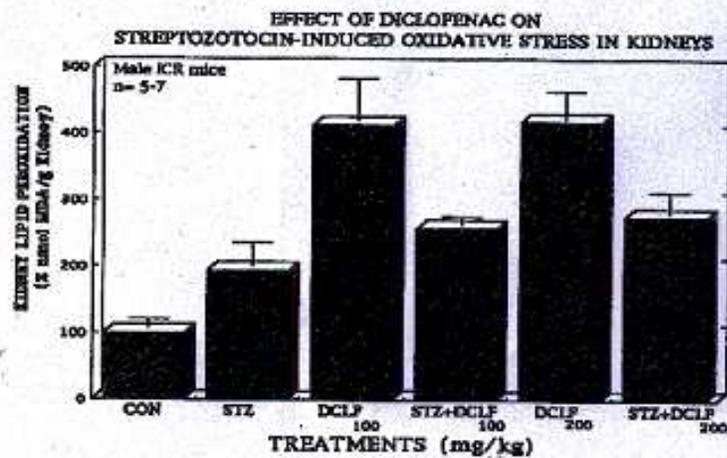


Fig. 5. Effect of Diclofenac on streptozotocin induced kidney DNA fragmentation

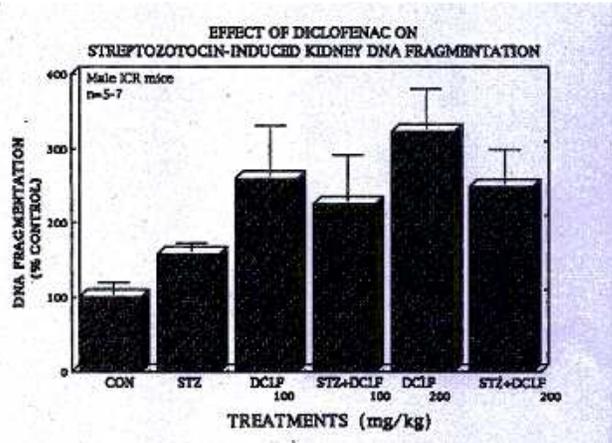


Fig. 6. Effect of Diclofenac on streptozotocin induced oxidative stress in kidneys

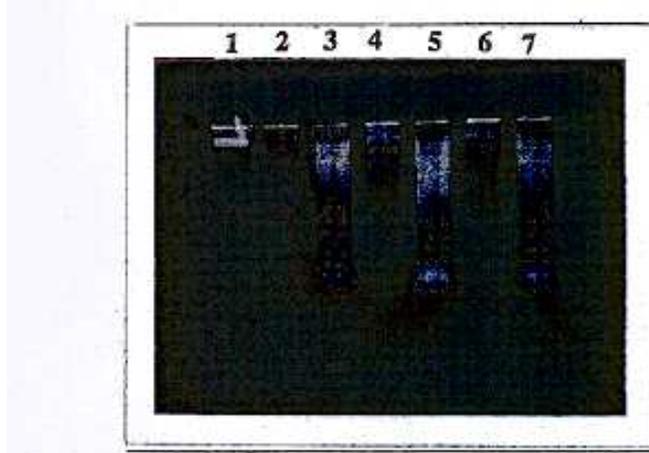


Fig. 7. Agarose electrophoresis assessment of genomic undamaged qualitative

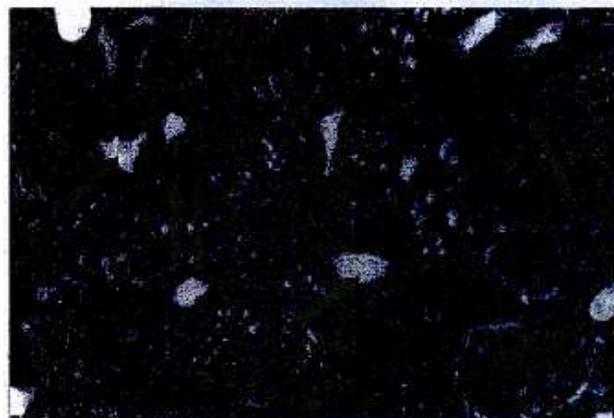


Fig. 8 A. Representative section of mouse kidney treated with vehicle .9% NaCl, no pathologic changes present. Normal architecture of kidney is vivid and there is total absence of apoptotic and necrotic cells.



Fig. 8-B. Representative section of mouse kidney treated with 200 mg/kg DCLF, showing random damage of all cellular compartment. Massive damage to macula densa areas.

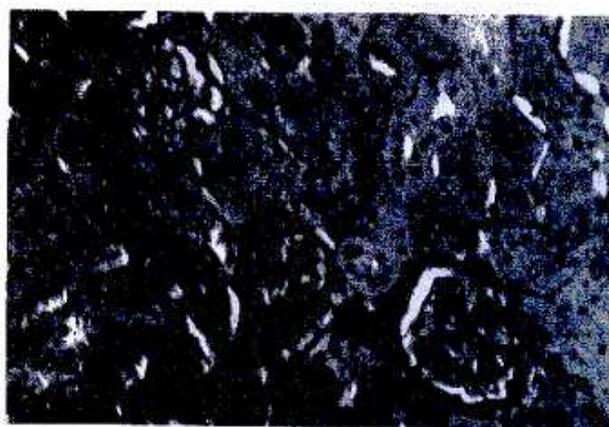


Fig. 8-C Representative section of mouse kidney treated with STZ+DCLF 200, showing nominal damage than DCLF alone. The changes were observed only in proximal and distal tubular cells.

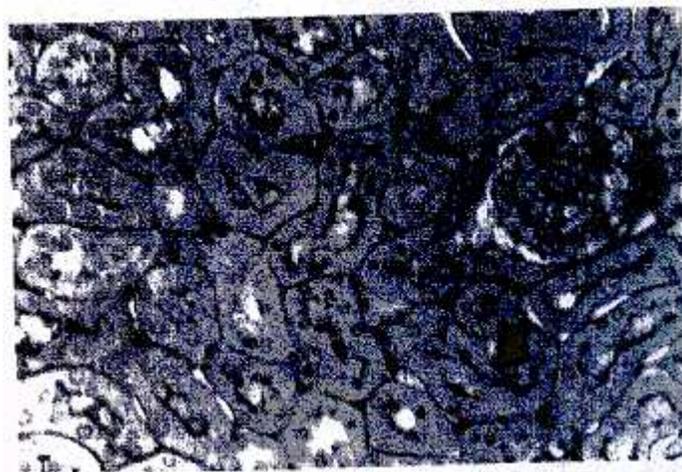


Fig. 8-D Representative section of mouse kidney treated with STZ alone, showing overall loss of glycogen and minor changes in the cortical areas.

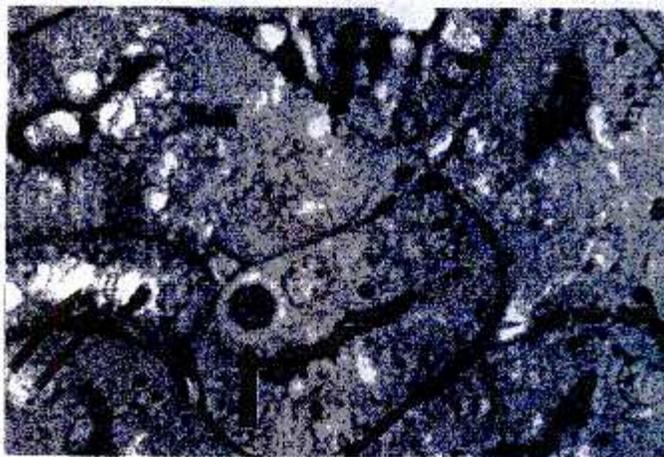


Fig. 9 Representative section of mouse kidney treated with DCLF 200, showing random damage of all cellular compartments. Magnified view of a kidney apoptotic nuclei, that are characteristic of an apoptotic cell.

Dose response of diclofenac-induced nephrotoxicity

Fig. 4 shows the dose-response effect of DCLF on ICR-mice at 24 hours after its administration. The nephrotoxicity was monitored by serum BUN levels. A positive correlation between the DCLF dose and proportionate increase in serum BUN is clear from Fig. 4. These doses of DCLF were highly effective in inducing kidney injury. The lowest dose was the least effective in inducing kidney injury and the highest dose was the most potent in inducing kidney injury.

Diclofenac-induced animal lethality

The two lowest doses of DCLF induced minimal death whereas the highest dose, 300 mg/kg, exceeded the limit of LD₅₀ and was not used in this investigation. The number of animals that died after DCLF exposure is given in Fig. 2. Based on these data, only the two lower doses of DCLF (100 and 200mg/kg) were used for the further studies.

Modulation of blood glucose by DCLF in diabetic and non-diabetic mice

Fig. 3 shows the modulatory influence of DCLF on normal and diabetic mice. STZ alone was effective in causing severe hyperglycemia. In contrast, DCLF alone was ineffective in elevating blood glucose levels; rather, it induced slight hypoglycemia (blood glucose less than 70mg/dl). Blood glucose levels in animals given DCLF alone were lower than control animals. Administration of DCLF to STZ-exposed mice did not significantly alter blood glucose levels except that these were slightly higher in STZ+DCLF administered animals as compared to STZ alone.

Modulation of nephrotoxicity by DCLF in diabetic and non-diabetic mice

Fig. 4 shows the degree of nephrotoxicity induced by DCLF in hyperglycemic and non-hyperglycemic mice as reflected by serum BUN levels. The toxicity in diabetic and non-diabetic mice did not reflect the trend observed with blood glucose levels (Fig. 3). Diabetic animals were extremely sensitive to DCLF and showed dramatic increase in blood glucose levels (Fig. 4) whereas normal animals were less vulnerable to nephrotoxic potential of DCLF. It is clear from

the data that hyperglycemia (or STZ exposure) did not potentiate DCLF-induced nephrotoxicity; rather impact of DCLF was significantly reduced by the presence of excessive glucose in circulation. BUN levels were lower in STZ+DCLF treated mice as compared to DCLF alone. This reduction in hyperglycemic state may due to rapid elimination of the drug. However, in control mice severe kidney injury was observed as reflected by sharp increase in BUN.

DCLF-induced oxidative stress in kidneys of diabetic and non-diabetic mice

Fig. 5 shows DCLF-induced oxidative stress-mediated lipid peroxidation in kidneys. The tissue levels of lipid peroxidation indirectly quantified by determining MDA levels in kidney homogenates (Fig. 5) showed that both doses of DCLF alone (100 mg/kg and 200 mg/kg) were able to induce significant lipid peroxidation and caused about 4-fold increase in MDA level. Similarly, STZ- alone was equally effective and nearly doubled its level in kidneys. However, hyperglycemia did not accentuate the degree of lipid peroxidation and levels of MDA were lower in STZ+DCLF group as compared to DCLF alone. Hyperglycemia exerted an opposing effect on DCLF-induced oxidative stress and pattern of oxidative stress mimicked the nephrotoxicity trend.

Quantitative DNA damage caused by DCLF in kidneys of normal and diabetic mice

Quantitative assay reliably predicts the extent of fragmented and intact DNA (Ray *et al.*, 1994) and Fig. 6 shows quantitative changes in integrity of genomic DNA during exposure to nephrotoxic doses of DCLF. The administration of DCLF alone caused massive kidney DNA fragmentation at 24 hours after the nephrotoxic dose. A 100 mg/kg dose of DCLF caused a 2.53 fold increase and 200 mg/kg dose caused a 3.57 fold increase in DNA fragmentation as compared to control group. Overall, the pattern of quantitative spectrophotometric DNA fragmentation mirrored the profiles of nephrotoxicity and oxidative stress discussed in Figs 4 and 5. In comparison to DCLF alone, STZ-induced hyperglycemia caused a moderate increase in DNA fragmentation (less than 2-fold). However, DCLF exposure to hyperglycemic mice did not potentiate DNA fragmentation. The percent fragmentation observed in STZ+DCLF groups was lower when compared to DCLF alone group. DCLF failed to take advantage of the diabetic condition and aggravated the slightly unstable integrity of genomic apparatus. Collectively, DNA fragmentation data also followed the changes observed in BUN (Fig. 4) and MDA (Fig.5) levels and suggests a link between these three events.

Qualitative DNA damage caused by DCLF in kidneys of normal and diabetic mice

Apoptotic type ladder-like DNA fragmentation dependent on Ca²⁺-endonuclease activity was evaluated the data obtained are shown in Fig. 7. The electrophoretogram generated by agarose gel electrophoresis showed that DCLF overdoses resulted in degradation of kidney DNA and a typical ladder of DNA fragments consisting of multiple repetitive bands diagnostic of Ca²⁺ -endonuclease activity (Hickey *et al.*, 2001). In addition, DCLF exhibited a dose-response effect on the damage. DNA isolated from control group (lane 2) remained totally intact and devoid of any fragmentation. Using the 1-kb DNA ladder as a marker size (lane 1), this fragmentation pattern presumably represents 200-bp multiples of DNA. The DCLF-induced loss of large molecular weight DNA (see fluorescence intensity at the top of the gel at wells 5 and 7) was readily apparent on the gels. The overall pattern of DNA fragmentation and a clear-cut ladder induced by toxic doses of DCLF suggested involvement of massive cell death via apoptosis. Compared to DCLF alone, STZ alone (lane 3) was far less active in fragmenting DNA. However,

mild laddering observed with STZ alone exposed DNA reflected a modest onset of apoptotic programmed death. Likewise, DCLF exposure in hyperglycemic mice resulted in a laddering which was far less pronounced as compared to DCLF alone. These changes agree with biochemical findings reported earlier (Hickey *et al.*, 2001).

Apoptotic and necrotic deaths in normal and diabetic mice

Histopathological examination of kidney sections vividly followed biochemical findings previously discussed. The photomicrographs of representative kidney sections from control and DCLF (200 mg/kg) treated mice at 24 hours are shown in Fig. 8. In order to assess the degree of nephrotoxicity, paraffin-embedded PAS stained mouse kidney sections from control and DCLF (200 mg/kg) treated groups were examined. PAS staining provided insight into the glycogen status of the tissue. Vehicle controlled (Fig 8, panel-A) kidney sections showed normal structure with no identifiable lesions in the absence of apoptotic and necrotic cells. In this section, most cells were uniformly stained, and intensity of coloration signified that nearly every cell was glycogen loaded with intact cytoplasm. A clear-cut undamaged dark blue stained nucleus was found in every cell and indicated an intact nuclear morphology. But sections from kidney tissues exposed to 200 mg/kg DCLF (Fig 8, panel B) demonstrated massive injury in almost all types of cells. The primary victims were proximal and distal tubular cells and macula densa of these cells sustained massive injury. Some tubular cells totally lost macula densa regions. The glomeruli showed shrinkage, hypervacuolation, and/or abnormal swelling.

Glycogen-depleted cells were scattered predominantly in the cortex. Cells of the ureter also suffered minor to moderate injury. Heavily drug impacted areas also showed the presence of inflammatory cells which signified ongoing necrosis. On a comparative basis, 200 mg/kg (Fig. 8B) was more damaging than 100 mg/kg (data not shown) which corroborated with previous biochemical findings. Representative section of mouse kidney treated with STZ+DCLF200 mg/kg (Fig.8C) showed less kidney cell damage than DCLF alone. STZ alone (Fig 8D) induced only minor changes and upon examination of the entire section, a negligible number of apoptotic cells were encountered. Fig.9 shows several apoptotic nuclei which were randomly present throughout section which reflect potency of DCLF to "turn on" apoptosis in the kidneys. Unlike DCLF, lack of numerous apoptotic nuclei in STZ+DCLF exposed kidney reflected down regulation of apoptotic process. Collectively, these data suggested that STZ-induced hyperglycemia did not potentiate DCLF nephrotoxicity in mice.

DISCUSSION

Diabetes mellitus alone ranks among top ten causes of death in western nations with a worldwide prevalence between 4 to 5 % (McGowan *et al.*, 2001). Despite important improvements in its clinical management, it is not yet possible to significantly control its consequences. Diabetes mellitus represents a group of metabolic disorders in which there is impaired glucose utilization manifested by persistent hyperglycemia, a relative or absolute lack of insulin production by the pancreas, and development of microvascular pathology in the retina and glomeruli. The morbidity associated with long-standing diabetes results from complications such as microangiopathy, retinopathy, nephropathy, neuropathy and their combinations. Hence the basis of these chronic long-term complications has been and still the subject of a great deal of research (Raptis and Viberti, 2001). Evidences suggest that complications of diabetes are consequence of

metabolic derangements, mainly hyperglycemia, from the finding that kidneys when transplanted into diabetics from normal individuals develop nephropathic lesions within five years after transplantation (Nam *et al.*, 2001). Conversely, kidneys with lesions of diabetic nephropathy demonstrate a reversal of the pathology when transplanted into non-diabetic individuals. In USA alone all end-stage chronic renal failure patients were diabetics (Hricik *et al.*, 1997). STZ-induced-diabetic hyperglycemia has been shown to aggravate cardiotoxicity of doxorubicin (Al-Shabanah *et al.*, 2000), thioacetamide-induced hepatotoxicity (El-Hawari and Plaa, 1983) and cyclosporine-A-induced renal tumorigenicity (Reddi *et al.*, 1991). The diabetes induces isoenzymes (CYP 450) known to participate in drug metabolism (Chang *et al.*, 2000). Since diabetes and NSAIDs, such as DCLF, share a common target in the kidney, the present study tested whether DCLF during hyperglycemia will synergize, potentiate or antagonize nephrotoxicity and the associated events. Thus several biochemical markers were measured including: (i) changes in blood glucose levels; (ii) changes in serum BUN levels, (iii) tissue MDA levels to indirectly determine ongoing lipid peroxidation associated with oxidative stress, (iv) quantitative and qualitative genomic DNA and (v) histopathological changes.

Data obtained showed that five ip doses of 100 mg/kg streptozotocin (administered every other day for 10 days) were sufficient to induce significant hyperglycemia. This condition was coupled with gradual loss of body weight (total loss of 3.6 grams in 10 days), which signified induction of acute diabetes mellitus. STZ doses in excess of this regimen either caused severe weight loss, sickness and/or death. These findings are similar to Ray *et al.* (2001). Similarly, DCLF alone was extremely effective in inducing severe nephrotoxicity. Lower doses (100 and 200 mg/kg) were sub-lethal, whereas, the highest dose (300 mg/kg) was extremely lethal and supertoxic. In fact, a 300 mg/kg dose caused 80% animal death. This corroborates with our earlier observations (Ray *et al.*, 1996).

Administration of DCLF to non-diabetics caused slight hypoglycemia but in diabetic animals there was no blood glucose lowering. This slight hypoglycemic effect of DCLF in non-diabetics may be due to nonspecific response of normal beta cells whereas its ineffectiveness in diabetics may be due to loss of beta cell responsiveness. In addition, at the time of sacrifice, DCLF-exposed kidneys were distinguishable from controls as they were pale and abnormally enlarged and significantly heavier. Animals in 100 mg/kg DCLF-treated group were less active than controls, whereas most animals in the 200 mg/kg group appeared sick and showed low level of activity. The appearances and activity levels of animals of STZ alone and STZ+DCLF groups were nearly identical. The color and texture of kidneys of these two groups were very similar. The impacts of STZ and STZ+ DCLF on kidneys were less severe as compared to kidneys exposed to DCLF alone. As regards nephrotoxicity, DCLF treated non-diabetic mice demonstrated massive kidney injury and showed sharp increase in BUN levels. This indicated a normal course of metabolism of DCLF as well as the after effects *in vivo*. However diabetic animals responded poorly to DCLF's toxic effects. Their BUN levels were lower than non-diabetics. This may be due to several reasons: (i) DCLF might have been excreted in urine very rapidly, (ii) hyperglycemia might have destroyed/inhibited various iso-forms of cytochrome P-450 enzymes responsible for its metabolism (iii) hyperglycemia might have interfered formation of toxic metabolites of DCLF and (iv) high glucose levels may have provided excessive energy and protected the cells when they were undergoing toxic assaults by DCLF or its reactive metabolites. Since these events were not examined systematically, it is very difficult to pinpoint

whether any or all of these contributed in reducing DCLF-nephrotoxicity in diabetic mice.

Histopathology of PAS-stained kidney sections followed the serum chemistry changes and strongly correlated with DNA damage and lipid peroxidation data. Kidney sections of control mice showed normal cellular structure with no evidence of injury. In contrast, DCLF-induced changes as manifested by numerous morphological alterations coupled with a lethal depletion of glycogen. Abnormal swelling of kidney cells and loss of nucleus or macula densa in numerous proximal and distal tubular cells were most commonly encountered. Numerous cells with apoptotic nuclear morphology ranging from fragmented bodies to fragmented nuclei (including fragmented chromatin) were also prominent in severely damaged areas. DCLF was also able to damage glomerular apparatus as its higher doses induced massive vacuolation to this filtration apparatus. In contrast to changes found in cortex area of the sections, medullary rays and ureter areas remained affected to a relatively lesser degree.

Apoptotic nuclei were predominantly localized to heavily injured proximal and distal tubular cells. The other striking feature was presence of numerous inflammatory cells. STZ alone caused depletion of glycogen and minor changes in macula densa. No gross changes were observed in the glomerular apparatus. Some apoptotic nuclei were seen but not many when compared to DCLF alone. Administration of STZ+DCLF combination was also very effective in inducing morphological alterations. However, overall changes were less complex than the changes exhibited in mice treated with DCLF alone. A significant reduction in both apoptotic and necrotic deaths was observed. Our histological findings suggest the incidence of apoptosis was random and lacked particular loci; most cells that demonstrated different stages of apoptosis were scattered throughout the heavily damaged areas.

Numerous findings now suggest that DNA becomes an easy target of both apoptosis and necrosis, and stability of the cellular genomic apparatus is constantly challenged by a wide-spectrum of exogenous toxicants and endogenous (oxidative damage, factor imbalance) threats, which generate DNA lesions. The present study also showed that STZ alone induced minor activation of endonuclease resulting in DNA fragmentation detectable by both methods. STZ+DCLF treated groups showed a slightly higher order of fragmentation but significantly lower than DCLF alone. DNA fragmentation assessed in this study is a total picture of mitochondrial and nuclear DNA fragmentation. Nagata *et al* (2000) have also reported that endonuclease, which is responsible for inducing DNA ladder is a caspase-activated DNAase (CAD) which preexists in living cells as an inactive complex with an inhibitory subunit, dubbed ICAD. Activation of CAD occurs by means of caspase-3-mediated cleavage of the inhibitory subunit, resulting in the release and activation of the catalytic subunit. Although, final activation of CAD may be or may not be Ca^{2+} - dependent, experiments are in progress to unravel DCLF's intervening role at such regulatory pathways. How DCLF modulated STZ-induced DNA fragmentation remains puzzling. Oxidant-induced injury to the plasma membranes of cells can evoke a wide variety of responses, including changes in transmembrane transport, receptor-ligand interactions, signal transduction, and enzyme activity regulation as well as differentiation and proliferation (Emerit *et al.*, 2001). It is suggested that lipid peroxidation plays a critical role in oxidant-induced renal cell injury; therefore, lipid peroxidation has been used as a sensor for renal injury (Nowzari *et al.*, 2000). Cellular macromolecules are highly vulnerable to oxidative injury by ROS, often leading to cell death by apoptosis and necrosis. Studies indicate that ROS or reactive nitrogen species (RNS) can have diverse effects on mammalian cell growth. In some

cases, small quantities of oxy radicals induce cells to undergo apoptosis whereas, in other cases, ROS promote cell proliferation and may function as intracellular second messengers. Evidences suggest that oxidative stress is a common mediator of apoptosis, perhaps via the formation of lipid hydroperoxides. It has been suggested that DCLF metabolism yields a very reactive quinoneimine species, which may be the prime suspect in generating oxidative stress (Tang *et al.*, 1999). DCLF is a powerful nephrotoxicant in addition to its hepatotoxic potential. In the present study, kidney MDA levels were determined to measure oxidative stress generated by DCLF. It caused dramatic increases (4 fold over control) in MDA, and STZ alone produced twice the amount detected in control tissues. DCLF-exposed diabetic tissues showed MDA levels close to STZ alone and significantly less compared to DCLF alone. The trends of elevated BUN levels and DNA fragmentation clearly resembled oxidative stress induced MDA accumulation data. How hyperglycemia reduced toxicity remains a question open for future investigation. Some studies have shown that glucose can act as a free radical scavenger. Weakening of DCLF toxicity, observed in these experiments in diabetics may be a reflection of this fact. There are a number of other mechanisms by which DCLF may have produced nephrotoxicity. Collectively, our results have suggested that DCLF is less effective in diabetics than non-diabetics. This implies that therapeutic doses usually prescribed for non-diabetics may not show equivalent analgesic action on diabetics. This conclusion is based on the fact that hyperglycemic condition failed either to potentiate or synergize DCLF-induced nephrotoxicity *in vivo*; showing that this drug cannot be considered as a problematic NSAID for diabetics.

REFERENCES

- A.E Raptis, G. Viberti, "Pathogenesis of diabetic nephropathy," Exp Clin Endocrinol Diabetes. 2001,109, S424-S437.
- A. K. Mohamed, A. Bierhaus, S. Schiekofer, H. Tritschler, H. Ziegler, and P.P. Nawroth, "The role of oxidative stress and NF- B activation in late diabetic complications," BioFactors. 1999,10,175-169.
- A. Carmo, V.J Cunha, A.P. Carvelho, C.M. Lopes, "Effect of Cyclosporin-A on the blood-retinal barrier permeability in streptozotocin-induced diabetes," Mediators-of-inflammation. 2000, 9, 243-248.
- A.S. Reddi, G.N. Jyothirmayi, K. Halka, and M.Y. Khan, "Potentiation of renal tumorigenicity by cyclosporin-A in the STZ diabetic rats," Cancer cell. 1991, 56,109-5.
- D.E. Hricik, M.S. Phinney, K.A. Weigel Knauss, J.A. Schulak, "Long-term renal function in type I diabetics after kidney or kidney-pancreas transplantation: influence of number, timing, and treatment of acute rejection episodes," Transplantation. 1997, 64, 1283-8.
- E. Rour, P. Beaune, J.P'. Leroux, "Immunoquantitation of some cytochrome P-450 isozyme in liver micro somes from streptozotocin-diabetic rats," Experientia 1986, 15,42 1162-3.

E.E. Blaak, B.H. Wolffenbuttel, W.H. Saris, M.M. Pelsers, A.1. Wagenmakers, "Weight reduction and the impaired plasma-derived free fatty acid oxidation in type 2 diabetic subjects," Clin Endocrinol Metab. 2001, 86, 1638-44.

E.J. Hickey, R.R. Raje, V.E. Reid, S.M. Grosss and S.D. Ray, "Diclofenac induced in vivo nephrotoxicity may involve oxidative stress-mediated massive genomic DNA fragmentation and apoptotic cell death," Free Radic. BioI & Med. 2001,31, 139-152.

F.B. Nowzari, S.D. Davidson, M. Eshghi, C. Mallouh, H. Tazaki, S. Konno, "Adverse effects of oxidative stress on renal cells and its prevention by antioxidants," Mol Urol. 2000, 4, 15-19.

H. Talke, G.E. Seubert, "Enzymatische Hamstoffbestimmung im Blut un serum im Optischen Test nach Warburg," Klin Wochschr. 1965, 43, 174.

Huma Naeem, Ashfaq Ahmad, Chaudhary Bakhtawar Khan, Raja Khawar Nawaz. Prognosis of uncontrolled diabetes and hypertension leads to secondary complications: a case report. J App Pharm 01(03): 144-148 (2011)

J.H. Zar, Biostatistical Analysis, 2nd ed., Prentice Hall, Englewood Cliffs, 1984.

J. Emerit, C. Beaumont, F. Trivin, "Iron metabolism, free radicals, and oxidative injury," Biomed Pharmacother. 2001, 55, 333-9.

J.H. Nam, J.I. Mun, S.I. Kim, S.W. Kang, K.H. Choi, K. Park, C.W. Ahn, B.S. Cha, Y.D. Song, S.K. Lim, K.R. Kim, H.C. Lee, K.B. Huh, "beta-Cell dysfunction rather than insulin resistance is the main contributing factor for the development of post-renal transplantation diabetes mellitus," Transplantation. 2001, 71,1417-23.

Jorns, A., Klemptner, J. M. Tiedge, S. Lenzen, "Recovery of pancreatic beta cells in response to long-term normoglycemia after pancreas or islet transplantation in severely streptozotocin diabetic adult rats," Pancreas. 2001,23, 186-96.

K. Burton, "A study of the conditions and mechanism of the Diphenylamine Reaction for the colorimetric estimation of Deoxiribonucleic Acid," Biochem. J. 1956, 62, 315-315-322.

M. Bagchir, E. A. Hassoum, D. Bagchi, and S. J. Stohs, "Endrin-induced increases in hepatic lipid peroxidation, membrane microviscosity, and DNA damage in rats," Arch Environ Contam Toxicol. 1992,23, 1-5.

M. El-Hawari, G.L. Plaa, "Potentiation of thioacetamide-induced hepatotoxicity in alloxin and streptozotocin-diabetic rats," Toxicol-Iett. 1983, 17,293-300.

O.A. Al-Shabanah, H.A. EL-Kashef, O.A. Badary, A.M. AI-Bekairi, and M.M. Elmazar, "Effect of streptozotocin-induced hyperglycaemia on intravenous pharmacokinetics and acute cardiotoxicity of doxorubicin in rats," Pharmacol Res. 2000,41, 31-7.

T.G. Chen, T.Y. Tai, H.C. Chang, C.T. Hong, R.M. Chen, T.L.Chen, "Modulation of cytochrome P450-dependent monooxygenases in streptozotocin-induced diabetic hamster:!! Reverse role of insulin in P450 activity and defluorination," Acta-Anaesthesiologica-Sin. 2000, 38, 65-72.

T. McGowan, P. McCue, K. Sharma, "Diabetic nephropathy," Clin Lab Med. 2001,21,111-146.

S.D. Ray, and M.W. Fariss, "Role of cellular energy status in tocopheryl hemisuccinate cytoprotection against ethylmethanesulfonate-induced toxicity," Arch. Biochem. Biophys. 1994,311,180-190.

S.D. Ray, R. Virgil, W. Marc W. Fariss, "Extracellular Calcium Dependent and Independent Mechanisms of Acetaminophen Hepatotoxicity," Toxicologist. 1986, 6, 187.

S.D. Ray, V.R. Mumaw, R.R. Raje, M.W. Farris, "Protectin of acetaminophen induced hepatocellular apoptosis and necrosis by cholesteryl hemisuccinate pretreatment," I: Pharmacol. Exp. Ther. 1996,279, 1470-83.

S.D. Ray, G. Balasubramanian, D. Bagchi, and C.S. Reddy, "Ca²⁺- Calmodulin antagonist Chlorpromazine and Poly (ADP-Ribose) Polymerase Modulators 4-Aminobenzamide and Nicotinamide Protect on Acetaminophen- Induced Hepatotoxicity and Oxidative Stress by Influencing Hepatic Expression of Bcl-XL and p53 in mice," Free Rad. Biol. Med. 2001,50, 146-51.

W. Tang, R.A. Steams, R.W. Wang, S.H. Chiu, T.A. Baillie, "Roles of human hepatic cytochrome P450s 2C9 and 3A4 in the metabolic activation of diclofenac," Chern Res. Toxicol. 1999, 12, 192-199.