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Author
Yang, Dong Yan

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DRUG-INDUCED HYPERSENSITIVITY: STUDIES WITH NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

by

Dong Yan Yang

DISSERTATION

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ABSTRACT

DRUG-INDUCED HYPERSENSITIVITY: STUDIES WITH NONSTEROIDAL ANTI-INFLAMMATORY DRUGS

Dong Yan Yang

We have hypothesized that the covalent binding of nonsteroidal anti-inflammatory drugs (NSAIDs) to proteins *via* reactive metabolites (such as acyl glucuronide or acyl CoA metabolites) *in vivo* may trigger an immune response and thereby cause the toxicity found in some patients with this class of drugs. Using diclofenac (D), a carboxylic acid drug that causes the most severe hypersensitivity reactions to NSAIDs, as a model compound, we examined the reactivity and covalent binding of diclofenac acyl glucuronide (DG) to plasma proteins both *in vitro* and *in vivo*, as well as the immunogenicity of diclofenac protein adducts, formed via reactive DG, in an animal model. We also investigated the immunologic aspect of the covalent binding in humans.

DG is reactive under physiological conditions and appears to follow first-order degradation kinetics, undergoing intramolecular acyl migration to other glucuronide isomers and hydrolysis to the parent compound. Higher pH and the presence of human serum albumin (HSA) decreased DG stability. DG was also found to react with HSA *in vitro*, with maximum covalent binding observed after 4 hr incubation with HSA.

Irreversible binding of D to plasma proteins was measurable in humans even after a single 75mg oral dose of D. The protein adducts accumulate in the body upon multiple dosing. The diclofenac protein conjugates exhibited biphasic elimination, with a long terminal half-life of 10.3 days.

Mouse serum albumin adducts of DG and D, formed *via* the imine mechanism or by the nucleophilic mechanism, were both immunogenic and could induce drug or acyl

glucuronide-specific antibodies in mice. These antibodies exhibited cross reactivity with other NSAIDs and their glucuronides to varying degrees.

Low titers of drug-specific IgG antibodies were detected in selective patients (4 of 18) who had had adverse reactions, mainly respiratory reactions, to NSAIDs and aspirin, and these antibodies exhibited cross-reactivity with other structurally similar NSAIDs. These findings suggest that reactions to NSAIDs in some patients may be due to an immune response to the drug and related drugs, rather than interference with the arachidonic acid metabolism pathway.

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GLOSSARY

AIA	Aspirin Induced Asthma
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
CFA	Complete Freund's Adjuvant
COX	Cyclooxygenase
D	Diclofenac
DG	Diclofenac Glucuronide
EDC	1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide
ELISA	Enzyme Linked Immunosorbent Assay
GST	Glutathione S-Transferase
HLA	Human Leukocyte Antigen
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
IFA	Incomplete Freund's Adjuvant
MSA	Mouse Serum Albumin
NSAID	Nonsteroidal Anti-inflammatory Drug
PBS	Phosphate Buffered Saline
PMSF	Phenylmethylsulfonyl Fluoride
UDPGA	Uridine Diphosphoglucuronic Acid
VA	Valproic Acid

1.0 INTRODUCTION

1.1 BACKGROUND

1.1.1 Nonsteroidal Anti-inflammatory Drugs

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the mostly commonly prescribed drugs worldwide for their analgesic and anti-inflammatory effects. It is estimated that up to 2% of the North American population use NSAIDs on a daily basis (Knodel *et al.*, 1992). About 50% of NSAID prescriptions are for people aged over 60; the number of NSAID users is still growing as a result of the increasing proportion of population aged over 60 years and the widespread use of NSAIDs for the prophylaxis of Alzheimer and thromboembolic complications of neurological and cardiological conditions.

NSAIDs currently available come from a variety of chemical classes (Table 1.1) (Boelsterli *et al.*, 1995). The vast majority of these drugs are weakly acidic, with ionization constant (pKa) ranging from 3 to 5. Acidic NSAIDs become sequestered preferentially in the synovial tissue of inflammed joints (Cummings and Nordby,1966), which may be of potential advantage during episodes of arthritis.

The six major classes of NSAIDs have the common property of inhibiting cyclooxygenase, the enzyme that catalyzes the synthesis of cyclic endoperoxide from arachidonic acid to yield prostaglandins, which are important mediators of pain and inflammation (Fig. 1.1). Our understanding of the mode of action of the NSAIDs as inhibitors of cyclooxygenase has been significantly expanded by the finding of different forms of this enzyme (DeWitt *et al.*, 1993; Meade *et al.*, 1993; Mitchell *et al.*, 1993; Furst, 1994; Patrignani *et al.*, 1994). Accumulated evidence

Table 1.1. Classification of nonsteroidal anti-inflammatory drugs according to their chemical structures (Boelsterli *et al.*, 1995).

Carboxylic Acids

Salicylic acids/-esters
- Aspirin [1]
- Sodium salicylate [1]
- Diflunisal [1]
- Benorilate

Acetic acids
- Phenylacetic acids
  - Diclofenac [1]
  - Alclofenac*
  - Fenclofenac*
  - Ibufenac*
- Carbo- and heterocyclic acetic acids
  - Indomethacin [1]
  - Clometacine
  - Sulindac [1]
  - Tolmetin [1]
  - Zomepirac*
  - Etodolac [1]
  - Fentiazac
  - Fenclozic acid

Propionic acids
- Ibuprofen [1]
- Naproxen [1]
- Flurbiprofen [1]
- Fenoprofen [1]
- Fenbufen [1]
- Benoxaprofen*
- Indoprofen*
- Ketoprofen [1]
- Pirprofen*
- Carprofen [1]
- Suprofen*
- Tiaprofenic acid
- Oxaprozin

Fenamic acids
- Flufenamic
- Mefenamic [1]
- Meclofenamic [1]
- Niflumic
- Tolfenamic

Enolic Acid

Pyrazolones
- Oxyphenbutazone [1]
- Phenylbutazone [1]
- Azapropazone [1]
- Feprazone

Oxicams
- Piroxicam [1]
- Sudoxicam
- Isoxicam
- Nebumetone [1]

This incomplete list encompasses the most frequently used NSAIDs, only compounds that are on the market or for which data have been reported from clinical trials, are listed. [1]Available in the USA. *Compounds have been withdrawn from the market (USA).

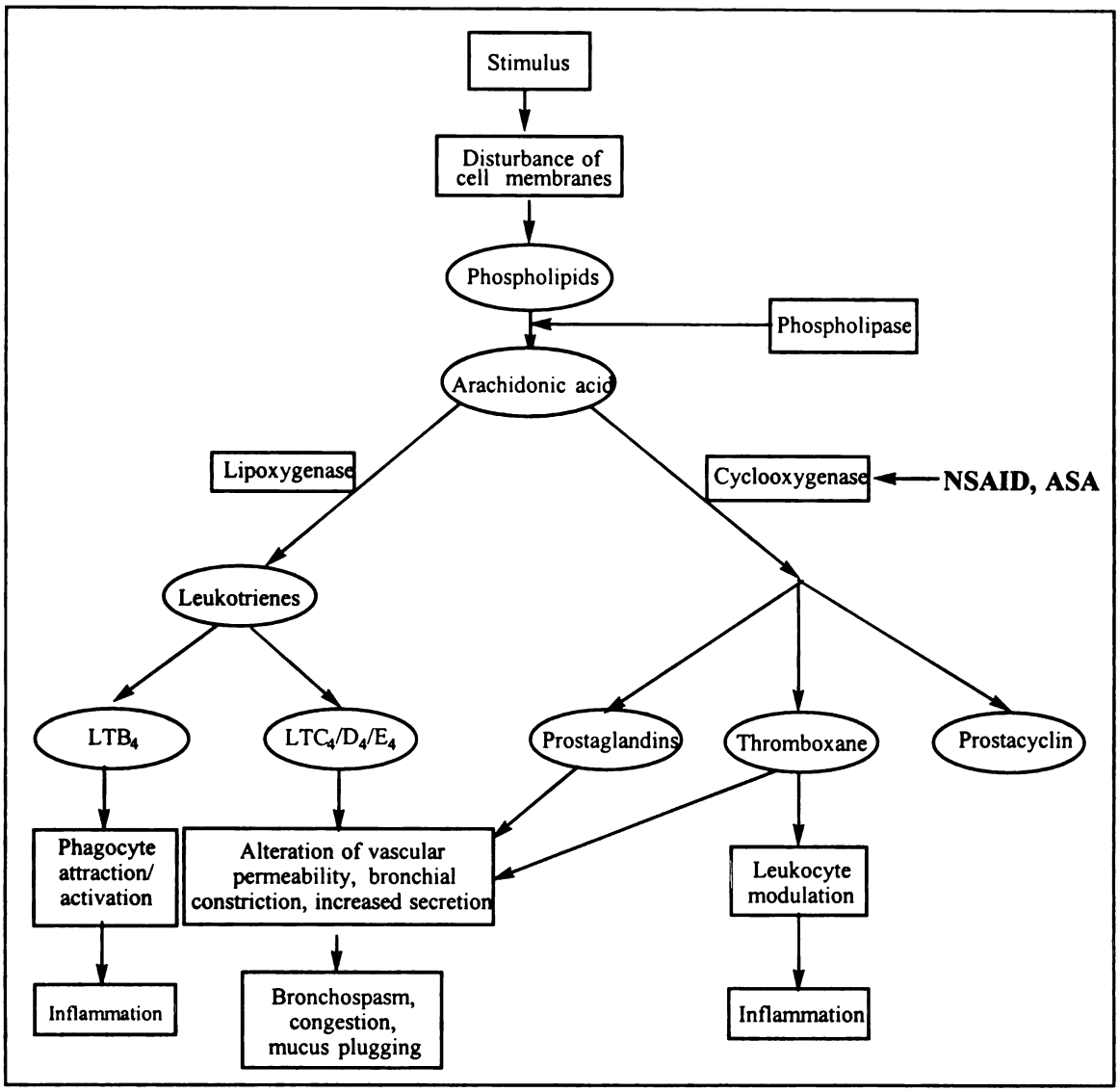

Fig. 1.1. Scheme for mediators derived from arachidonic acid and sites of drug action. (ASA = aspirin)

suggests that there at least two isoforms of cyclooxygenase. Cyclooxygenase 1 (COX-1) is a constitutive form expressed at all cell types, functioning as a "housekeeping" enzyme. Cyclooxygenase 2 (COX-2) is an inducible form only expressed at sites of tissue inflammation. Both COX-1 and COX-2 can be inhibited by all of the available NSAIDs, though evidence suggests that there may

be difference in the way certain NSAIDs affect the constitutive (COX-1) and the inducible (COX-2) enzyme *in vitro*.

1.1.2 Toxicity of Nonsteroidal Anti-inflammatory Drugs

As a group, NSAIDs rank first among commonly prescribed drugs for serious adverse reactions. NSAIDs were responsible for 25% of all adverse drug reactions reported to the Committee on Safety of Medicines in the UK and 21% of all suspected adverse drug reactions reported to the US Food and Drug Administration (Committee on Safety of Medicines, 1986; Rossi *et al.*, 1987). Even though the most common adverse reaction of NSAIDs is gastrointestinal toxicity, the most significant life-threaten reactions that hamper the continuing usage of NSAIDs are the less common organ toxicities (such as liver, kidney, and hematological diseases) or allergic reactions (such as asthma, urticaria, and anaphylaxis) (Kenny, 1992). Cross-reactivity among NSAIDs have also been observed (Stevenson, 1984).

It is striking that of 26 drugs withdrawn from the U.S. and British markets during the past 30 years, six were NSAIDs of the carboxylic acid class (Bakke *et al.*, 1984; Strom *et al.*, 1989). They are alclofenac, benoxaprofen, ibufenac, indoprofen, suprofen and zomepirac, all of which were withdrawn because of organ toxicities and/or severe allergic reactions. Many other currently available on the market acid NSAIDs, such as aspirin (Amos *et al.*, 1971), diclofenac (Helfgott *et al.*, 1990; Boelsterli *et al.*, 1995; van der Klauw *et al.*, 1996), tolmetin (McCall and Cooper, 1980; Rake and Jacobs, 1983), ibuprofen (van der Klauw *et al.*, 1996), fenoprofen (Porile *et al.*, 1990), diflunisal (Cook *et al.*, 1988), naproxen (Boelsterli *et al.*, 1995), and ketoprofen (Boelsterli *et al.*, 1995; van der Klauw *et al.*, 1996), have been implicated as the cause for rare hypersensitivity reactions. As the usage of NSAIDs increases, the numbers of adverse reactions will increase in parallel.

Up to now, the pathogenesis of hypersensitivity to NSAIDs has not yet been clearly understood.

1.1.3 Proposed Mechanisms of Immunotoxicity to Nonsteroidal Anti-inflammatory Drugs

Currently, the widely accepted hypothesis is that toxicities to aspirin and other NSAIDs are caused by inhibition of cyclooxygenase activity by these compounds, resulting in an overproduction of cysteinyl leukotrienes from arachidonic acid (Szczeklik *et al.*, 1977; Szczeklik, 1987; Stevenson and Lewis, 1987). Leukotrienes are potent brochoconstrictors (Dahlen *et al.*, 1980); they increase microvascular permeability (Rinkema *et al.*, 1984), stimulate mucus secretion and plasma exudation (Marom *et al.*, 1982), and also play an important role in the chemotaxis and activation of inflammatory cells (Bousquet *et al.*, 1990; Kowalski *et al.*, 1996). This hypothesis is supported by many clinical observations in susceptible individuals, where taking aspirin and other NSAIDs precipitate bronchial asthma and sensitivity (Stevenson, 1984; Szczeklik, 1987) and by the cross-reactivity to various NSAIDs which seems to correlate with these drugs' potency in inhibiting cyclooxygenase (Stevenson and Lewis, 1987; Settipane *et al.*, 1995). Antagonism of leukotrienes could inhibit bronchoconstriction after aspirin and allergen challenge (Chung, 1995; Holgate *et al.*, 1996).

However, the prostaglandin inhibition hypothesis cannot explain all of the adverse reactions to NSAIDs. Many patients with NSAID sensitivity do not show cross-reactivity with other NSAIDs that are not structurally related, even though they are potent inhibitors of cyclooxygenase *in vitro* (Szczeklik *et al.*, 1977). This selective phenomena was also observed in other patients with angioedema (Blanca *et al.*, 1989a; Carmona *et al.*, 1992; Katz *et al.*, 1993; Quiralte *et al.*, 1996), urticaria, and anaphylactoid reactions (Carmona *et al.*, 1992; Fernandez-Rivas *et*

al., 1993; Quiralte *et al.*, 1997). These observations indicated that cyclooxygenase inhibition was not involved in these adverse reactions to NSAIDs.

Our laboratory has speculated that hypersensitivity reactions to NSAIDs of the carboxylic acid class may occur *via* an immune-based mechanism (Benet and Spahn, 1988). As described in detail in the following section, acid NSAIDs can be metabolized to reactive intermediates, such as acyl glucuronides or acyl-CoA metabolites, which can act as haptens and become immunogenic when covalently bound to endogenous macromolecules. There is a great deal of evidence that suggests an immunologic mechanism may be implicated in the adverse reactions to NSAIDs in humans. The clinical presentations of anaphylactoid reactions to diclofenac, tolmetin, zomepirac and other NSAIDs are very similar to type I IgE-mediated hypersensitivity reactions (McCall and Cooper, 1980; Rake and Jacobs, 1983; Fernandez-Rivas *et al.*, 1993; Mori *et al.*, 1997). Aspirin-specific IgE antibodies have been observed in aspirin-hypersensitive patients (Phills and Perelmutter, 1974; Blanca *et al.*, 1989a; Zhu *et al.*, 1997). Other studies have shown that acetylsalicylic anhydride or acetylsalicyl-salicylic acid are highly reactive compounds capable of binding to proteins and inducing anti-aspiryl IgE antibodies (Bundgaard, 1974; Bundgaard and de Weck, 1975). NSAIDs have also been reported to cause the type IV mechanism of hypersensitivity and patients have exhibited positive patch tests to NSAIDs (Romano *et al.*, 1994; Romano and Pietrantonio, 1997). Furthermore, organ toxicity caused by NSAIDs may result from covalent binding of drug to tissue proteins and subsequent antigen-antibody complex formation (Williams *et al.*, 1992). Studies have shown that protein conjugates of tolmetin (Zia-Amirhosseini *et al.*, 1995), diclofenac (Yang *et al.*, 1998) and diflunisal (Williams *et al.*, 1995) formed *via* their reactive acyl glucuronide metabolites could induce a drug-specific antibody response in animals. Recent reports have demonstrated the presence of drug and metabolite-specific

antibodies in patients with diclofenac-induced immune hemolytic anemia (Salama *et al.*, 1996; Bougie *et al.*, 1997). The finding of a positive association between human leukocyte antigen (HLA) and aspirin induced asthma (AIA) highly suggest immunity as the underlying mechanism of the disease (Dekker *et al.*, 1997).

1.2 REACTIVITY OF CONJUGATION METABOLITES

1.2.1 Reactivity of Acyl Glucuronides

In vivo, many carboxylic acid drugs, including most NSAIDs on the market and all of those withdrawn from the market, are conjugated with D-glucuronic acid to yield hydrophilic acyl glucuronides. These conjugated metabolites were previously assumed to be pharmacologically inactive species, since they are readily excreted in urine. But in the last decade, many studies have shown that they are reactive. Acyl glucuronides, unlike ether glucuronides, are labile under physiological conditions because of the susceptibility of the ester group to nucleophilic attack. The reactivity of acyl glucuronide is manifest *via* hydrolysis, acyl migration, transacylation reaction, and irreversible binding to proteins.

1.2.1.1 Hydrolysis and Acyl Migration

Acyl glucuronides of NSAIDs undergo spontaneous chemical hydrolysis, and enzymatic hydrolysis by β-glucuronidase and nonspecific esterases, releasing the parent aglycone. They also undergo intramolecular rearrangement *via* an acyl migration of the drug moiety from the 1-O-β position to the 2-O-, 3-O- and 4-O-positions among the hydroxyl groups of the glucuronic acid ring, leading to β-glucuronidase-resistant isomers (Fig. 1.2) (Spahn-Langguth and Benet, 1992).

The stability of acyl glucuronides of a number of NSAIDs has been investigated (Spahn-Langguth and Benet, 1992). It is quite variable among

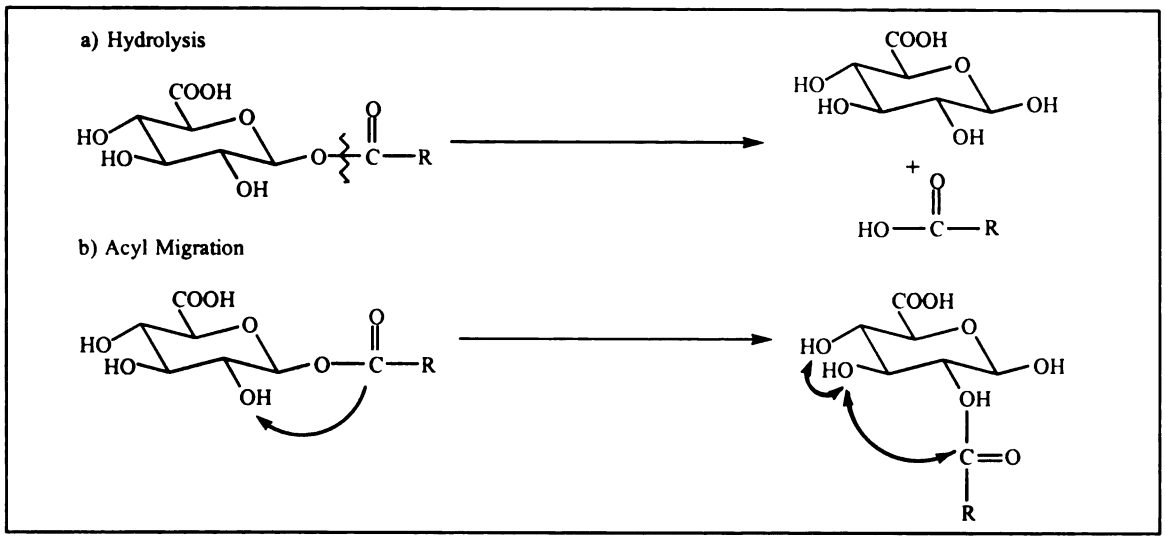

Fig. 1.2. Reactivity of acyl glucuronide. a) Hydrolysis of acyl glucuronide to parent aglycone and glucuronic acid; b) Migration of acyl group of the β-1-O-acyl glucuronide from C1 to C2. The acyl group can then migrate to C3 and subsequently to C4.

different compounds and influenced by many factors. The rates of hydrolysis and acyl migration depend on the pH, temperature and composition of the solution (Smith *et al.*, 1985; Hyneck *et al.*, 1988a; Munafo *et al.*, 1990). For most acyl glucuronides, hydrolysis is substantial in an aqueous alkaline environment and intramolecular rearrangement *via* acyl migration appears to be the predominant reaction under neutral or slightly alkaline conditions. The stability of acyl glucuronide can be significantly enhanced by low pH (pH 2-4), low temperature and esterase inhibitors. The presence of protein also influences the stability of acyl glucuronide, though the effect is inconsistent among NSAIDs. For example, human serum albumin (HSA) was found to accelerate the degradation of oxaprozin glucuronide (Ruelius *et al.*, 1986), R- and S-fenoprofen glucuronide (Volland *et al.*, 1991), and R-and S-naproxen glucuronide (Bischer *et al.*, 1995). However, degradation of tolmetin glucuronide was decreased in the presence of HSA while

bovine serum albumin (BSA) increased the rate of hydrolysis (Munafo *et al.*,1990). Both fatty-acid-free and fraction-V HSA increase the degradation rate of S-carprofen at 37^{0}C and pH 7.4. However, fatty-acid-free HSA was found to stabilize R-carprofen, while fraction-V HSA has no effect on the stability (Iwakawa *et al.*, 1988). The reason why the presence of protein causes variable effects on the stability of different acyl glucuronides is not clear.

1.2.1.2 Reaction with Small Nucleophiles

Acyl glucuronides are also capable of undergoing transacylation reactions with small nucleophiles. Clofibrate acyl glucuronide was found to react covalently with ethanethiol to form the clofibrate thioester (Stogniew and Fenselau, 1982). Clofibrate acyl glucuronide was shown to be a substrate of glutathione S-transferase (GST), and reacts with the reactive metabolite scavenger glutathione (GSH) to form the thioester conjugate of clofibrate and GSH both *in vitro* and *in vivo* (Shore *et al.*, 1995). GSH was unreactive with isomers of clofibrate acyl glucuronide formed by acyl migration, indicating that β-1-O-acyl glucuronide itself was the preferred substrate.

1.2.1.3 Irreversible Binding to Proteins

In addition, drugs, in the form of their acyl glucuronides, can bind covalently to endogeneous macromolecules. Such irreversible binding with plasma and tissue proteins has been reported *in vitro* for a number of carboxylic acid drugs (Spahn-Langguth and Benet, 1992). To name a few, NSAIDs (aspirin, diflunisal, tolmetin, zomepirac, benoxaprofen, fenoprofen, ibuprofen, naproxen, indomethacin, carprofen, oxaprozin, flufenamic acid, and suprofen), hypolipidemic agent (beclobrate, clofibric acid, gemfibrozil) (van Breemen and Fenselau, 1985; Sallustio *et al.*, 1997), an antiepileptic drug (valproic acid) (Williams *et al.*, 1992),

and a diuretic agent (furosemide). Irreversible binding also has been demonstrated in humans on administration of tolmetin (Hyneck *et al.*, 1988b), zomepirac (Smith *et al.*, 1986), fenoprofen (Volland *et al.*, 1991), beclobric acid (Mayer *et al.*, 1993), diflunisal (McKinnon *et al.*, 1989), ibuprofen (Castillo *et al.*, 1995), and diclofenac (Chapter 3).

The *in vitro* covalent binding of the isomers of acyl glucuronides to proteins was also observed. The isomers of diflunisal glucuronide (Williams and Dickinson, 1994), suprofen glucuronide (Smith and Liu, 1993) and valproic acid glucuronide (Williams *et al.*, 1992) were more reactive and exhibited higher covalent binding toward the protein than that of their respective β-1-O-acyl glucuronide. In perfused liver, tissue protein adduct concentrations were higher after perfusions of diflunisal glucuronide isomers than with diflunisal glucuronide, suggesting isomers of acyl glucuronide may play a major role in the formation of protein adducts in liver (Wang and Dickinson, 1997).

Benet *et al.* (1993) observed that degradation rates (intramolecular rearrangement and hydrolysis) for 9 carboxylic acid drug glucuronide metabolites show an excellent correlation (r^2=0.995) with the extent of drug covalent binding to albumin *in vitro*. Furthermore, for those drugs examined, there appears to be a structure relationship between the degradation rate constant of glucuronide and covalent binding. The covalent binding capacity appears to depend on the degree of substitution at the carbon alpha to the carboxylic acid.

Two irreversible binding mechanisms have been proposed (Fig. 1.3). The first involves a nucleophilic displacement of the glucuronosyl moiety by -NH_2, -SH, or -OH groups of the protein. At the end of the reaction, only the drug, minus glucuronic acid, remains irreversibly bound to the protein (Faed, 1984). The second mechanism involves a ring opening in the glucuronic acid moiety after migration of the acyl group to the 2-, 3-, or 4-position of the hydroxyl groups of the

sugar. The aldehyde structure then reacts with a lysine residue to form an imine. Further, Amadori rearrangements lead to more stable 1-amino-2-keto products in which the drug is covalently bound to the protein through a glucuronic acid link (Smith *et al.*, 1990).

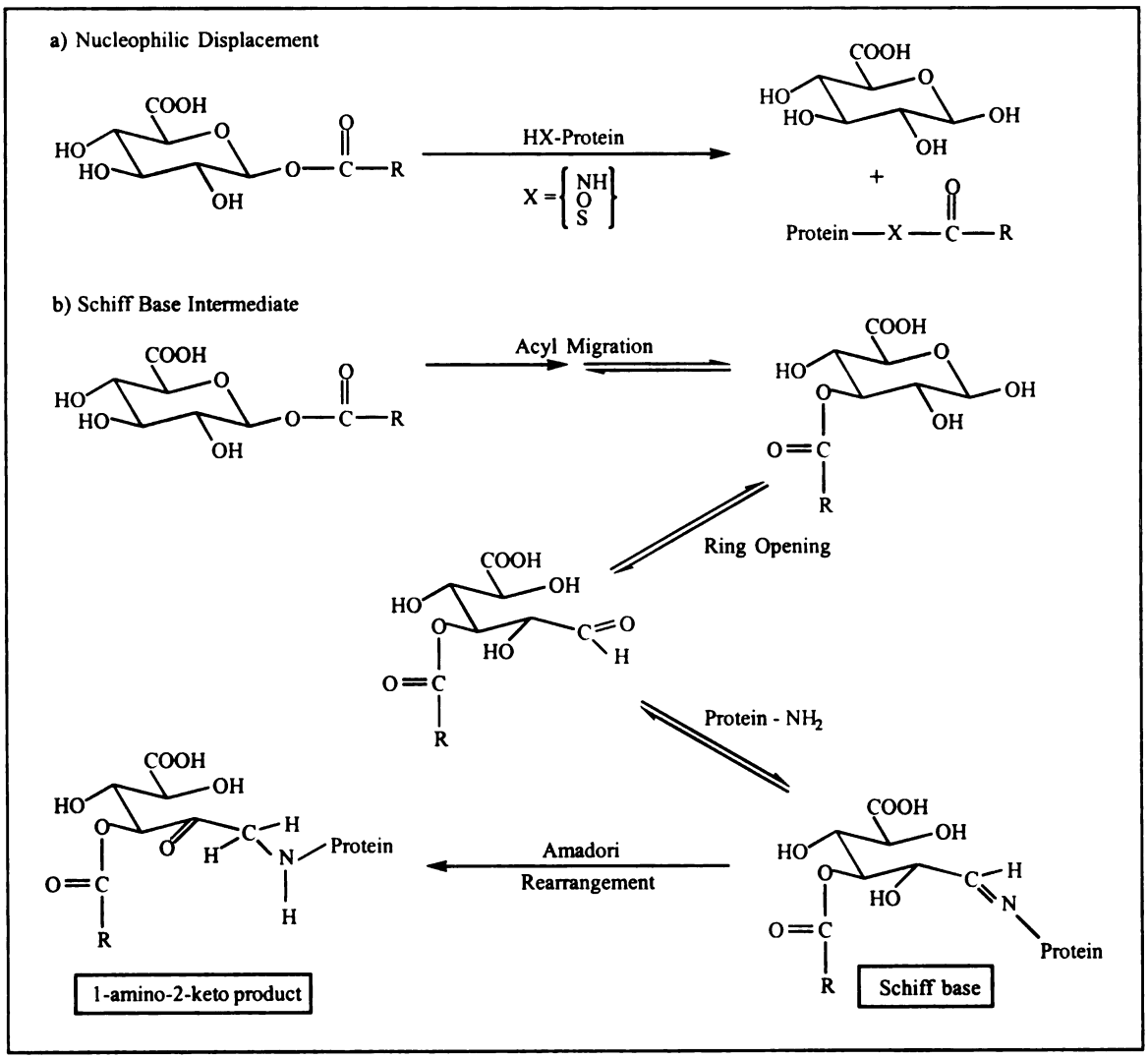

Fig. 1.3. Proposed mechanisms for the irreversible binding of carboxylic acids to proteins *via* their glucuronides. a) Nucleophilic displacement mechanism; b) Schiff base intermediate mechanism.

1.2.2 Reactivity of Other Metabolites

Formation of reactive acyl-CoA thioesters by acyl-CoA ligases is another potential mechanism of adduct generation for carboxylic acid drugs. Acyl-CoA intermediates have been implicated in the chiral inversion of ibuprofen and fenoprofen, and formation of hybrid triglycerides has been well documented (Hutt and Caldwell, 1983). This pathway has been implicated in alkylation of proteins by several other xenobiotic amphipathic carboxylic acids (Hertz and Bar-Tana, 1988); it is possible that it could also mediate covalent modification of proteins by NSAIDs. Alternatively, adduct formation might involve cytochrome P450-mediated oxidation, which has been described for both diclofenac and ibuprofen (Miyamoto *et al.*, 1997) and which has been implicated in formation of a 50 KDa diclofenac adduct in rat liver homogenates *in vitro* (Kretz-Rommel and Boelsterli, 1994b). In the future it will be important to determine whether these metabolic pathways play a role in formation of any of the adducts we have detected.

1.2.3 Toxicological Implications

The reactivity of drug acyl glucuronide toward proteins may be directly associated with the perplexing toxicity associated with many carboxyl-containing drugs.

1.2.3.1 Covalent Binding and Drug Hypersensitivity

Irreversible binding of carboxylic acid drugs, *via* their reactive acyl glucuronides, to plasma proteins *in vitro* has been well documented and has also been detected following administration of drugs to animals and humans. In humans, accumulations of protein adducts of tolmetin (Zia-Amirhosseini *et al.*, 1994), diflunisal, probenecid (McKinnon *et al.*, 1989), and diclofenac (Yang and Benet, 1998) were observed after multiple dosing of each drug to healthy

volunteers. The protein adducts are long-lived, with terminal half-lives from 5 to 10 days. Covalent binding of drug to plasma proteins *in vivo* should be related to acyl glucuronide reactivity and exposure to acyl glucuronide; an excellent (r^2=0.873) linear correlation was observed between the *in vitro* degradation rate constants for five carboxylic drugs and the extents of adduct formation in humans after normalizing the bound drug concentrations to the measured plasma glucuronide concentrations (Benet *et al.*, 1993).

Adduct synthesis has been thought to induce toxicity and anaphylactic or anaphylactoid reactions to drugs *via* hapten formation. Haptens are small chemical groups that cannot elicit antibody responses themselves because they cannot recruit T helper cells. When coupled to a carrier protein, however, they become immunogenic, because T cells can be primed to peptides derived from the protein. This effect is responsible for the allergic responses of many people to many popular small molecule drugs, such as penicillin and sulfonamides, which react with proteins to form haptens that can stimulate an antibody response (Blanca *et al.*, 1989b; Shepherd, 1991; Rieder *et al.*, 1991). Protein conjugates of tolmetin (Zia-Amirhosseini *et al.*, 1995), diflunisal (Williams *et al.*, 1995), and diclofenac (Yang *et al.*, 1998) had been show to be immunogenic and to generate drug-specific antibodies in animals.

1.2.3.2 Covalent Binding and Organ Toxicities

The reactivity of acyl glucuronide is not limited to plasma components; a number of carboxylic acid drugs have also been shown to covalently bind to tissue proteins both *in vitro* and *in vivo*. NSAIDs have been implicated in rare organ toxicities, including hepatitis, nephrotic syndrome and renal insufficiency, and hematological disorders (hemolytic anemia, thrombocytopenia, *etc.*). Clinical and

histopathological observations suggest that the immune system may be involved in causing organ toxicities by this class of compounds (Tarazi *et al.*, 1993).

Several protein adducts of carboxylic acid drugs, including sulindac, ibuprofen, diclofenac, zomepirac, diflunisal, clofibric acid, and valproic acid, have been detected immunochemically in the livers of mice and rats treated with these compounds (Bailey and Dickinson, 1996; Wade *et al.*, 1997). Kretz-Rommel and Boelsterli (1993, 1994a) observed that covalent binding of diclofenac to hepatocellular proteins was dependent on acyl glucuronide formation in cultured rat hepatocytes. Hargus *et al.* (1994) also reported that UDP-glucuronosyltransferase (UGT) catalyzed the formation of diclofenac metabolites that were bound selectively to 110-, 140-, and 200-kDa hepatic plasma membrane proteins. The 110-kDa plasma membrane protein was identified as rat liver dipeptidyl peptidase IV (CD26) (Hargus *et al.*, 1995), suggesting that the hepatotoxicity associated with diclofenac might be in part due to the covalent modification of this membrane protein. While drug modified hepatic proteins were observed, markedly different patterns of protein adducts were detected in immunoblotting studies undertaken in different laboratories, and the pattern of modification varied from drug to drug (Hargus *et al.*, 1994; Kretz-Rommel and Boelsterli, 1994b; Bailey and Dickinson 1996). The reasons for the different patterns found in the different laboratories are unclear, but contributing factors could include differences in model systems (*in vivo* rat vs. cultured hepatocytes), samples (liver homogenate vs. subcellular factions), and specificity of antibodies. Acyl glucuronides also can covalently bind to amino acid residues of a number of target proteins in renal (Smith and Liu, 1995), intestine (Ware *et al.*, 1998), and other tissues. Smith and Liu (1995) observed that covalent binding of suprofen to renal tissues of rat correlated with excretion of its acyl glucuronide by the kidney.

1.3 OBJECTIVES

This newly recognized reactivity of acyl glucuronide has an important, but still poorly defined, bearing on biological distribution and metabolism of a widely prescribed class of drugs. The overall objective of these studies is to further define the pharmacokinetics and biochemical significance of acyl glucuronide reactivity and covalent binding with diclofenac, a widely used NSAID, and to clarify the immunologic aspects of the covalent binding in humans.

The specific aims of the studies presented here were to:

1. Characterize the *in vitro* reactivity of diclofenac acyl glucuronide and measure formation and degradation of the covalently-bound drug *in vitro*.
2. Examine the disposition and stability of diclofenac protein conjugates in the plasma after administration of diclofenac to humans.
3. Evaluate, in a mouse model, the immunogenicity of diclofenac protein adducts formed via the imine mechanism, as well as by the nucleophilic displacement mechanism.
4. Assess the factors involved in the observed cross-reactivity with other NSAIDs.
5. Investigate the relationship between irreversible binding *in vivo* and immunologic toxicity in humans by a) searching for drug-specific antibodies in sera of patients who had an allergic reaction to a NSAID and b) examining lymphocytes of allergic patients for drug-specific antibodies secreting B cells.

2.0 REACTIVITY OF DICLOFENAC ACYL GLUCURONIDE AND IRREVERSIBLE BINDING TO PLASMA PROTEINS *IN VITRO*

2.1 BACKGROUND

In 1988 the Food and Drug Administration approved marketing of the NSAID diclofenac sodium (Voltaren®, Ciba-Geigy) for use in the treatment of rheumatoid arthritis, osteoarthritis, and ankylosing spondylitis (Sallmann, 1986). Diclofenac is the first NSAID to be approved that is a phenylacetic acid derivative. It is a sodium salt with the chemical name sodium [o-(2,6-dichlorophenyl)amino] phenylacetate and the chemical structure is shown in Fig. 2.1. Compared to other NSAIDs, it is structurally unique, since it includes a phenylacetic acid group, a secondary amino group, and a phenyl ring containing chlorine atoms, which cause maximum twisting of the ring (Small, 1989). Diclofenac competes with arachidonic acid *in vitro* and *in vivo* in a dose-dependent manner for binding to cyclooxygenase, which results in decreased formation of prostaglandin $F_{2\alpha}$, prostacyclin and thromboxane A_2 (see Fig.1.1). By inhibiting the production of these prostaglandins, diclofenac reduces the inflammation, swelling, and pain that accompany arthritis. Diclofenac has negligible effects on lipoxygenase but prevents the formation of leukotrienes, which cause inflammation at high concentrations (Ku *et al.*, 1986).

Diclofenac is well tolerated compared with other NSAIDs. As with other NSAIDs, gastrointestinal problems are the most frequent effects. Rare adverse reactions including blood dyscrasias, erythema multiforme, hepatitis, anaphylaxis, and urticaria (O'Brien, 1986) have been noted. Allergic reactions have been reported in 0.5% of patient taking diclofenac (Willkens, 1985). According to the reports of drug-associated anaphylaxis received in the years 1974 to 1994 in The

Netherlands (van der Klauw *et al.*, 1996), diclofenac is one of the most frequently reported drugs (30 of 773 reports). Diclofenac also produces a mild hepatotoxicity in 15% of patients, and in rare instances, causes a fulminant hepatic necrosis. The presence of fever, rash, or eosinophilia suggests hypersensitivity is the underlying mechanism (Ouellette *et al.*, 1991; Boelsterli *et al.*, 1995). In 1993, the Australian Adverse Drug Reactions Advisory Committee reported that diclofenac was implicated in 45% of the reported severe hypersensitivity reactions to NSAIDs (Adverse Drug Reactions Advisory Committee, 1993).

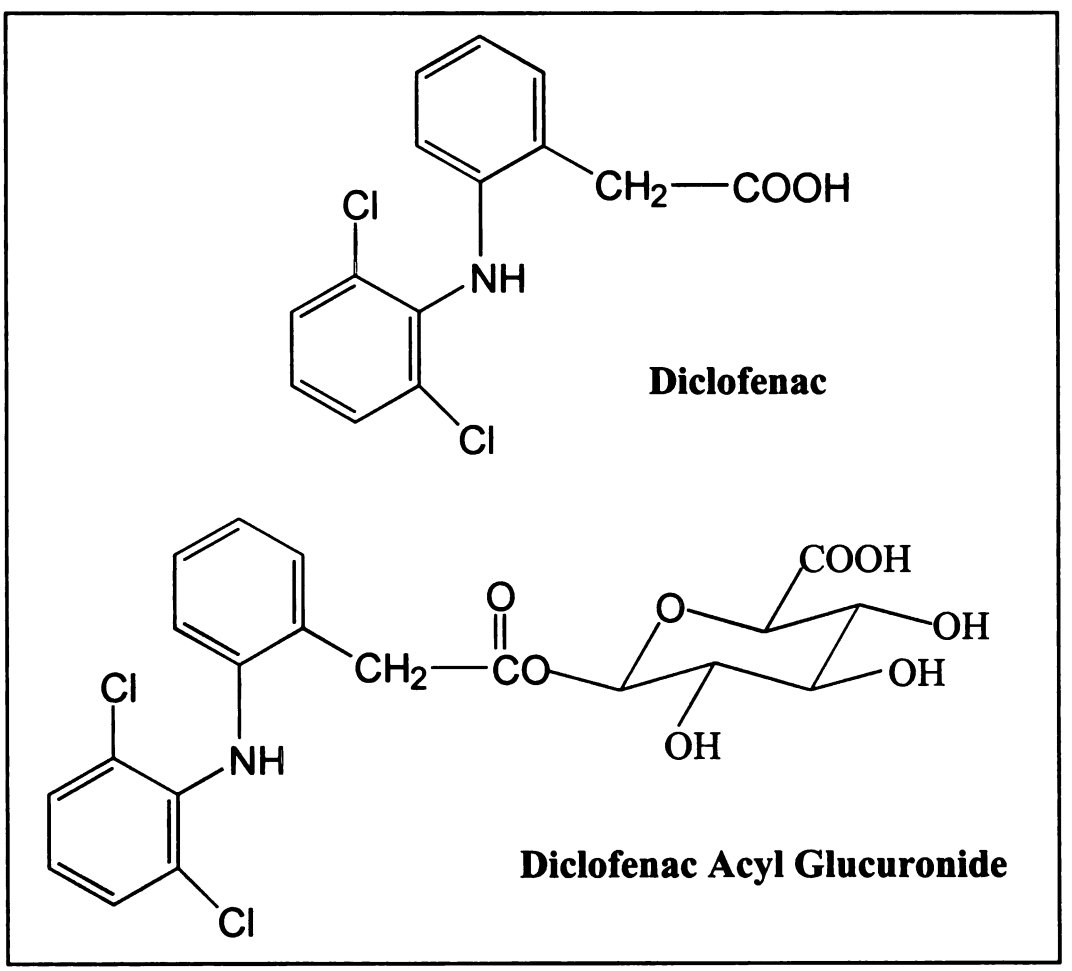

Fig. 2.1 Chemical structure of diclofenac and its acyl glucuronide.

As for many other NSAIDs, diclofenac contains a carboxylic acid group. In humans, the drug is extensively metabolized in liver to form an acyl glucuronide, as well as hydroxyl-diclofenac and its subsequent glucuronide metabolites, which are excreted from the bile and urine. Acyl glucuronides of carboxylic acid drugs have been considered to be inactive and rapidly excreted, however, in the past several years it has been shown that some acyl glucuronides are potentially reactive intermediates that not only undergo hydrolysis and acyl migration but also bind covalently to plasma proteins *in vitro* and *in vivo* (Faed, 1984; Spahn-Langguth and Benet, 1992). Our laboratory has speculated that the reactivities of acyl glucuronides and the covalent binding of these conjugates to endogenous macromolecules may cause organ toxicity and allergic reactions to drugs and/or their metabolites (Benet and Spahn, 1988). Here, the reactivity of diclofenac acyl glucuronide and the *in vitro* covalent binding to plasma proteins were investigated.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Diclofenac sodium (D), fenoprofen calcium, phenylmethylsulfonyl fluoride (PMSF), uridine diphosphoglucuronic acid (UDPGA), saccharic acid 1,4-lactone, Human Serum Albumin (HSA) (fraction V, fatty acid free) were obtained from Sigma Chemical Co. (St. Louis, MO). Diclofenac glucuronide (DG) was prepared *in vitro* with human liver microsomes as described by Volland *et al.* (1991). Analytical grade acetic acid, phosphoric acid and potassium phosphate were purchased from Aldrich (Milwaukee, WI). HPLC grade solvents were obtained from Fisher (Santa Clara, CA).

2.2.2 Instruments

HPLC analyses were performed using a Shimadzu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadzu Corp., Kyoto, Japan) with a Shimadzu SCL-10A controller, a Shimadzu SPD-10A UV/Vis detector (detection at 280 nm), and a Shimadzu RF-5353 fluorescence monitor (excitation 280 nm, emission 355 nm). The detector signal was processed by a Shimadzu EZCHROM chromatography data system, version 3.1. A post-column photoderivatization method was chosen for the bioanalysis of diclofenac (Wiese and Hermansson, 1991). A UV Pen-Ray lamp (1/4x21/8", wavelength 254 nm, Upland, CA) was used as a light source. A PTFE tube (1/16"O.D. x 0.010"I.D., Alltech, San Jose, CA) was wound directly against the glass of the lamp. The length of the PTFE capillary was 1.5 m. The reactor was then connected between the outlet of the column (5-m ultrasphere ODS, 4.6 x 250mm, Beckman Instruments, Berkeley, CA) and the fluorescence detector.

2.2.3 Stability of Diclofenac Glucuronide (DG)

The method utilized is similar to that described previously (Volland *et al.*, 1991). Briefly, DG was dissolved in 0.1 M phosphate buffer (pH 7.4, 6.8, or 5.5, 37^{0}C) with or without 0.5 mM HSA to give a final concentration of 0.1 mM conjugate. Aliquots (100 µl) were taken at specified times; after addition of 200 µl of acetonitrile containing 20 µg/ml internal standard, mixtures were vortexed and centrifuged at 4^{0}C for 10 minutes, and aliquots of supernatant (50 µl) were analyzed by HPLC. An isocratic HPLC system was used. The mobile phase contains 50% methanol in 50 mM ammonium acetate buffer, pH 4.5, at a flow rate of 1.0 ml/min and ambient temperature. Concentrations of acyl migration isomers were calculated using the standard curve of the glucuronide, assuming that the

molar extinction coefficient of the isomers are the same as DG, as described in the studies of zomepirac glucuronide isomers (Smith *et al.*, 1985).

2.2.4 Irreversible Binding of Diclofenac (D) to Plasma Proteins *In Vitro*

DG solution (0.1 mM in 0.1 M sodium phosphate buffer, pH 7.4) containing 0.5 mM HSA was incubated at 37°C. Aliquots (0.5 ml) were taken at 0, 0.5, 1, 2, 3, 4, 6, 8, and 24 hr after incubation for measurement of covalent bound drug. Irreversible binding of D to proteins was determined as described by Zia-Amirhosseini *et al.* (1994) and is defined as the amount of diclofenac that remains bound to plasma proteins after an exhaustive washing procedure, which is then liberated after treatment with strong base. Proteins were precipitated from aliquots and controls by addition of 0.5 ml of cold isopropanol, followed by 1.5 ml of cold acetonitrile. After centrifugation, the protein pellet was washed 8 times with 3 ml of methanol:ether (3:1) to remove reversibly bound D and DG. The protein pellet, which contains covalently bound D, was dried under nitrogen and incubated with 1 ml of 0.25 M KOH at 80°C for 1.5 hours to release D from the adduct. The protein digest was acidified to pH 3 with 50 µl of concentrated phosphoric acid and, after addition of internal standard (150 µl of 2 µg/ml fenoprofen), extracted with 5 ml of methylene chloride. The organic phase was evaporated under nitrogen and reconstituted with 300 µl of mobile phase for HPLC analysis. A gradient HPLC system was used for the measurement of released D. The mobile phase initially contained 38% methanol, gradually increasing to 85% methanol over 23 mins, in 50 mM ammonium acetate buffer, pH 4.5, at a flow rate of 1.0 ml/min and ambient temperature. Standard curves were constructed by spiking precipitated HSA protein pellets with D before base hydrolysis. Protein concentrations after base hydrolysis were determined using bicinchoninic acid protein assay (BCA assay). Concentrations of irreversibly bound drug are expressed as percentage bound of

total conjugate present at the start of the incubation or the amount of diclofenac (in moles) bound per mole of protein (x 10^3).

2.3 RESULTS

2.3.1 Stability of Diclofenac Glucuronide (DG)

Stability of DG in phosphate buffer and buffer containing HSA was studied separately at 37^{0}C. During incubation the amount of DG decreased and appeared to follow first-order kinetics. The amount of parent drug increased with time due to hydrolysis from DG. Three additional peaks, acyl migration products formed by the aglycone migration from position 1 of the glucuronic acid to positions 2, 3 and 4, were observed. These isomers are resistant to β-glucuronidase but can be easily degraded in alkaline solution. Since the specific identities of these peaks have not been definitively established, the acyl migration products in this study are reported as the sum of all three isomers, assuming extinction coefficients identical to that of the parent, as previously demonstrated for zomepirac (Smith *et al.*, 1985).

Figure 2.2 shows the time-dependent degradation of DG in phosphate buffer (a) and buffer containing HSA (b) at pH 7.4. In phosphate buffer at pH 7.4 (Fig. 2.2a), DG was rapidly degraded, and after ~3 hr incubation when almost all of the parent DG was degraded the formation of glucuronide isomers was about three times more than the extent of the hydrolysis to parent drug. DG isomers exhibited a much slower degradation rate compared to DG and gradually hydrolyzed to diclofenac over the 24-hr study period. In the presence of HSA (Fig. 2.2b), DG was readily hydrolyzed to parent drug. At the 3-hr incubation time point, the hydrolysis product exceeded the acyl migration isomers by more than 2-fold. At pH 6.8 both in the absence and presence of albumin, as compared to the pH 7.4 results, hydrolysis was almost unchanged but acyl migration decreased about 25%,

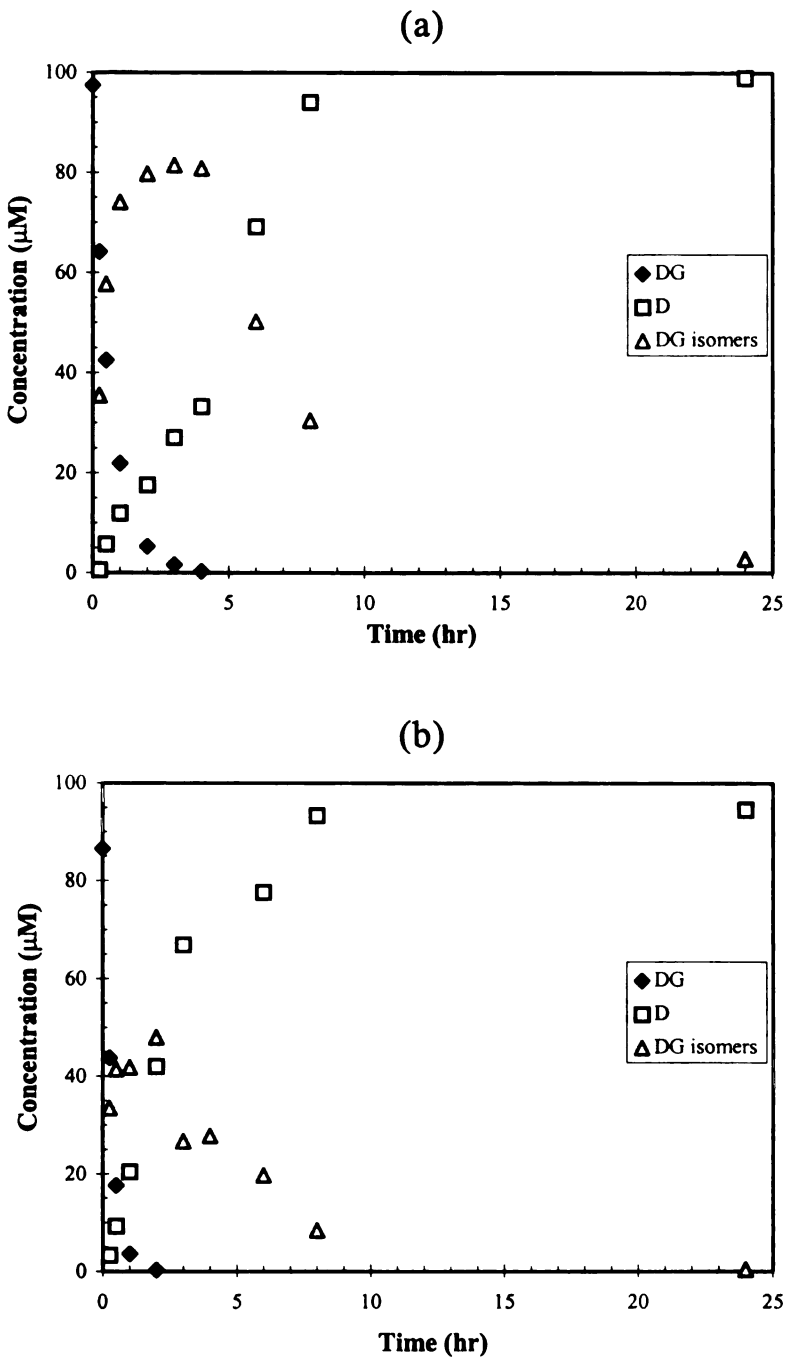

Fig. 2.2. Time-dependent degradation of diclofenac glucuronide (0.1 mM) at pH 7.4 in (a) sodium phosphate buffer at 37°C; (b) sodium phosphate buffer containing 0.5 mM HSA at 37°C.

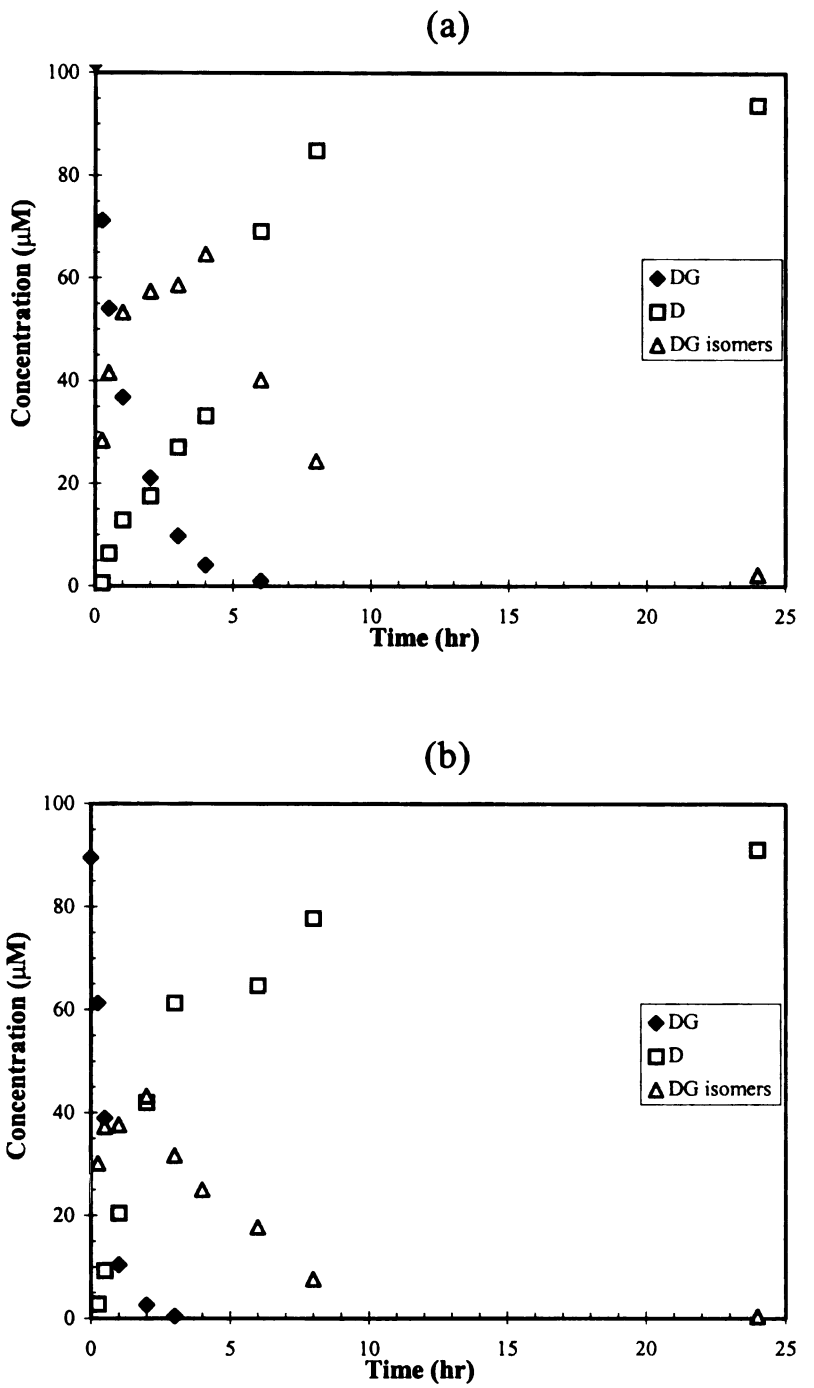

Fig. 2.3. Time-dependent degradation of diclofenac glucuronide (0.1 mM) at pH 6.8 in (a) sodium phosphate buffer at 37°C; (b) sodium phosphate buffer containing 0.5 mM HSA at 37°C.

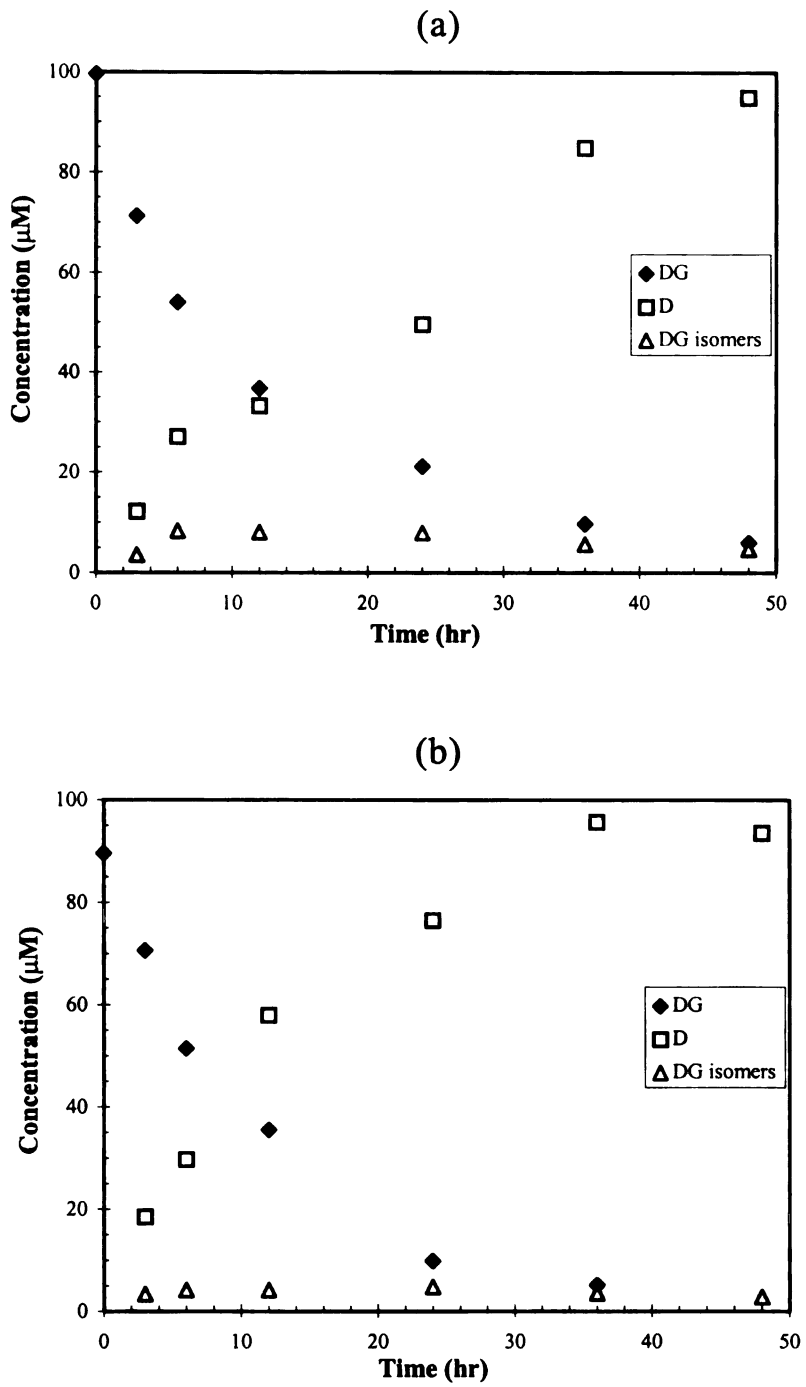

Fig. 2.4. Time-dependent degradation of diclofenac glucuronide (0.1 mM) at pH 5.5 in (a) sodium phosphate buffer at 37°C; (b) sodium phosphate buffer containing 0.5 mM HSA at 37°C.

which results in a greater stability of DG (Fig. 2.3). At pH 5.5, both hydrolysis and acyl migration were significantly decreased as compare to the pH 7.4 and 6.8 results. Negligible acyl migration (< 8%) occurred after 6 hr, the slow rate (k_d=0.06 hr^{-1}) of DG hydrolysis was similar to the rate of formation of diclofenac (Fig. 2.4).

The apparent half-lives and the first order degradation rate constants of DG (0.1 mM) at pHs 7.4, 6.8, and 5.5 in phosphate buffer (Table 2.1a), and in phosphate buffer containing 0.5 mM HSA (Table 2.1b) at 37°C are listed. Degradation rate constants reflect the sum of hydrolysis and acyl migration rate constants plus a possible rate constant for the formation of covalently bound drug which may result directly from the β-1-O-acyl glucuronide. At a higher pH the β-1-O-acyl glucuronide is less stable; the degradation rate constant of DG in phosphate buffer increased approximately 26 fold at pH 7.4 as compared to pH 5.5. When DG was incubated in the presence of HSA, the degradation rate constants increased (Table 2.1).

Table 2.1. Apparent half-lives and first order degradation rate constants of diclofenac glucuronide (0.1 mM) at various pH values in (a) sodium phosphate buffer at 37°C; (b) sodium phosphate buffer containing 0.5 mM HSA at 37°C. Values are the mean ± SD, n=3.

(a)

pH	$t_{1/2}$ (hr)	k_d (hr^{-1})
7.4	0.47 ± 0.05	1.49 ± 0.18
6.8	0.92 ± 0.10	0.76 ± 0.09
5.5	12.1 ± 2.17	0.06 ± 0.01

(b)

pH	$t_{1/2}$ (hr)	k_d (hr^{-1})
7.4	0.22 ± 0.03	3.23 ± 0.48
6.8	0.39 ± 0.05	1.79 ± 0.21
5.5	8.43 ± 0.98	0.08 ± 0.01

2.3.2 Irreversible Binding of Diclofenac to Proteins *via* Glucuronide *In Vitro*

Irreversible binding of diclofenac to proteins occurred after incubation of purified DG with HSA in buffer (pH 7.4). No irreversible binding was observed when diclofenac was incubated under the same conditions. The amount of diclofenac that was irreversibly bound to protein was calculated as a percentage of the amount of DG added at the start of incubation (0.1 mM). Concentrations of diclofenac covalently bound to HSA *in vitro* at pH 7.4 vs. time are shown in Fig. 2.5. The maximum covalent binding (8.7%) occurred after 4 hours of incubation, and then gradually decreased to 2% after 24 hours. Similar instability of the covalent adducts *in vitro* has previously been reported (Volland *et al.*, 1991) reflecting the lability of these adducts, presumably to hydrolysis.

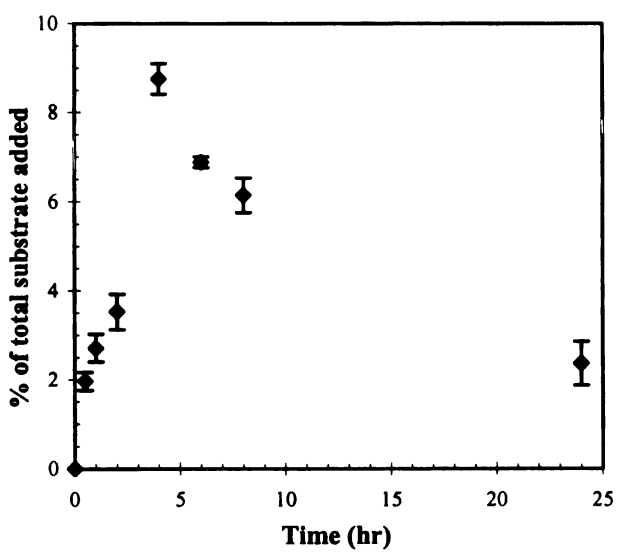

Fig. 2.5. Concentration of diclofenac irreversibly bound to HSA (0.5 mM in sodium phosphate buffer, pH 7.4 and 37°C) vs. time. (Values are mean ± SD, n=3.)

2.4 DISCUSSION

As previously reported in studies of several acyl glucuronide of carboxylic acid-containing NSAIDs (Musson *et al.*, 1985; Smith *et al.*, 1985; Hyneck *et al.*, 1988a; Spahn *et al.*, 1988; Volland *et al.*, 1991; Bischer *et al.*, 1995; Castillo *et al.*, 1995), DG is reactive under physiological conditions, undergoing intramolecular acyl migration to other glucuronide isomers and hydrolysis to the parent compound. Lower pH increased DG stability. Degradation rate constants of the β-1-O-acyl glucuronide also depend on temperature, and composition of the medium. Acyl glucuronides are most stable at a pH between 2-4, at low temperatures, and in the presence of esterase inhibitors (Munafo *et al.*, 1990).

The stability of acyl glucuronides is also influenced by proteins in the media. When DG was incubated in the presence of HSA, the degradation rate constant increased. The esterase-like activity of the albumin molecule has been reported (Kurono *et al.*, 1987). Volland *et al.* (1991) also reported that HSA decreased R- and S- fenoprofen glucuronide stability at pH 7.4 and 6.8, reflecting the esterase-like activity of HSA. At pH 5.5 no significant difference was observed by Volland *et al.* (1991), probably due to the minimal esterase activity at this pH. Ruelius *et al.* (1986) also observed the catalytic activity of HSA at physiologic pH, finding that the hydrolysis rate of oxaprozin glucuronide was increased in the presence of plasma proteins and albumin. Here, the presence of HSA significantly decreased DG stability not only at pH 7.4 and 6.8 but also at pH 5.5, suggesting the contribution of both esterase-activity and the catalytic activity of HSA on DG stability. The effects of protein on glucuronide stability are not consistent. Munafo *et al.* (1990) observed that HSA stabilized tolmetin glucuronide, while bovine serum albumin (BSA) caused an increase in the rate of hydrolysis.

In vitro irreversible binding of diclofenac to HSA occurred after incubation of DG with HSA in buffer, with maximum binding observed after 4 hr incubation. The *in vitro* irreversible binding of acyl glucuronide was shown to be depend on glucuronide concentration (Munafo *et al.*, 1990), pH (Smith *et al.*, 1986), and time (van Breemen and Fenselau, 1985). Mass spectrometric studies have confirmed that *in vitro* covalent modification of HSA by tolmetin glucuronide occurs *via* two different mechanisms (Schiff base formation and nucleophilic displacement) and binding sites of tolmetin and tolmetin glucuronide on HSA have also been identified (Ding *et al.*, 1993, 1995). For different drugs, the mechanisms for covalent binding are the same, however, the binding patterns are very different. When compared to the binding sites on HSA for tolmetin glucuronide, only a limited number were also found for benoxaprofen glucuronides (Qiu *et al.*, 1998). Most hydrophobic aromatic NSAIDs bind to the IIA and IIIA regions of HSA both *in vitro* and *in vivo*; different binding orientation of the drug glucuronides may contribute to different binding site preferences within these two regions.

Benet *et al.* (1993) observed an excellent correlation between the *in vitro* moles of drug maximally bound irreversibly per mole of protein (epitope density) of covalently bound drug (for 6 carboxylic acid drugs, for 3 of which both isomers were evaluated) versus the *in vitro* degradation rate constant (acyl migration and hydrolysis) for the β-1-O-acyl glucuronide conjugates of each compound. Furthermore, a structure activity relationship between the facility for glucuronide degradation and covalent binding for these drugs was also observed. Reexamination of the previous correlation (Benet *et al.*, 1993) and the ability of albumin to stabilize degradation for some glucuronides and accelerate degradation for others (with the difference being most pronounced for DG), led us to utilize the maximum degradation rate observed in testing *in vitro* and *in vivo* correlations with the extent of covalent binding observed. We believe that this is justified, since we

cannot characterize the microenvironment at the protein when covalent binding occurs, but we do know that acyl migration facilitates this reaction. Thus, we believe that the maximum rate of degradation at pH 7.4, 37^{0}C (primarily reflecting acyl migration) best reflects the ability of the glucuronide to facilitate covalent binding.

Our results show that DG, a carboxylic acid with an unsubstituted α-carbon, exhibits the largest degradation rate and the most covalent binding *in vitro* of any NSAID investigated to date in the presence of HSA (Fig. 2.6). These results are consistent with and added to the data obtained with other acyl glucuronides reported previously by our laboratory. Furthermore, a structure activity relationship between the facility for glucuronide degradation and covalent binding for these drugs was also observed. As the number of substituents on the carbon alpha to the carboxylic acid of the aglycone increased, the degradation rate constant of its glucuronide and the maximum covalent binding to proteins *in vitro* decreased. For diclofenac, tolmetin and zomepirac, the three drugs exhibiting the greatest degree of binding, the carbon in the alpha position to the carboxyl group is unsubstituted, containing two hydrogen atoms. In contrast, the compounds with an intermediate degree of binding, fenoprofen and carprofen, have a single substituent on the α-carbon, whereas the compounds exhibiting the least binding, furosemide and beclobric acid, have a completely substituted α-carbon.

The covalent binding of NSAIDs to proteins *via* reactive glucuronide metabolites may trigger an immune response and cause the toxicity found in some patients with this class of drugs. Kretz-Rommel and Boelsterli (1994a) demonstrated that diclofenac can be bioactivated to a reactive acyl glucuronide, which covalently binds to hepatocellular proteins in rat hepatocytes. Diclofenac dependent IgG autoantibodies were detected in the plasma of patients who had diclofenac-induced hemolytic anemia (Salama *et al.*, 1991, 1996). Protein

conjugates of tolmetin glucuronide were shown by our laboratory to stimulate an antibody response in mice (Zia-Amirhosseini *et al.*, 1995). We also describe (Chapter 4) an antibody response in mice to diclofenac adducts formed by both the nucleophilic displacement and the imine mechanisms.

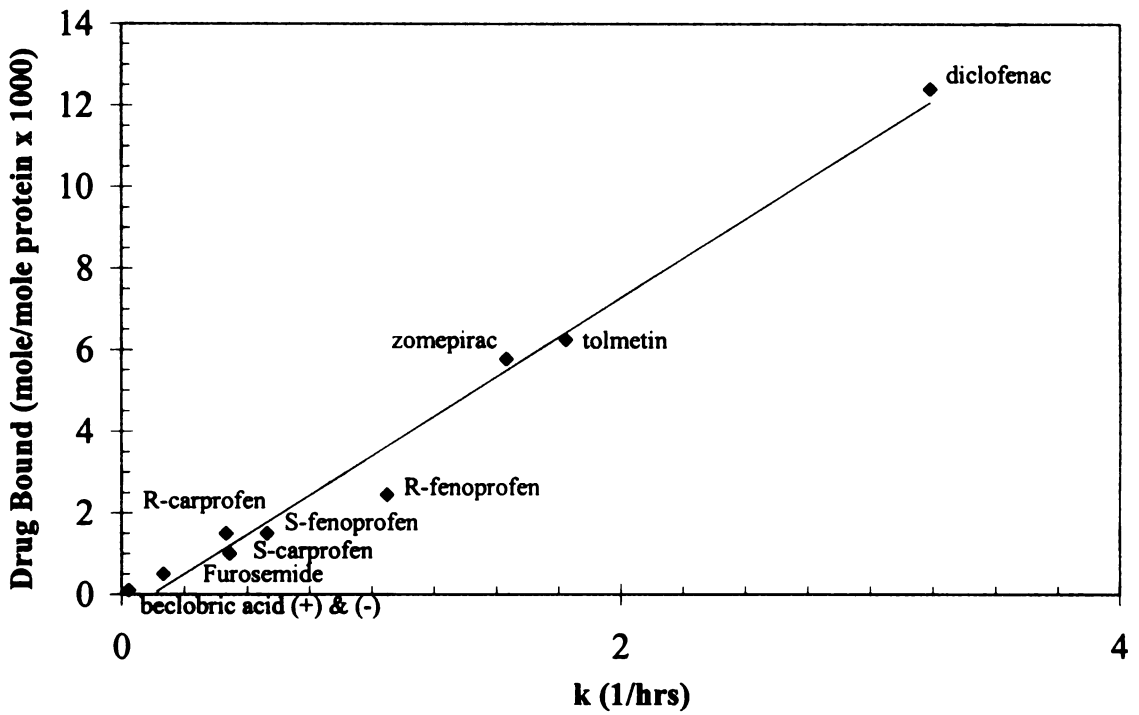

Fig. 2.6. Plot of maximum epitope density (moles drug irreversibly bound per mole of protein x 10^3) versus the maximum degradation rate constant (hr^{-1}) for the pH 7.4, 37°C *in vitro* incubation of various acyl glucuronides (0.1 mM). Degradation rates reflect both acyl migration and hydrolysis. Results are obtained from seven different studies utilizing purified β-1-O-acyl glucuronide of diclofenac, zomepirac, tolmetin, carprofen, fenoprofen, furosemide and beclobric acid (Benet *et al.*, 1993). The data points for the (+) and (-) enantiomers of beclobric acid are indistinguishable on the scale used. An r^2 of 0.983 was obtained for this regression.

3.0 HUMAN *IN VIVO* STUDIES ON THE DISPOSITION AND STABILITY OF DICLOFENAC PROTEIN CONJUGATES

3.1 BACKGROUND

Diclofenac is a potent nonsteroidal anti-inflammatory drug (NSAID) of the phenylacetic acid class, used mostly as an analgesic and in the treatment of rheumatic diseases. Like other commonly used NSAIDs, diclofenac can cause rare hypersensitivity reactions, including allergic reactions and organ toxicities. In 1993, the Australian Adverse Drug Reactions Advisory Committee reported that diclofenac was implicated in 45% of the reported severe hypersensitivity reactions to NSAIDs (Adverse Drug Reactions Advisory Committee, 1993).

A growing body of evidence suggests that hypersensitivity reactions to NSAIDs may occur *via* an immune response to drug covalently modified self-protein *via* a reactive metabolite (Fernandez-Rivas *et al.*,1993; Kretz-Rommel and Boelsterli,1993; Salama *et al.*, 1996; Bougie *et al.*, 1997). A common metabolite of acidic NSAIDs is the acyl glucuronide conjugates of the drug. It has been shown that the acyl glucuronides of carboxylic acid containing NSAIDs are reactive intermediates and will undergo isomerization and hydrolysis under physiological conditions, and are capable of reacting with protein nucleophiles to form covalent adducts both *in vitro* and *in vivo* (Spahn-Langguth and Benet, 1992).

Diclofenac, a compound that causes the most hypersensitivity reactions among NSAIDs, is mainly metabolized in the liver by conjugation (Small, 1989). The principle metabolites are 4'-hydroxydiclofenac and its glucuronide conjugate. About 5-10 percent of the dose is excreted in urine and bile as the acyl glucuronide, which can be readily hydrolyzed to parent drug by chemical (base) or enzymatic (esterase and glucuronidase) reactions. *In vivo*, the production of acyl glucuronide

of diclofenac may be underestimated due to hydrolysis in the blood and enterohepatic circulation. As shown in Chapter 2, diclofenac glucuronide exhibits the largest degradation rate in the presence of human serum albumin and the most covalent binding *in vitro* of any NSAID investigated to date. Here, we investigated the formation and stability of diclofenac protein adducts after oral administration of a single dose and after ten-day twice daily multiple dosing of diclofenac to six healthy volunteers.

3.2 MATERIALS AND METHODS

3.2.1 Materials

For human studies, the commercial diclofenac sodium product (Voltaren®, 75 mg tablets, Ciba-Geigy Corp, Summit, NJ) was obtained from the UCSF Hospital Pharmacy. BCA protein assay kit were obtained from Pierce (Rockford, IL). Analytical grade acetic acid, phosphoric acid and potassium phosphate were purchased from Aldrich (Milwaukee, WI). HPLC grade solvents were obtained from Fisher (Santa Clara, CA).

3.2.2 Instruments

HPLC analyses were performed using a Shimadzu gradient system (autosampler model SIL-10A, HPLC pumps model LC-10AT, Shimadzu Corp., Kyoto, Japan) with a Shimadzu SCL-10A controller, a Shimadzu SPD-10A UV/Vis detector (detection at 280 nm), and a Shimadzu RF-5353 fluorescence monitor (excitation 280 nm, emission 355 nm). The detector signal was processed by a Shimadzu EZCHROM chromatography data system, version 3.1. A post-column photoderivatization method was chosen for the bioanalysis of diclofenac (Wiese and Hermansson, 1991). A UV Pen-Ray lamp (1/4x21/8", wavelength 254 nm,

Upland, CA) was used as a light source. A PTFE tube (1/16"O.D. x 0.010"I.D., Alltech, San Jose, CA) was wound directly against the glass of the lamp. The length of the PTFE capillary was 1.5 m. The reactor was then connected between the outlet of the column (5-μ ultrasphere ODS, 4.6 x 250mm, Beckman Instruments, Berkeley, CA) and the fluorescence detector.

3.2.3 Study Design

Covalently bound diclofenac protein adduct was measured in plasma samples from a clinical study. The clinical study was performed in the UCSF General Clinical Research Center after receiving approval from the UCSF Committee on Human Research. Six healthy volunteers (three women and three men), aged between 25 and 38 years, who had normal physical and laboratory examinations and no history of adverse reaction to any NSAID, participated. All volunteers were not allowed to take any medications, including over-the-counter drugs, one week before and during the study. On the first study day, a 75 mg oral dose of diclofenac was administered to each volunteer. Blood samples (12 ml) were collected at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 36, 48, 60, 72 hours after the drug administration. On the fourth study day, chronic dosing began; the volunteers took 75 mg of diclofenac twice daily on days 4 through 13 for 10 days. On the 14th study day, after a last morning 75 mg dose of diclofenac, blood samples were taken at 0, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 24, 26, 36, 48, 60, 72, 96 and 120 (day 19) hours. Additional samples were drawn on the mornings of days 21, 24, 28, 35, 42, 49, 56, and 63. The subjects were asked to fast for 10 hours prior to the study on days one and fourteen of dosing and food was served two hours after the dosage. No adverse reactions were observed during the study period.

3.2.4 Sample Handling

Blood samples were collected in precooled, heparinized Vacutainers (Becton Dickinson, Rutherford, NJ). After centrifugation at 2000 g for 10 mins at 4°C, plasma was adjusted to pH 2-4 to stabilize diclofenac glucuronide and stored at -20°C until analysis.

Concentrations of diclofenac (D) and its β-1-O-acyl glucuronide (DG) were determined by HPLC. Briefly, 0.5 ml of plasma samples and controls was spiked with 100 µl of a 2 µg/ml solution of fenoprofen. Proteins were precipitated by addition of 1.5 ml cold acetonitrile and vortexed. After centrifugation at 2250 g for 10 min, the supernatant was then transferred to another test tube and evaporated to dryness under N_2 at 30°C. The residue was reconstituted into 300 µl of mobile phase, and a 200 µl aliquot was injected onto the HPLC. In order to separate the glucuronide from other metabolites, the mobile phase was adjusted in a gradient to contain methanol 35% to 90% over 50 mins in 10 mM ammonium phosphate, pH 2.4, at a flow rate of 1 ml/min and room temperature. All samples were prepared and analyzed on the same day.

Covalent binding of diclofenac to plasma proteins was determined as described for the *in vitro* studies (see Section 2.2.4).

3.3 RESULTS

The pharmacokinetic parameters of diclofenac and its glucuronide after a single oral dose of 75 mg diclofenac are summarized in Table 3.1. For subject 2, an unidentified interfering peak in all plasma samples obscured the diclofenac glucuronide peak, and this subject's data was excluded from Table 3.1 (although measurable adduct concentrations were analyzed). Irreversible binding of diclofenac to plasma proteins occurred in all subjects and was detectable in early

samples even after a single dose. Figure 3.1 depicts the plasma concentration-time profile of diclofenac, diclofenac glucuronide and irreversible bound diclofenac in subject 1 after a single dose of 75 mg diclofenac. The protein adducts were eliminated much slower than the drug and its glucuronide metabolite.

The maximum concentration of covalently bound diclofenac after a single dose was 1.88 ± 0.84 ng drug/mg protein (Table 3.2). The mean covalent binding of diclofenac after multiple dosing is depicted in Fig. 3.2. Much higher concentrations of drug-protein adduct (ranging from 5.9 to 10.4 fold increases) are present in plasma after 10 days of twice daily dosing of diclofenac. The mean maximum concentration reached was 12.7 ± 3.5 ng drug/mg protein. Figure 3.2 shows that the diclofenac protein adduct underwent an initial, more rapid decline with a mean half-life of 28 hr, followed by a much slower decline (mean terminal half-life 10.3 ± 2.0 days).

Table 3.1. Maximum concentration, time to peak and AUCs of diclofenac and diclofenac glucuronide. Data were obtained from 5 healthy volunteers after a single oral dose of 75 mg diclofenac. (Subject 2 concentrations were obscured by an interfering peak in all plasma samples.)

Subject	**Diclofenac**			**Diclofenac Glucuronide**		
	C_{max} (μg/ml)	t_{max} (hr)	AUC (μg x hr/ml)	C_{max} (μg/ml)	t_{max} (hr)	AUC (μg x hr/ml)
1	2.12	2.0	3.56	0.42	2.0	0.76
3	3.43	1.5	4.16	0.17	1.5	0.22
4	2.00	1.0	2.75	0.18	1.0	0.38
5	2.79	1.0	3.45	0.23	1.0	0.53
6	1.97	2.0	3.91	0.36	2.0	0.44
Mean ± SD	2.46 ± 0.52	1.5 ± 0.4	3.56 ± 0.38	0.27 ± 0.10	1.5 ± 0.4	0.46 ± 0.14

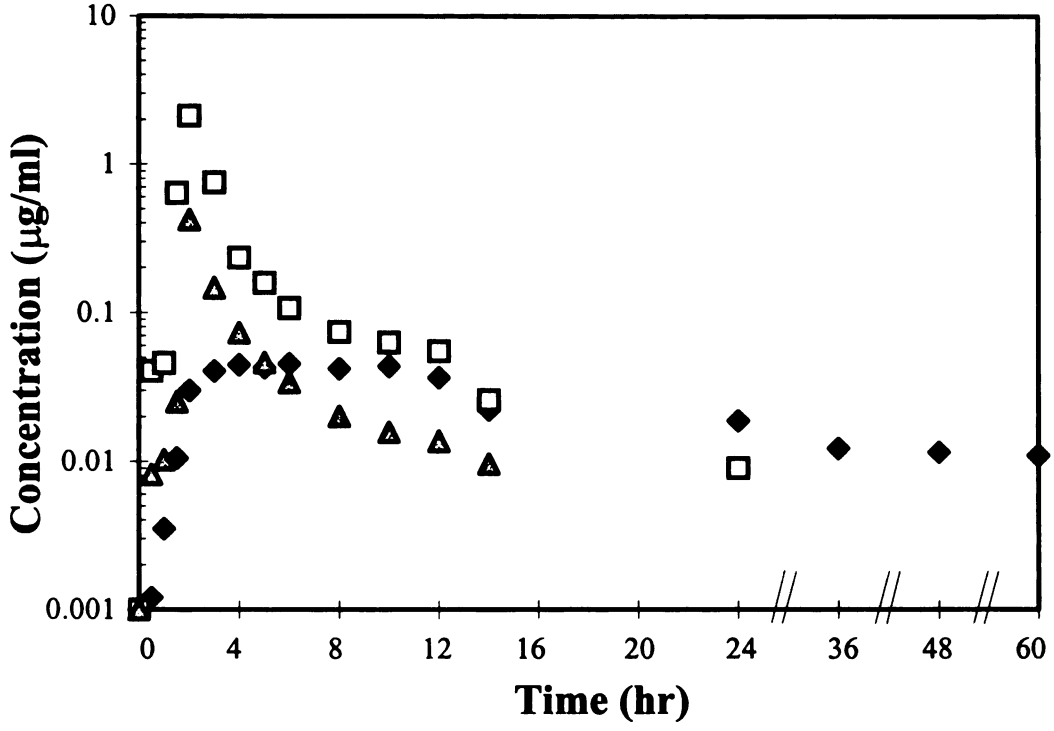

Fig. 3.1. Concentration-time profiles of diclofenac (squares), diclofenac β-1-O-acyl glucuronide (triangles), and covalently bound diclofenac (diamonds) after a single oral dose of 75 mg diclofenac in subject 1. Time zero represent the time the dose was administered. Concentrations of diclofenac, the glucuronide and covalent bound drug were below the limit of analytical sensitivity in the 24, 36 and 72 hr samples, respectively.

Table 3.2. Maximum concentrations (C_{max}) of covalently bound diclofenac per milligram of protein after single and multiple dosing of diclofenac and terminal half-lives of the protein adduct following multiple dosing.

Subject	Single Dose	Multiple Doses	
	C_{max} (ng/mg protein)	C_{max} (ng/mg protein)	Half-life (days)
1	2.68	15.7	10.2
2	1.75	11.3	8.92
3	0.78	7.56	9.41
4	0.99	10.3	14.1
5	2.65	16.0	8.36
6	2.43	15.6	10.6
Avg. ± SD	1.88 ± 0.84	12.7 ± 3.5	10.3 ± 2.0

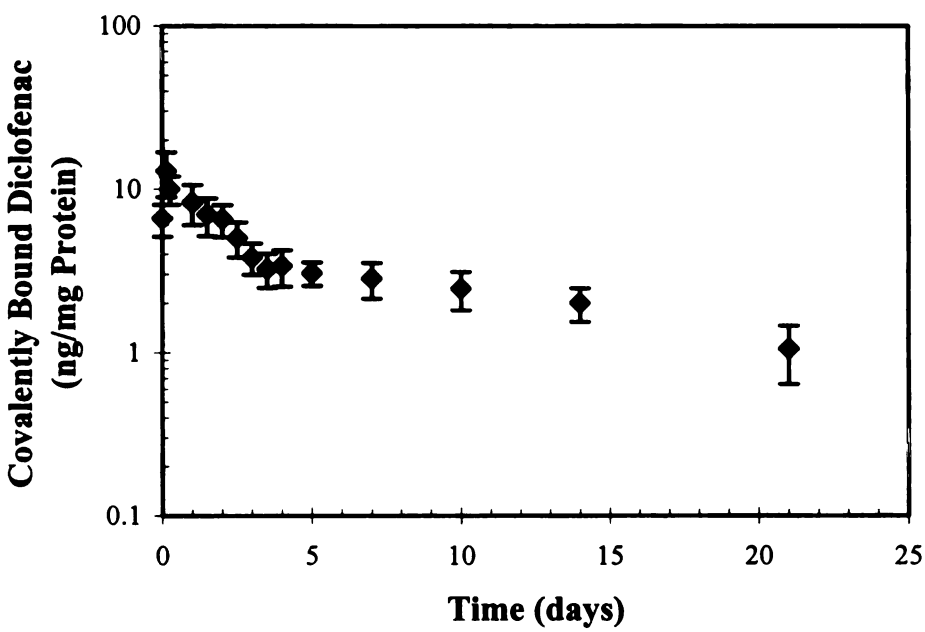

Fig. 3.2. Concentration-time profiles of covalently bound diclofenac after ten day twice-daily oral dosing of diclofenac. Time zero represents the time when the last dose of the multiple dosing regimen was administered. (Values are mean ± SD, n=6.)

3.4 DISCUSSION

Measurement of diclofenac glucuronide in plasma samples has not been reported before, most probably, due to the instability of acyl glucuronide and the esterase-like activity of albumin which could accelerate its degradation. However, utilizing procedures (low pH and low temperature) to stabilize diclofenac glucuronide, this metabolite could be detected in plasma of volunteers even after administration of a single 75 mg oral dose of diclofenac.

Irreversible binding of diclofenac to plasma proteins occurred in all subjects and was detectable in early samples even after a single 75 mg dose of diclofenac. Much higher concentrations of drug-protein adduct (average 6.7 fold increase) were present in plasma after 10 days of multiple dosing of diclofenac. Accumulation of tolmetin protein adducts was also observed in a human study (Zia-Amirhosseini *et al.*, 1994); the maximum concentration of covalently bound tolmetin after 10 days of multiple dosing was 10 fold higher than that observed after a single dose. Since NSAIDs are usually prescribed for the management of chronic conditions, therefore the accumulation of protein adducts after multiple doses would generally be expected. The diclofenac protein adducts exhibited biphasic elimination, with an average terminal half-life of 10.3 days. Kitteringham *et al.* (1985) observed a similar biphasic elimination profile for protein conjugates of dinitrofluorobenzene in rabbits. The rapid early elimination phase was attributed to accelerated uptake and degradation of modified proteins by the liver, and was dependent on the density of bound hapten. The second phase was consistent with the normal turnover rate of albumin. Biphasic elimination of protein adducts of diflunisal (another NSAID) in humans was also observed (McKinnon *et al.*, 1989). An average terminal half-life of 10 days was reported for the protein adducts of diflunisal and 13.5 days for adducts of probenecid after a 6-day oral dosing of

diflunisal with oral co-administration of probenecid during the last 2 days of the regimen. The biphasic decline of covalently-bound drug in plasma could reflect different *in vivo* stabilities of different adducts (McKinnon *et al.*, 1989), or the chemical rearrangement of more reactive to more stable protein adducts. As described in Chapter 1, two mechanism have been proposed to describe the covalent binding of acyl glucuronides to proteins. The nucleophilic displacement mechanism leads to formation of an amide, ester, or a thioester bond between the protein and the drug. The thioester, ester, and amide linkages so formed may have quite different stabilities to hydrolysis (Faed, 1984). The Schiff base mechanism involves the generation of an imine (-C=N-), which can undergo rearrangement to a more stable 1-amino-2-keto product. Both mechanisms occur simultaneously *in vitro*. Ding *et al.* (1995) observed that the Schiff base mechanism, in which the glucuronide acid moiety of the acyl glucuronide is retained within the adduct, is favored at lower (closer to physiological) glucuronide metabolite concentrations *in vitro*. Both mechanism may also occur *in vivo*. It is not clear which mechanism would produce the most stable adduct *in vivo*.

Many factors could contribute to drug protein adduct concentrations in a certain tissue at a particular time *in vivo*, including the amount of the dose and the ratio of the drug undergoing glucuronidation, the intrinsic reactivity of the acyl glucuronide, the duration of exposure of the acyl glucuronide to target proteins, the availability of protein binding sites, and the stability of the adducts formed. Glucuronidation occurs mainly in the endoplasmic reticulum of the liver. Subsequent transport of the glucuronide preferentially across the sinusoidal membrane into blood or preferentially across the canalicular membrane into bile will surely influence the overall extent of exposure to plasma proteins, as well as intrahepatic exposure. Such preferential transport is determined by a number of factors including molecular size (Hirom *et al.*, 1976; Klaassen and Watkins, 1984).

In rats, preferential biliary excretion occurs for compounds with molecular masses of >300-350 Da, which includes most drug glucuronides. For humans, however, the molecular weight threshold for preferential biliary excretion is higher (about 500 Da), and many glucuronides are eliminated primarily into blood. The amount of acyl glucuronide recovered in urine is the portion which survives chemical and enzymatic degradation in the systemic circulation prior to renal excretion. Impaired renal function affects those NSAIDs with metabolites requiring renal clearance. Retained acyl glucuronide metabolites of benoxaprofen (Aronoff *et al.*, 1982), diflunisal (Verbeeck *et al.*, 1979; Dickinson *et al.*, 1991), etodolac (Ogiso *et al.*, 1997), naproxen (Vree *et al.*, 1992), and ketoprofen (Hayball *et al.*, 1993) are readily hydrolyzed back to the parent NSAIDs *in vivo*, potentially reducing the total body clearance of the parent compound and increasing the extent of synthesis of protein adducts. Disease states such as renal failure that compromise elimination of salicyl acyl glucuronide can increase the exposure to the reactive metabolites, resulting in enhanced covalent binding to plasma and perhaps other tissue proteins (Liu *et al.*, 1996). Biliary excretion of acyl glucuronide is usually followed by its enzymatic (and chemical) hydrolysis in the gut and absorption of liberated parent drug into portal blood (enterohepatic circulation). Subsequent reglucuronidation in the liver enhances exposure of the body to the reactive glucuronide. A good estimate of *in vivo* exposure of plasma proteins to an acyl glucuronide can be obtained by measuring the area under the concentration-time curve (AUC) of the glucuronide in plasma.

Previously, our laboratory observed a highly significant linear correlation ($r^2 = 0.873$) between the *in vitro* drug glucuronide degradation rates and the *in vivo* covalent binding in humans for these drugs when the extent of adduct formation was corrected for the measured plasma glucuronide concentrations from five human drug studies (Benet *et al.*, 1993). As shown in Table 3.3, following oral

administration of diclofenac to five healthy volunteers, the covalent binding of diclofenac to plasma proteins was the highest compared to the five other drugs. Though the linear correlation was slightly decreased ($r^2 = 0.804$), our data still support our hypothesis that the degree of covalent binding of carboxylic acid drugs in humans may depend on the reactivity of their acyl glucuronide metabolites.

Since HSA accounts for 90% of all proteins in plasma, we assume that adduct formation is primarily involved with albumin in plasma. The half-life of the diclofenac protein adduct is shorter than the turnover rate of albumin (half-life 17-23 days) (Lentner, 1984). This could be due to either hydrolytic cleavage of irreversible-bound drug in the circulation or accelerated proteolytic degradation of drug modified albumin *in vivo*. It is also possible that diclofenac could covalently bind to some other plasma proteins that have shorter half-lives than albumin.

Here, we demonstrated that diclofenac could covalently bind to plasma proteins, presumably *via* diclofenac acyl glucuronide, following administration to humans. Diclofenac could also covalently modify tissue macromolecules. Immunochemical approaches have been used recently by several groups to investigate the formation of hepatic protein adducts derived from diclofenac (Kretz-Rommel and Boelsterli, 1993; Pumford *et al.*, 1993; Hargus *et al.*, 1994; Gil *et al.*, 1995). Studies undertaken with specific polyclonal antisera, produced by immunization of rabbits with synthetic diclofenac-protein adducts were expressed in livers of mice and rats treated with the drug *in vivo* and also in rat and human hepatocytes incubated with diclofenac *in vitro*. Immunoblotting studies suggest that many different hepatic proteins could become covalently modified by reactive metabolites derived from the drug. Our data, as well as those of others described above, support the hypothesis that the covalent binding of diclofenac and other NSAIDs to macromolecules *in vivo* may contribute to the high incidence of

Table 3.3. *In vivo* irreversibly bound drug, area under the plasma drug glucuronide concentration time curve and maximum *in vitro* acyl glucuronide degradation rates.[a]

Parent Compound	Bound Drug (mole/mole protein) x10^4	AUC Glucuronide (mole x hr/L) x 10^6	Bound/AUC 10^{-2}	k (hr^{-1})
Diclofenac[b]	3.88 ± 0.44	0.96 ± 0.27	3.95	3.24
Tolmetin	2.77 ± 1.54	3.72 ± 0.95	0.75	1.78
Zomepirac	2.33 ± 0.45	6.41 ± 2.14	0.36	1.54
R-Fenoprofen	1.02 ± 0.32	6.31 ± 5.65	0.16	1.06
S-Fenoprofen	3.23 ± 0.85	60.4 ± 24.7	0.054	0.58
Racemic Carprofen	1.92 ± 1.28	40.9 ± 7.30	0.047	0.43
(-) - Beclobric Acid	0.12 ± 0.03	8.16 ± 1.34	0.015	0.031
(+) - Beclobric Acid	0.20 ± 0.11	8.31 ± 1.63	0.024	0.027

[a]Measurement of maximum amount of drug covalently bound to plasma proteins and area under the plasma concentration time curve (AUC) for the glucuronide conjugates measured in six different groups of healthy volunteers following oral dosing of either 75mg of diclofenac ([b]values for 5 subjects only), 400mg of tolmetin, 100mg of zomepirac, 600mg of racemic fenoprofen, 50mg of racemic carprofen or 100mg of racemic beclobric acid. When covalently bound drug is normalized to area under the curve for the respective glucuronide conjugates, a good correlation with the maximum *in vitro* degradation rate constant (k) is obtained with an r^2 of 0.804.

unexplained side-effects associated with these drugs (Spahn-Langguth and Benet, 1992).

Covalent binding of reactive chemicals or metabolites to proteins has been a popular hypothesis as the mechanism to explain the toxicities observed for many xenobiotics (Hinson and Roberts, 1992; Chang *et al.*, 1994). A feature of the hypothesis is that reactive chemicals covalently modify specific proteins, thereby altering their function, or disrupting some regulatory pathway leading to cell death (Boelsterli, 1993). Another important consequence of covalent modification of proteins can be activation of the immune system. Covalent binding of chemicals to proteins can initiate an immune response against the chemical through covalent binding of a reactive intermediate to proteins, which may change the three-dimensional structure whereby the immune system recognizes the protein as foreign, leading to the production of hypersensitivity. It has been shown that the covalent binding of xenobiotics to proteins also can cause an autoimmune response (Beaune *et al.*, 1994). In the future, identification of the proteins covalently modified by reactive intermediates of NSAIDs will be important in gaining a better understanding of the relationship between covalent binding and immunotoxicity of NSAIDs.

4.0 IMMUNOGENICITY OF DICLOFENAC PROTEIN CONJUGATES FORMED *VIA* A DRUG-GLUCURONIDE INTERMEDIATE

4.1 BACKGROUND

Hypersensitivity reactions, ranging from mild organ toxicity or skin rash to severe organ injury, asthma or anaphylaxis, have been described in patients treated with many different acidic NSAIDs and cross-reactivity among NSAIDs has also been observed (Helfgott *et al.*, 1990; Bosso *et al.*, 1992; Tarazi *et al.*, 1993). These adverse reactions are relatively rare but are an important clinical issue due to the widespread use of this class of drugs, the idiosyncratic nature of the toxicity, and the potential for progression to fulminant hepatic, renal or congestive heart failure. Moreover, individual NSAIDs differ markedly in the incidence, severity, and type of organ toxicity or allergic reactions that they generate (Szczeklik *et al.*, 1977; Szczeklik, 1987). Several NSAIDs have been withdrawn from the market because of severe hepatotoxicity, and many others remain in general use despite being associated with liver damage, while some appear to cause little hepatic injury (Lewis, 1984; Boelsterli *et al.*, 1995).

Currently, the mechanisms underlying NSAID-induced hepatotoxicity are not fully understood. The presence of rashes, fever, and/or eosinophilia in some patients, which are indicative of hypersensitivity reactions, suggest that the immune system may be involved (Tarazi *et al.*, 1993). However, the absence of these markers in other patients suggest the possibility that direct toxicities could be caused by the parent drugs and /or their metabolites (Scully *et al.*, 1993; Banks *et al.*, 1995). Metabolism of NSAIDs *in vivo* occurs *via* several enzymatic pathways that may be involved in the generation of reactive metabolites capable of binding to plasma or cellular macromolecules. Acyl glucuronides of NSAIDs, which are

chemically labile, have been identified in the systemic circulation and have been shown to mediate binding of several NSAIDs to plasma proteins (Spahn-Langguth and Benet, 1992). Other potential routes of bioactivation of these drugs are *via* cytochrome P450 or acyl-CoA ligases (Hertz and Bar-Tana, 1988; Miyamoto *et al.*, 1997).

The reactivity and ability of acyl glucuronides of NSAIDs to covalently bind to plasma and tissue proteins have been well documented. Two mechanisms have been proposed to describe the covalent binding of acyl glucuronides to proteins (see Fig. 1.3) (Benet *et al*, 1993). The first mechanism involves nucleophilic displacement of the glucuronosyl group by a protein nucleophile (-NH, -SH, or -OH) to form an adduct with the drug covalently bound to the protein (drug-protein) and the glucuronic acid liberated. The second mechanism involves the isomerization of the acyl glucuronide and the formation of an imine (Schiff base intermediate) with an amino group of the protein which can then undergo Amadori rearrangement to a more stable 1-amino-2-keto protein adduct in which the drug is covalently bound to the protein *via* an opened glucuronic acid ring (drug glucuronide-protein). The imine mechanism may lead to shared functional group as the potential hapten, i.e., the glucuronic acid moiety, which may be consistent with the immunologic cross-reactivity observed for NSAIDs.

It was reported in 1993 that diclofenac, a widely used NSAID for the treatment of rheumatoid arthritis, osteoarthritis, or ankylosing spondylitis, was involved in the most severe hypersensitivity reactions to NSAIDs in humans (Adverse Drug Reactions Advisory Committee, 1993). We have shown that diclofenac glucuronide conjugate is the most reactive acyl glucuronide in buffer containing human serum albumin (HSA) exhibiting the most extensive covalent binding to HSA *in vitro* and to plasma proteins *in vivo* of any NSAID investigated to date (Chapters 2 and 3). Diclofenac-modified plasma proteins could be detected

even after a single 75 mg oral dose of diclofenac to humans, and the protein adduct was found to accumulate to much higher concentrations of adduct after ten-day twice daily multiple dosing of the drug (Chapter 3). Here, we investigated the immunogenicity of diclofenac protein adducts formed *via* the reactive acyl glucuronide in a mouse model and explored the link between the covalent binding of the glucuronide and the immunotoxicity of the parent drug. The immunogenicities of two different drug protein adducts (D-protein and DG-protein) formed *via* two different binding mechanisms were examined individually to differentiate the significance of each protein adduct in the pathogenesis of the toxicity caused by NSAIDs. Our results indicate that both protein adducts caused a drug specific antibody response in mice.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Diclofenac sodium(D), mouse serum albumin (MSA), human serum albumin (HSA), Complete Freund's Adjuvant (CFA), Incomplete Freund's Adjuvant (IFA), 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), and sodium cyanoborohydride (NaCNBH) were obtained from Sigma Chemical Co. (St. Louis, MO). ELISA plates (96 wells) was obtained from Applied Scientific (San Francisco, CA). ELISA starter kit and BCA protein assay kit were obtained from Pierce (Rockford, IL).

Diclofenac glucuronide (DG) was purified from the bile of rats. Briefly, bile catheters were placed in Sprague Dawley rats (300g, B&K Inc., Fremont, CA) under general anesthesia. Each rat was injected twice with a 100 mg/kg i.v. diclofenac dose (dissolved in water for injection) separated by four hours. Bile was collected into cold microcentrifuge tubes from the time of the first injection till 5

hours after the last injection and immediately acidified with acetic acid to a pH between 2 to 4. After centrifugation at 16000 g for 2 min, the supernatant was extracted three times with equal volumes of ethyl acetate. The organic phase was dried under N_2 and reconstituted in mobile phase. Diclofenac glucuronide was purified by HPLC; structural characterization of glucuronide was performed by mass spectrometry.

4.2.2 Preparation of Protein Adducts

Two types of diclofenac protein adducts were prepared *in vitro*. MSA was used as a model self-protein. Diclofenac-modified MSA (D-MSA) was synthesized following a general method described previously (Hayase *et al.*, 1989) with minor modifications. Briefly, diclofenac sodium (2 mg/ml) was incubated with MSA (2 mg/ml) and EDC (2mg/ml) (in H_2O, pH 6.0) at room temperature for two hours. Diclofenac glucuronide-modified MSA (DG-MSA) was prepared by a method described previously by our laboratory (Zia-Amirhosseini *et al.*, 1995). Briefly, MSA (0.69 mM) was reacted with DG (6.9 mM) in the presence of $NaCNBH_3$ (30 mM), and the reaction was carried out at 37°C in phosphate buffer (1ml, 0.1M, pH 7.4) for six hours.

After each reaction, one fifth of the mixture (200 µl) was used for quantitation of covalently bound drug released from modified protein after alkaline hydrolysis as described previously (Zia-Amirhosseini *et al.*, 1994). The second part of the reaction mixtures (800 µl) were washed 8 times by centrifugal filtration through a 30-KDa-cutoff membrane (Amicon centricons, Beverly, MA) and the remaining solution was rinsed with phosphate buffered saline (PBS). After lyophilization, the final product, containing both modified and unmodified protein, was stored at 4°C and used for immunization in mice. The yield of D-MSA and DG-MSA was 13.2 and 14.5 µg bound diclofenac/mg MSA, respectively.

D-modified and DG-modified human serum albumin (D-HSA and DG-HSA) were prepared using similar methods to those described above and the yield of the two protein adducts was 22.6 and 17.6 μg bound diclofenac/mg HSA, respectively.

4.2.3 Immunization

Our research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23). Female Balb/c mice (20-23g, B&K Inc., Fremont, CA) were used for the immunization study as described by Zia-Amirhosseini *et al.* (1995). Mice were divided into three groups (12 mice/group) and each group was immunized i.p. with D-MSA, DG-MSA, or a mixture of MSA and diclofenac in a 72:1 (wt/wt) ratio. Each mouse received 250 μg of antigen in 100 μl of PBS in combination with CFA for the first immunization and in combination with IFA for subsequent immunizations. Antigen was injected once every two weeks and blood samples were taken one week after each immunization. Blood was collected into a Samplette serum separator (Sherwood, St. Louis, MO), incubated at 37°C for 2.5 hours and centrifuged at 2000 g for 20 minutes at 4°C. Aliquots of plasma (5 or 30 μl) were stored at -20°C.

4.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

The ELISA assay was utilized at room temperature to test the reactivity of antisera from immunized mice against protein conjugates of diclofenac and diclofenac glucuronide. The 96 wells of the ELISA plate were coated with 100 μl/well of coating buffer (0.2 M sodium bicarbonate buffer, pH 9.4) containing 100 μg/ml of D-HSA or DG-HSA or HSA and shaken for 2 hours. After rinsing three times with PBS and three times with washing buffer, 150 μl/well of blocking buffer (10% BSA solution in PBS) was added and incubated for 1 hr. Then a series of

antiserum dilutions were incubated for 1 hr. After repeating the washing procedure, 100 µl/well of alkaline phosphatase labeled anti-mouse IgG and IgM (H+L) (1:800 dilution in the blocking buffer) was added and incubated for two hours, followed by washing and the incubation of 100 µl of substrate solution (5 mg of *p*-nitrophenyl phosphate disodium salt in 10 ml of a 1M diethanolamine solution) for 15 to 20 mins. Absorbance of the colored *p*-nitrophenol was read by an ELISA reader (BT2000, Fisher Biotech, Pittsburgh, PA) at 405 nm.

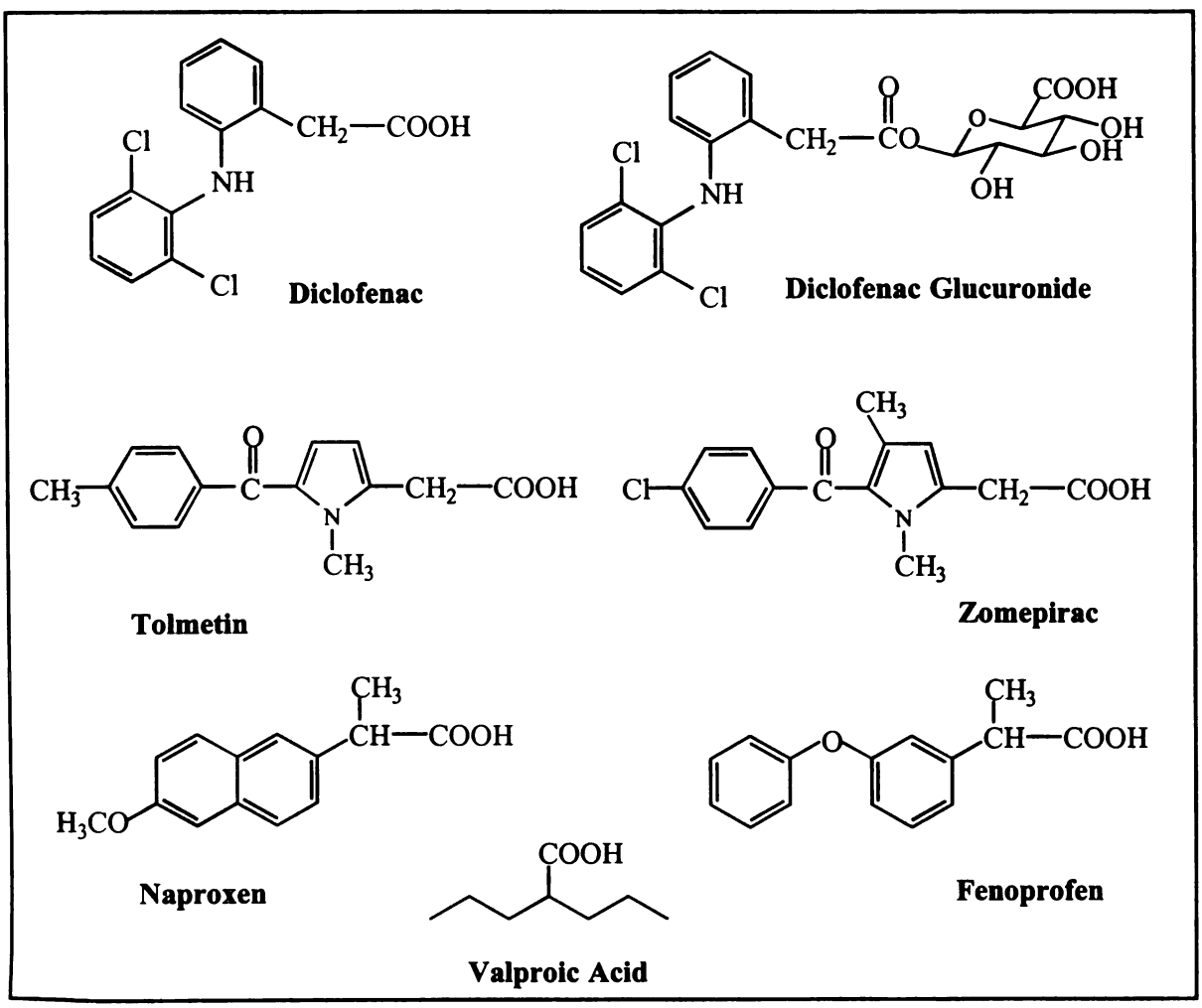

Fig. 4.1. Chemical structures of diclofenac, diclofenac glucuronide, and some inhibitors used for the inhibition studies.

4.2.5 Inhibition Studies

The same ELISA system as described above was used for inhibition studies. Anti-sera from immunized mice were diluted 1:1000 and were incubated with the same volume of solutions, each containing different compounds (potential inhibitors) at various concentrations for 15-20 minutes prior to the addition of the anti-sera to the coated ELISA plate. If the anti-sera reacted with these compounds, the binding of the anti-sera to the drug-protein conjugates coated ELISA plate would be decreased, leading to a decreased absorbance when compared to anti-sera preincubated with buffer. The anti-sera were diluted with the blocker solution and the compounds were dissolved in phosphate buffer (0.1 M, pH 7.4). The potential inhibitor compounds tested including: D-MSA, DG-MSA, D, DG, tolmetin, tolmetin glucuronide, zomepirac, fenoprofen, fenoprofen glucuronide, naproxen, naproxen glucuronide, valproic acid, glucuronic acid, glucose, and MSA (Fig. 4.1) . Percent inhibition at each inhibitor concentration was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Absorb. (without inhibitor) - Absorb. (with inhibitor)}}{\text{Absorb. (with inhibitor)}} \times 100$$

4.3 RESULTS

4.3.1 Immunization with DG-MSA

After four immunizations, three of the twelve mice immunized with DG-MSA developed antibodies that can bind to DG-HSA. This response rate is consistent with that observed for tolmetin protein conjugate (Zia-Amirhosseini *et al.*, 1995). As shown in Fig. 4.2, the anti-sera of the responsive mice exhibit higher reactivity against the DG-modified proteins than the unmodified protein.

The antibody reactivity against non-conjugated HSA, at low titer, likely reflects the presence of heterologous cross-reactive antibody since HSA but not free drug will bind to the ELISA plate. The high titer binding reflects the specific reactivity of the antibodies produced with the drug-conjugate. Antibodies reactive with drug-protein conjugates were not present in the anti-sera of the remaining mice of each group. No protein conjugate specific antibodies were detected in any of the mice immunized with a mixture of unmodified MSA and diclofenac (Fig. 4.3).

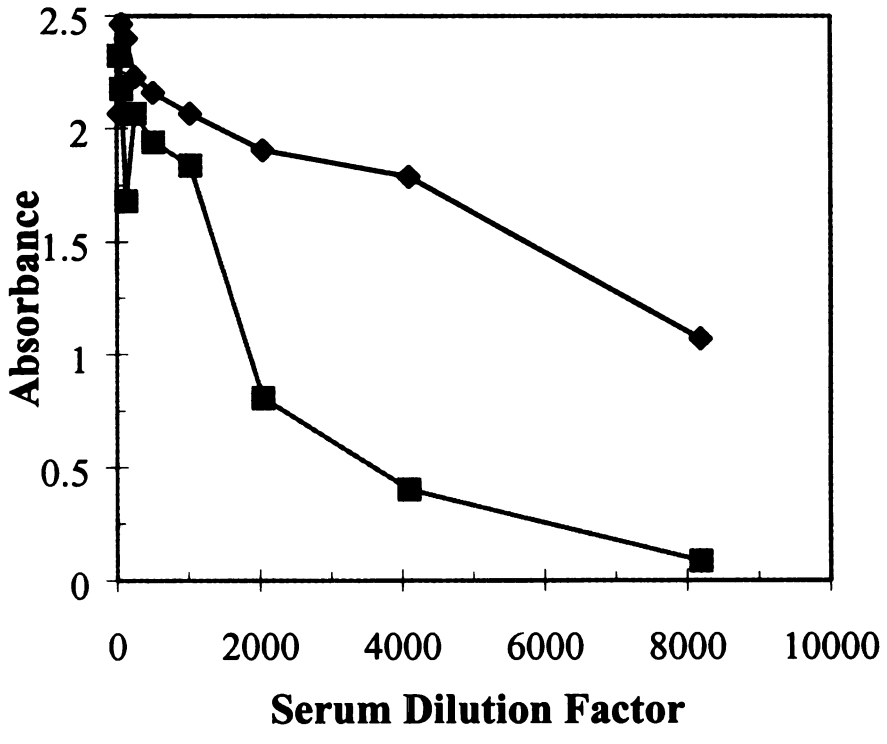

Fig. 4.2. Reactivity of antiserum against DG-HSA (diamonds) and D+HSA (squares). The antiserum was obtained from a mouse (No. 1) immunized four times with DG-MSA. This plot represents typical results of the three mice that developed antibodies against DG-HSA after DG-MSA immunizations.

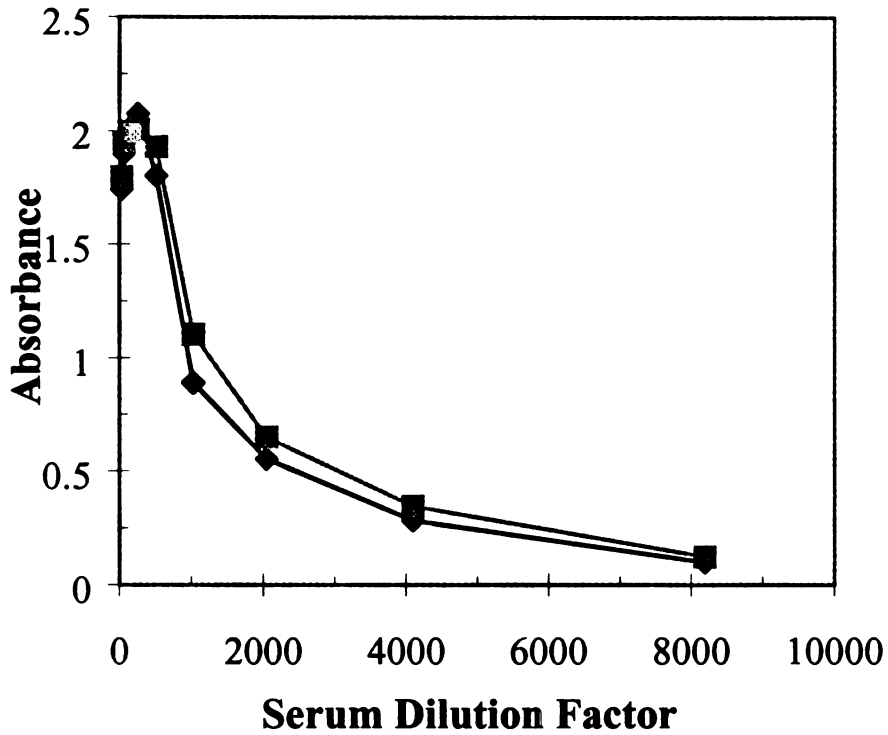

Fig. 4.3. Reactivity of antiserum against DG-HSA (diamonds) and D+HSA (squares). The antiserum was obtained from a mouse (No. 3) immunized four times with unmodified MSA. This plot represents results of all the twelve mice after immunizations with a mixture unmodified MSA and diclofenac (ratio 72:1).

In order to test the specificity of the antibodies to the drug protein conjugates, antiserum was pre-incubated with DG-MSA or D-MSA prior to ELISA assay. Both DG- MSA and D-MSA could completely inhibit the antigen-antibody interaction (Fig. 4.4). DG-MSA was more effective than D-MSA in inhibiting the binding of the antisera from mice immunized with DG-MSA. Diclofenac and DG also exhibited good inhibition of the antibody binding (Fig. 4.5). The fact that diclofenac alone could contribute about 60% of inhibition indicates that antibodies specifically recognizing diclofenac were present in the sera of responder mice. However, the inhibition by DG was always greater than the inhibition by diclofenac, suggesting that the sera of responding mice also contained antibodies that specifically recognize the diclofenac glucuronide metabolite. The inhibition studies with DG-MSA, D-MSA, DG, and D indicate that DG-MSA is immunogenic and can induce the production of antibodies recognizing D, DG, and DG-MSA in mice.

At much higher concentrations, other NSAIDs and their glucuronides could also inhibit the antibody-antigen interaction to various degrees. As shown in Fig. 4.5, tolmetin, fenoprofen and naproxen exhibited 15%, 12%, and 5% inhibition of antibody binding, respectively. The glucuronide metabolites of tolmetin, fenoprofen and naproxen inhibited the antibody-antigen interaction to a greater magnitude than the corresponding aglycone drug. This further demonstrates the presence of antibodies recognizing features of the glucuronide metabolite. The antibodies did not cross-react with glucose and glucuronic acid (data not shown).

4.3.2 Immunization with D-MSA

After four immunizations, four out of the twelve mice immunized with D-MSA developed antibodies which bind to D-HSA. As shown in Fig. 4.6, the anti-sera of the responding mice have higher reactivity against diclofenac modified

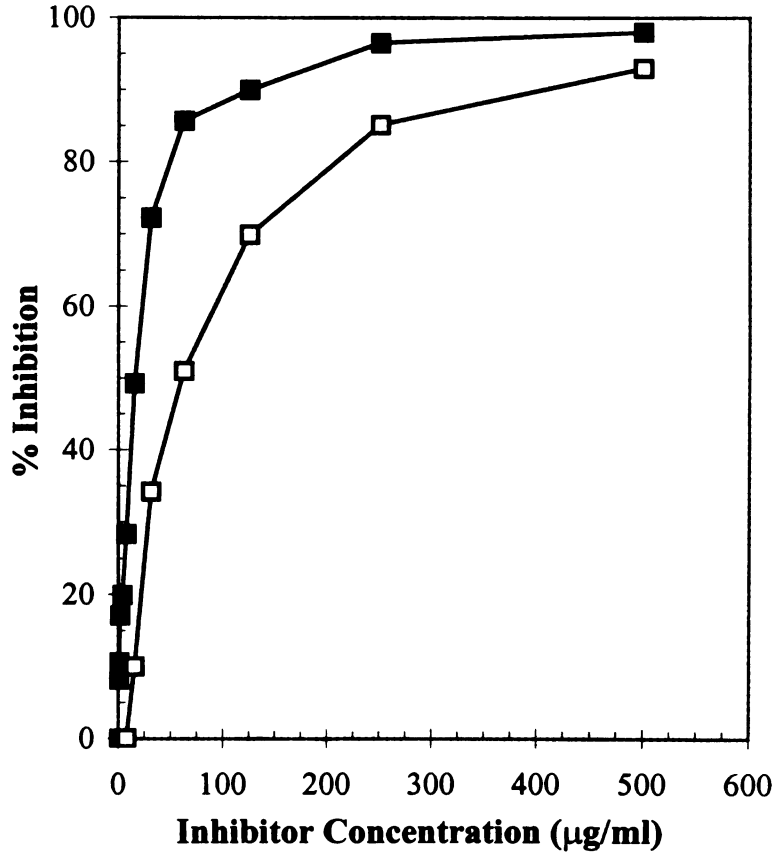

Fig. 4.4. Inhibition of antibody-antigen interaction by DG-MSA (solid squares) and D-MSA (open squares). The inhibition curves for mouse 1 which was immunized four times with DG-MSA are shown.

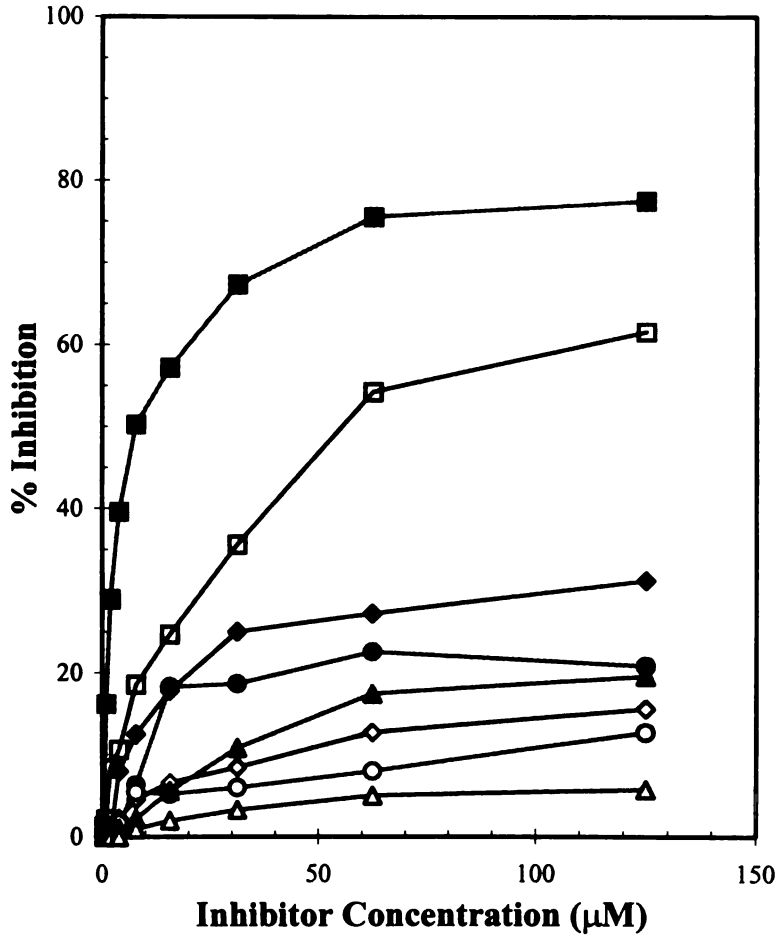

Fig. 4.5. Inhibition of antibody-antigen interaction by DG (solid squares), D (open squares), tolmetin glucuronide (solid diamond), tolmetin (open diamond), fenoprofen glucuronide (solid circle), fenoprofen (open circle), naproxen glucuronide (solid triangle), and naproxen (open triangle). The inhibition curves for mouse 1 which was immunized four times with DG-MSA are shown.

proteins than against unmodified protein. Antibodies reactive with D-HSA were not present in the anti-sera of the remaining mice of the group. No protein conjugate specific antibodies were detected in any of the mice immunized with unmodified MSA (Fig. 4.3).

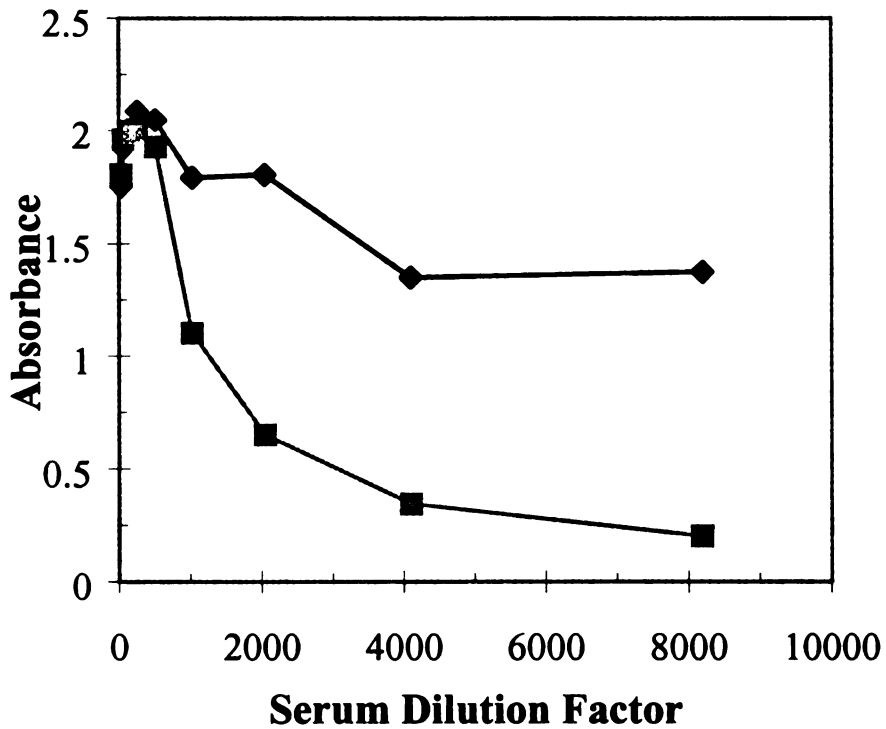

Fig. 4.6. Reactivity of antiserum against D-HSA (diamonds) and D+HSA (squares). The antiserum was obtained from a mouse (No. 2) immunized four times with D-MSA. This plot represents typical results of the four mice that developed antibodies against D-HSA after D-MSA immunizations.

The specificity of the antibodies to the drug protein conjugates was confirmed by inhibition studies with drug protein conjugates, D-MSA and DG-MSA (Fig. 4.7). DG-MSA and D-MSA could effectively inhibit the antigen-antibody interaction, and there was no difference in the degree of inhibition by DG-MSA compared to D-MSA at various inhibitor concentrations. D and DG both exhibited the same high level of inhibition of antibody binding (Fig. 4.8) suggesting that antibodies only specifically recognizing the diclofenac moiety were present in the antisera of responding mice. These findings indicate that D-MSA is also immunogenic and can induce drug and drug protein specific antibody production in mice.

Low level of cross-reactivity of the diclofenac specific antibodies with other NSAIDs were observed. At much higher concentrations, tolmetin, zomepirac, fenoprofen, naproxen (Fig. 4.8) and their glucuronides inhibited the antibody-antigen interaction from 5 to 30%. The inhibitions by glucuronide conjugates of NSAIDs were similar to those of the drugs themselves (data not shown). Tolmetin and zomepirac, the two compounds which like diclofenac possess an unsubstituted α-carbon to the carboxylic acid group, showed greater inhibition of the antibody binding than fenoprofen and naproxen (Fig. 4.8). Valproic acid, an acidic anti-epileptic drug that has a very different structure from the NSAIDs, did not cross-react with diclofenac specific antibodies.

4.4 DISCUSSION

In vivo covalent binding of NSAIDs to proteins *via* glucuronide may result in two different classes of protein adducts; in one the drug is linked directly to the amino acid of the protein and in the other the drug is bound to the protein via a glucuronic acid ring. The intrinsic immunotoxicity of each class of protein adduct

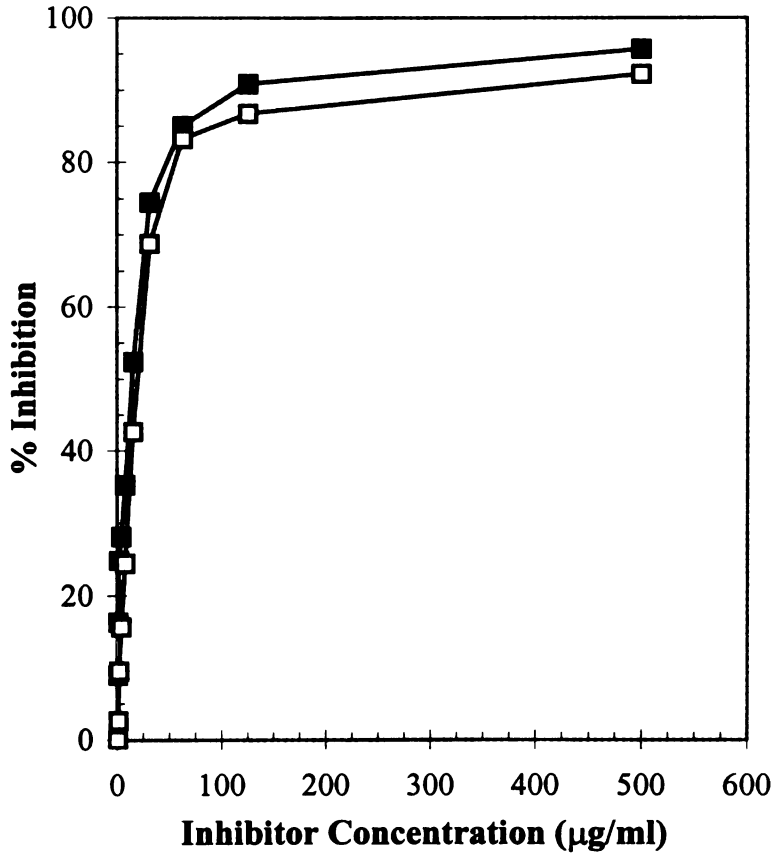

Fig. 4.7. Inhibition of antibody-antigen interaction by DG-MSA (solid squares) and D-MSA (open squares). The inhibition curves for mouse 2 which was immunized four times with D-MSA are shown.

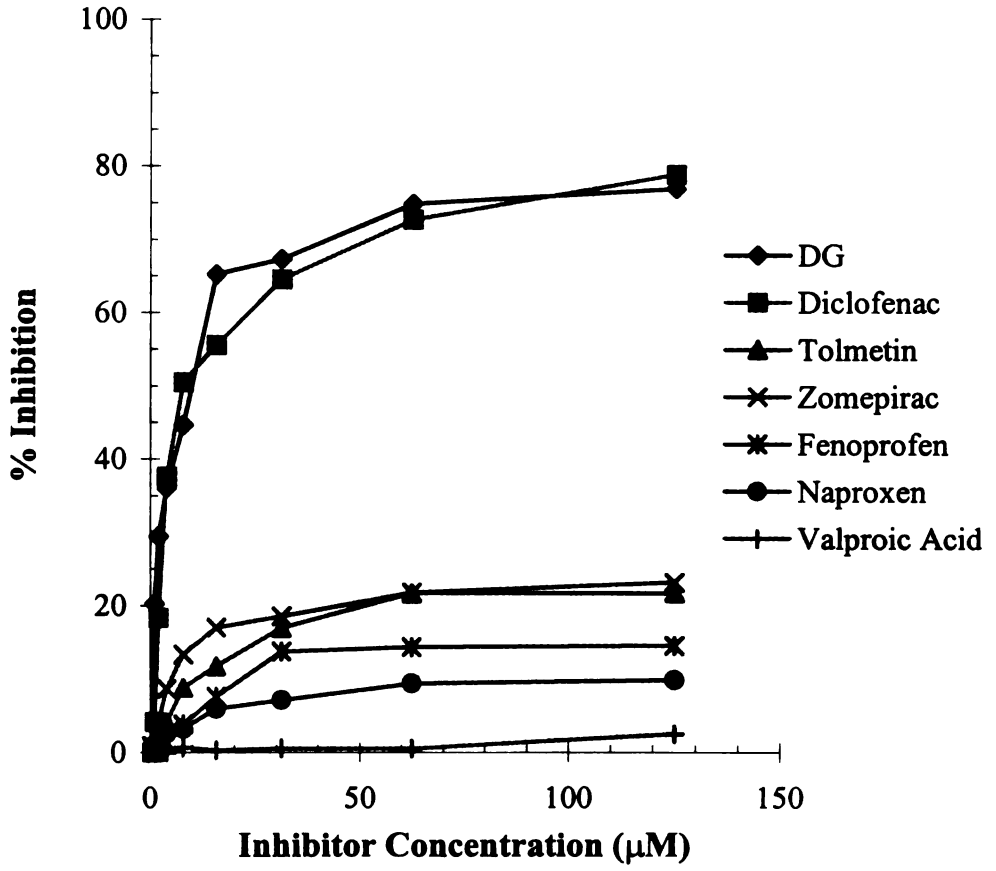

Fig. 4.8. Inhibition of antibody-antigen interaction by NSAIDs and valproic acid. The inhibition curves for mouse 2 which was immunized four times with D-MSA are shown.

may depend on the chemical and biochemical stability of the covalent bond between the drug and protein moiety. The two classes of products would be expected to have markedly different stabilities, and therefore different plasma half-lives, under physiologic conditions. Though the amide products would be anticipated to be relatively stable, the imine products would be expected to undergo spontaneous hydrolysis or slower rearrangement to more stable Amadori products. Our laboratory has shown that the imine mechanism is favored at lower (closer to physiological) glucuronide metabolite concentrations (Ding *et al.*, 1995). Here, we examined the immunogenicity of the two classes of protein adducts separately in a mouse model to explore possible differences in immunotoxicity and cross-reactivity related to their chemical structures.

Protein adducts were synthesized *in vitro*. Diclofenac itself was incapable of chemically modifying proteins. Diclofenac-modified MSA was obtained after incubation of diclofenac with MSA in the presence of EDC. This procedure allows diclofenac to directly link to protein by an amide group. DG-modified protein was obtained after the reaction of DG with MSA in the presence of $NaCNBH_3$. As reported for other NSAIDs, e.g. tolmetin (Ding *et al.*, 1994) and benoxaprofen (Qiu *et al.*, 1998), the extent of covalent binding of diclofenac was 15 fold higher in the presence of $NaCNBH_3$ than in its absence, and the imine-based mechanism was dominant in this reaction.

After four immunizations with D-MSA or DG-MSA, 33% and 25%, respectively, of the mice developed protein adduct specific antibodies. In the sera of responding mice that received DG-MSA, the majority of the antibodies recognized the diclofenac moiety, another portion of the antibodies recognized DG, a third small portion could recognize the MSA. Cross-reactivity of the antibodies with other NSAIDs and their glucuronides was observed. The antibodies consistently showed higher cross-reactivity to the glucuronide conjugates of

NSAIDs than to the parent drugs themselves, confirming the presence of drug-glucuronide specific antibodies in the sera. However, the antibodies did not cross react with valproic acid, which although a carboxylic acid possesses a structure markedly different from NSAIDs. Similarly, the majority of the antibodies developed in the responding mice immunized with D-MSA, recognize diclofenac and a small portion identify a combinatorial epitope dependent on the structure of MSA. Diclofenac glucuronide specific antibodies were not present in the sera of these mice. Low cross-reactivity with other acidic NSAIDs at much higher concentrations was observed. The cross-reactivity of antibody with NSAIDs appears to depend on the chemical structure of the drug, especially the substitution at the α-carbon to the carboxylic acid. The antisera had properties suggesting that antibodies against the structure of the α-carbon were present. This is suggested by the fact that tolmetin and zomepirac, similar in structure to diclofenac with respect to the unsubstituted α-carbon, exhibit the greatest inhibition of the antibody-antigen interaction as compared to fenoprofen and naproxen, both of which have a single substitution at the α-carbon of the carboxylic acid group.

Both DG-MSA and D-MSA were immunogenic and could induce drug-specific antibodies in mice. The titer of antibody produced against DG-MSA was similar to D-MSA indicating that the two classes of protein adducts could elicit an equally strong immune response *in vivo*. Interestingly, both immunogens only produced a response in a subset of animals immunized. This parallels the variable instance of NSAID reactivity in humans. The antibodies produced in mice could cross react with other NSAIDs and the cross-reactivity seems to depend on the similarity of the chemical structure of acidic NSAIDs. The antibodies reactive to DG had higher cross-reactivity to glucuronide metabolites of other NSAIDs than to the drugs themselves. These data support the hypothesis that a hypersensitivity reaction to NSAIDs may be initiated by an immune response to drug or drug-

glucuronide modified proteins and that cross-reactivity among NSAIDs may be caused by cross-reacting antibodies.

A limited number of studies have been conducted to study the immunotoxicity of covalent binding of NSAIDs to proteins *in vivo*. Zia-Amirhosseini *et al.* (1995) also observed stimulation of an antibody response in three out of twelve mice after immunization with tolmetin glucuronide-modified MSA. Antibodies specific for tolmetin and tolmetin glucuronide were generated and these antibodies cross-reacted with other NSAIDs and their glucuronides. Diflunisal-specific antibodies were obtained in all of six rats (Worrall *et al.*, 1995) and three rabbits (Williams *et al.*, 1995) immunized with diflunisal-modified proteins. Three of six rats injected with rat serum albumin modified by incubation with diflunisal acyl glucuronide and its rearrangement isomers generated similar antibodies (Worrall *et al.*, 1995). In that study, injected rat serum albumin contained a low level mixture of modifications via diflunisal acyl glucuronides by both the nucleophilic displacement mechanism and the imine mechanism. Cross reactivity of antibodies raised against the protein adduct mixture with the other NSAID glucuronides were not assessed in that study. The antibodies generated against diflunisal did not cross react with naproxen and ketoprofen (as the free drugs). These results are not surprising to us, since there is little structural similarity between diflunisal and naproxen and ketoprofen. However, all these reports suggest that covalent binding to proteins may be involved in hypersensitivity responses to acid NSAIDs.

5.0 DETECTION OF DRUG SPECIFIC ANTIBODIES IN SERA OF PATIENTS WHO HAD HYPERSENSITIVITY REACTIONS TO A NONSTEROIDAL ANTI-INFLAMMATORY DRUG

5.1 INTRODUCTION

Many nonsteroidal anti-inflammatory drugs (NSAIDs) that contain a carboxylic acid group, to name a few such as aspirin, ibuprofen, fenoprofen, naproxen, diflunisal, tolmetin, and diclofenac, can cause hypersensitivity reactions in humans (Szczeklik, 1986; Quiralte *et al.*, 1997). Adverse reactions are rare but are higher than that of most other commonly used drugs (Irey, 1976; Faich, 1986) and may have various clinical manifestations including urticaria, angioedema, rhinoconjuctivitis, asthma, anaphylactic reactions, and organ toxicities. Cross-reactivities among acid NSAIDs have also been observed (Quiralte *et al.*, 1996, 1997).

It was proposed that an immunologic pathogenesis inferred in some patients with hypersensitivity reactions to acidic NSAIDs (Spahn-Langguth and Benet, 1992). A common metabolite of acidic NSAIDs is the glucuronide conjugate and a number of studies have showed that acyl glucuronide metabolites of acidic NSAIDs are reactive intermediates and capable of forming protein adducts with plasma and tissue proteins *in vivo* (Spahn-Langguth and Benet, 1992; Bailey and Dickinson, 1996). The resulting modified protein could be recognized by the immune system as a non-self protein and act as an immunogen to induce production of drug-specific antibodies. Thus acyl glucuronides of acidic NSAIDs could act as haptens which become immunogenic when covalently bound to proteins. Studies have confirmed that protein conjugates of tolmetin, diflunisal and diclofenac formed *via* their reactive acyl glucuronide metabolites could induce a

drug-specific antibody response in animals (Williams *et al.*, 1995; Zia-Amirhosseini *et al.*, 1995; Chapter 4).

Recently, a strong positive association between the presence of HLA-DPB1*0301 and aspirin-induced asthma (AIA) in a relatively large sample of patients has been reported (Dekker *et al.*, 1997). The character of AIA is a persistent inflammation of the airway with marked eosinophilia, epithelial disruption, cytokine production (Nasser *et al.*, 1996; Szczeklik *et al.*, 1977). Such a pathologic process could result from a non-IgE-mediated reaction to an endogenous or exogenous antigen (Szczeklik, 1997). The class II human leukocyte antigens (HLA) play a central role in the regulation and ignition of an immune response to an antigen. Finding of an HLA association with AIA suggests immunity as the underlying mechanism of the disease.

In this study we detect the presence of drug-specific IgG antibodies in select patients who experienced adverse reactions to a NSAID. The specificity and cross-reactivity of the antibodies was confirmed by inhibition ELISA studies. An immunologic mechanism is implicated in the adverse reactions to NSAIDs.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Diclofenac sodium, ibuprofen, tolmetin, fenoprofen, naproxen, valproic acid, ovalbumin (Grade VI), and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC) were obtained from Sigma Chemical Co. (St. Louis, MO). ELISA plates (96 wells) were obtained from Applied Scientific (San Francisco, CA). ELISA starter kit and BCA protein assay kit were obtained from Pierce (Rockford, IL).

5.2.2 Plasma Samples

Sera of eighteen patients who had hypersensitivity reactions to a NSAID manifested as cross-reacting respiratory reactions were used to detect drug-specific antibodies. Among these patients, thirteen had a prior history of respiratory reaction to ibuprofen, two to diclofenac, and three to naproxen. Eighteen control sera samples were obtained from young healthy volunteers.

5.2.3 Preparation of Protein Adducts

Diclofenac, ibuprofen, and naproxen protein adducts were prepared *in vitro*. Chicken ovalbumin (Ova) was used as a carrier protein. Drug-modified ovalbumin (D-Ova) was synthesized as described by Hayase *et al.* (1989). Briefly, each drug (6.7 mM) was reacted with ovalbumin (30 μM) in the presence of EDC (2mg/ml); the reaction was carried out at room temperature in water (1ml, pH 6.0) for two hours. After each reaction, one fifth of the mixture (200 ml) was used for quantitation of covalently bound drug released from modified protein after alkaline hydrolysis as described in Chapter 4. The other part of reaction mixtures (800 ml) was washed 8 times by centrifugal filtration through a 30-KDa-cutoff membrane (Amicon centricons, Beverly, MA) and the remaining solution was rinsed with phosphate buffered saline (PBS). After lyophilization, the final product, containing both modified and unmodified protein, was stored at 4^0C and used for coating ELISA plate. The yield of diclofenac, ibuprofen, and naproxen drug-modified protein adduct was 22.6, 16.7, and 17.7 μg/mg protein, respectively.

5.2.4 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was utilized to test the reactivity of antisera of each patient against protein conjugates. ELISA assay was performed at room temperature. The 96 wells of the ELISA plate were coated with 100 μl/well of coating buffer (0.2 M

sodium bicarbonate buffer, pH 9.4) containing 2.5 mM of D-ovalbumin or ovalbumin and shaken for 2 hours. After washing three times with PBS and three times with washing buffer, 150 µl/well of blocking buffer (10% BSA solution in PBS) was added and incubated for 1 hr. Then a series of dilutions of antiserum were incubated for 1 hr. After the washing procedure, 100 µl/well of alkaline phosphatase labeled anti-mouse IgG and IgM (H+L) (1:800 dilution in the blocking buffer) was added and incubated for two hrs, followed by washing and the incubation of 100 µl of substrate solution (5 mg of *p*-nitrophenyl phosphate disodium salt in 10 ml of a 1M diethanolamine solution) for 15 to 20 mins. Absorbance of the colored *p*-nitrophenol was read by an ELISA reading machine (BT2000, Fisher Biotech) at 405 nm.

5.2.5 Inhibition Studies

The same ELISA system as described above was used for inhibition studies. Anti-sera from each patient were diluted 1:4 and were incubated with the same volume of solution for a number of compounds (potential inhibitors) at various concentrations for 15-20 minutes prior to the addition of the anti-sera to the coated ELISA plate. If the anti-sera reacted with these compounds, the binding of the anti-sera to the drug-protein conjugates coated ELISA plate would be decreased, leading to a decreased absorbance when compared to anti-sera preincubated with buffer. The anti-sera were diluted with the blocker solution and the compounds were dissolved in phosphate buffer (0.1 M, pH 7.4). The compounds used were: diclofenac, ibuprofen and naproxen modified protein conjugates, diclofenac, diclofenac glucuronide, ibuprofen, ibuprofen glucuronide, tolmetin, zomepirac, fenoprofen, naproxen, valproic acid, and ovalbumin. Percent inhibition at each inhibitor concentration was calculated as:

$$\% \text{ Inhibition} = \frac{\text{Absorb. (without inhibitor) - Absorb. (with inhibitor)}}{\text{Absorb. (with inhibitor)}} \times 100$$

5.3 RESULTS

Sera of eighteen patients who had hypersensitivity reactions to one or two NSAIDs were examined by ELISA for the drug specific antibodies. Low levels of drug protein conjugate-specific antibodies were present in four patients' samples. As shown in Fig. 5.1, sera from subject 3, who had allergic reactions to ibuprofen and tolmetin, Subject 8, and Subject 10, who both had allergic reactions mainly to ibuprofen., exhibited higher reactivity toward the ibuprofen protein adduct at lower titers. Sera from subject 14, who had allergic reactions to diclofenac, showed higher reactivity against diclofenac-protein conjugates than unmodified proteins (Fig. 5.2). No conjugate specific antibodies were observed in any of 18 control sera (Fig. 5.3). Drug protein conjugate specific antibodies were not found at measurable levels in the remaining 14 patients (Fig. 5.4).

As shown in Fig. 5.5 and Fig. 5.6, both ibuprofen-ovalbumin and ibuprofen inhibit the reactivity of the antibodies to drug protein conjugates which indicates that the antibodies are specific to the drug protein conjugate and can recognize the ibuprofen moiety. The antibodies cross-reacted with other NSAIDs having some degree of structural similarity (Fig. 5.7), but did not recognize valproic acid, which has totally different chemical structure. The antibodies in the sera of subject 14 are specific to diclofenac-modified protein (Fig. 5.8) and most of them could only recognize the diclofenac structure since diclofenac and diclofenac glucuronide inhibit antibody- antigen interactions to the same level (Fig. 5.9). The antibodies

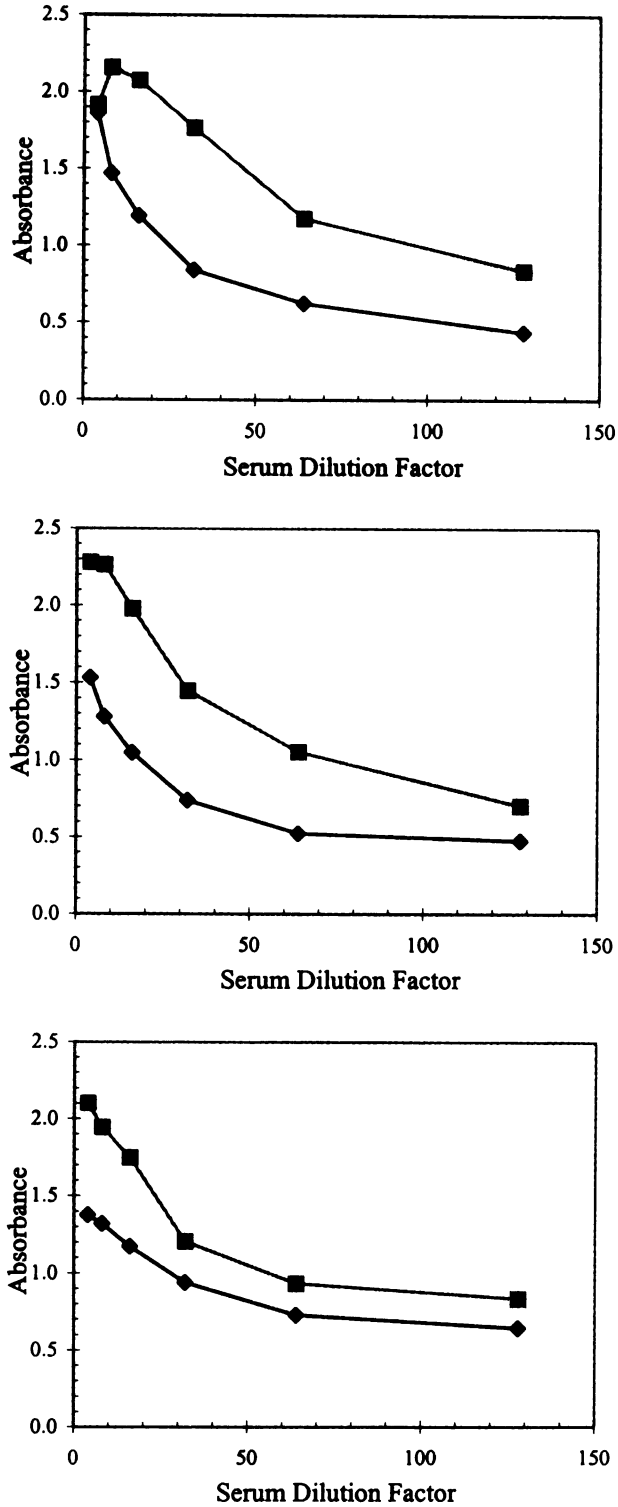

Fig. 5.1. Reactivity of plasma samples from Subject 3 (upper), who had allergic reactions to ibuprofen and tolmetin, Subject 8 (middle), and Subject 10 (bottom), who both had allergic reactions to ibuprofen. The squares show the reactivity against ibuprofen-ovalbumin, the diamonds show the reactivity against unmodified ovalbumin.

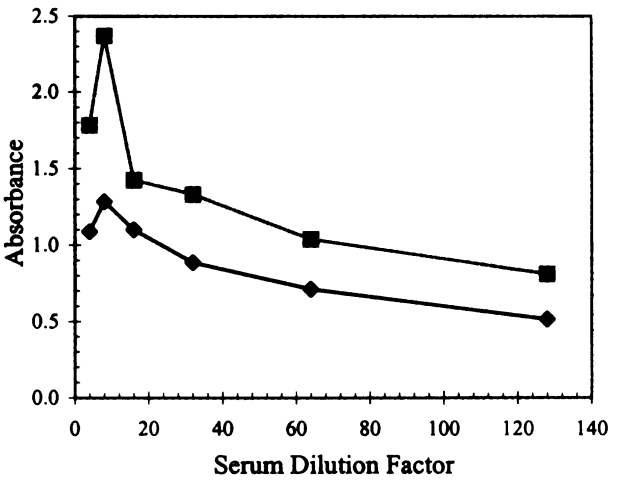

Fig. 5.2. Reactivity of plasma samples from Subject 14, who had allergic reactions diclofenac. The squares show the reactivity against diclofenac-ovalbumin, the diamonds show the reactivity against unmodified ovalbumin.

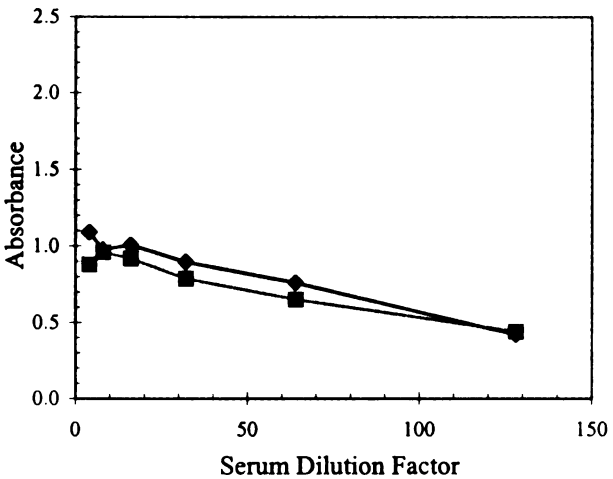

Fig. 5.3. Reactivity of plasma samples from a control, who had no allergic reaction to any NSAID. The squares show the reactivity against ibuprofen-ovalbumin, the diamonds show the reactivity against unmodified ovalbumin.

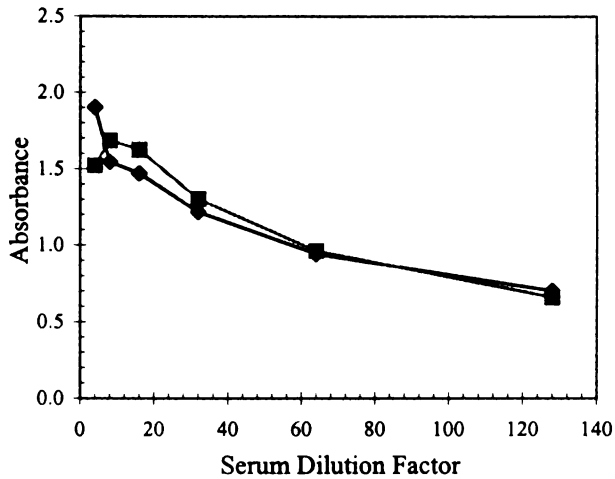

Fig. 5.4. Reactivity of plasma samples from Subject 5, who had allergic reactions ibuprofen. The squares show the reactivity against ibuprofen-ovalbumin, the diamonds show the reactivity against unmodified ovalbumin.

have lower cross-reactivity toward other NSAIDs (Fig. 5.10) and don't cross-react with valproic acid. Cross-reactivity among NSAIDs was not observed in any control sample (Fig. 5.11). These data indicate that drug protein conjugate specific antibodies can be detected in sera of selected patients who have had respiratory reactions to NSAIDs and aspirin, and that these antibodies show some degree of cross reactivity with other structurally similar drugs.

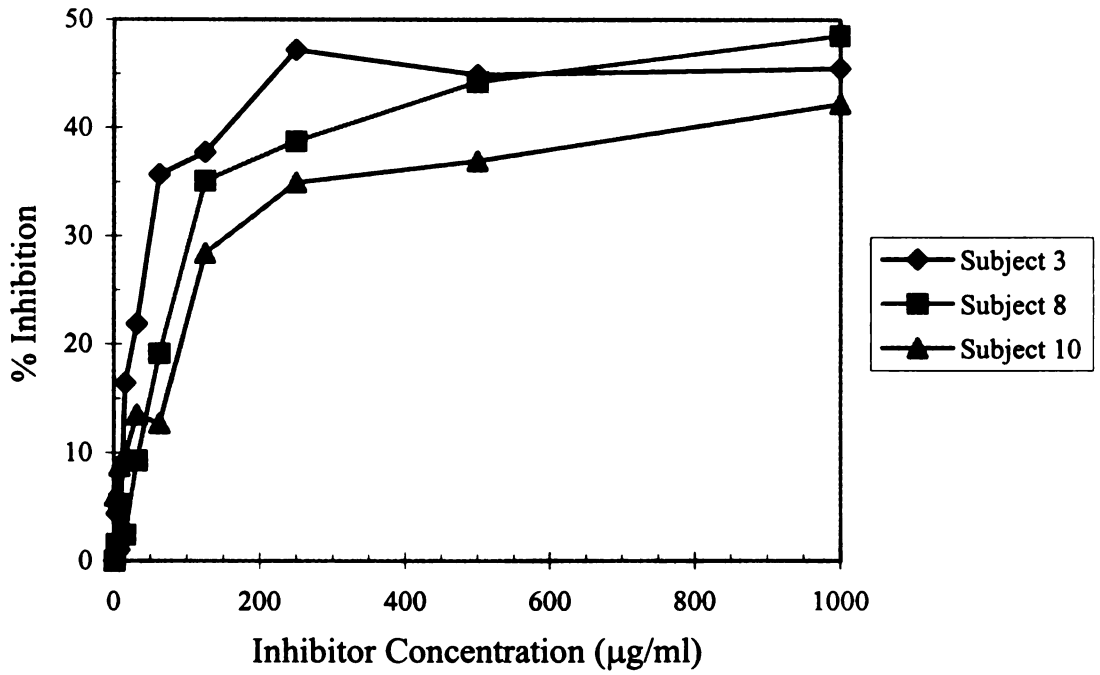

Fig. 5.5. Inhibition of antibody-antigen interaction by ibuprofen-ovalbumin conjugate. The inhibition curves for subject 3 (diamonds), subject 8 (squares), and subject 10 (triangles) are shown.

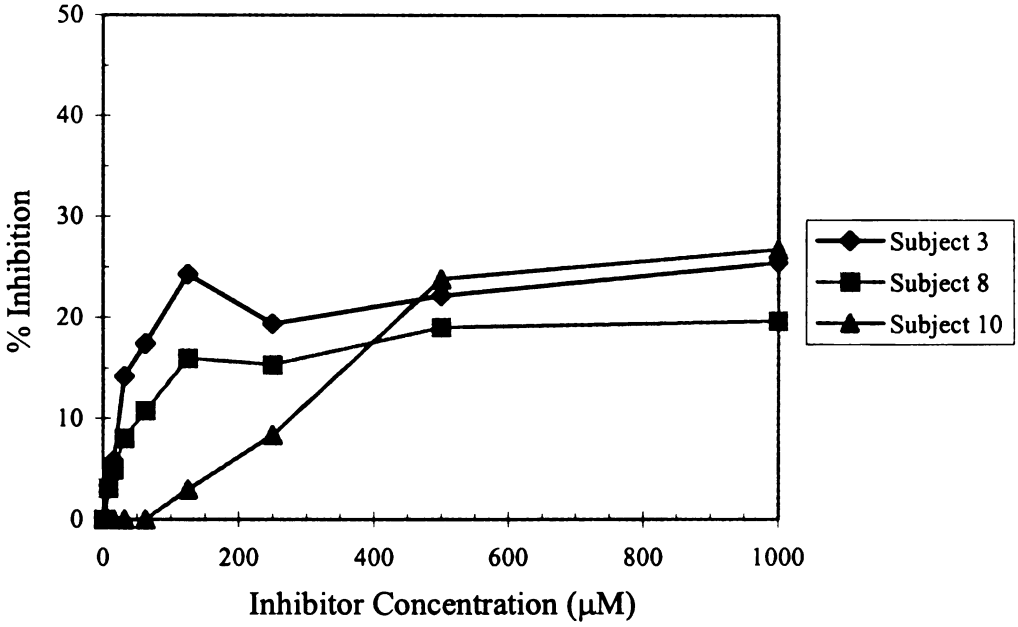

Fig. 5.6. Inhibition of antibody-antigen interaction by ibuprofen. The inhibition curves for Subject 3 (diamonds), Subject 8 (squares), and Subject 10 (triangles) are shown.

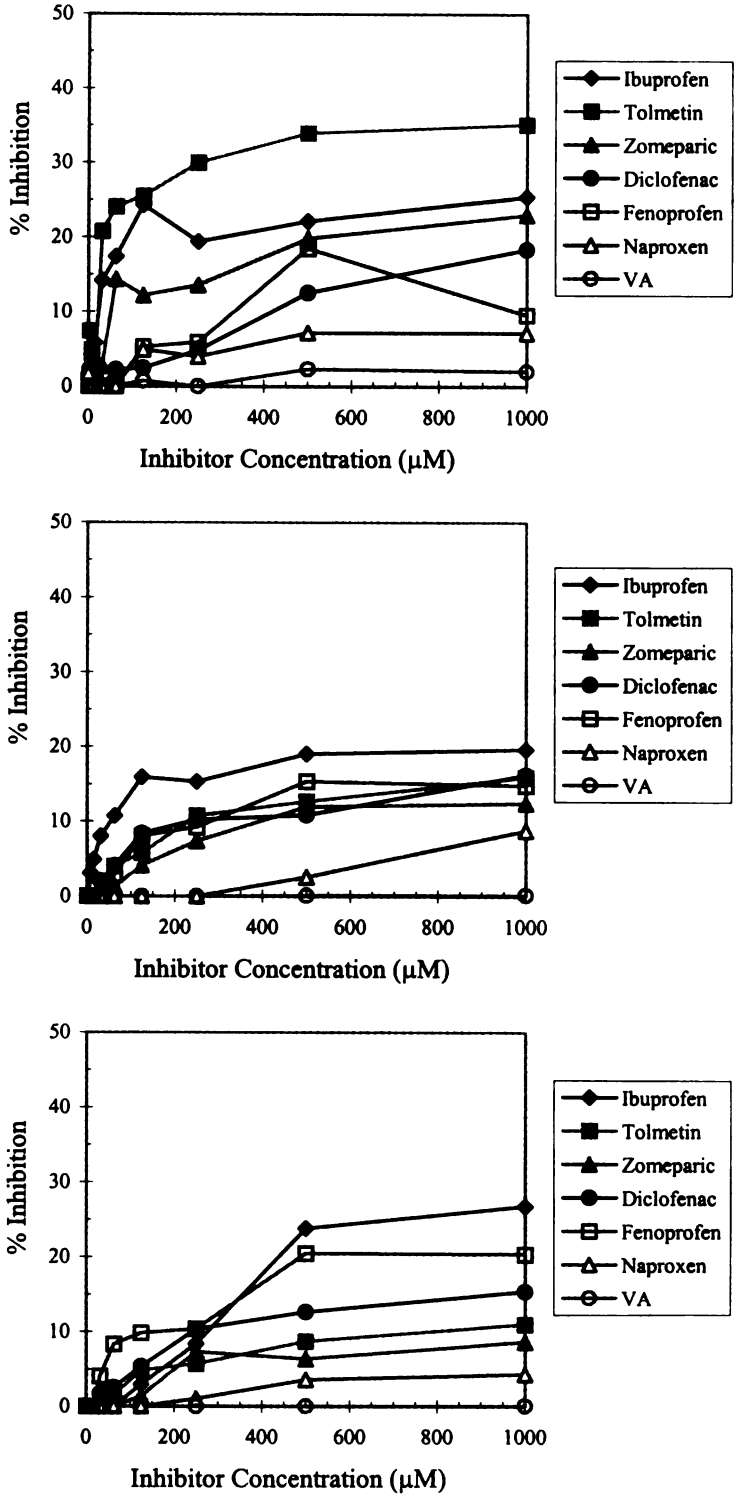

Fig. 5.7. Inhibition of antibody-antigen interaction by NSAIDs and valproic acid (VA). The inhibition curves for Subject 3 (upper), Subject 8 (middle), and Subject 10 (bottom) are shown.

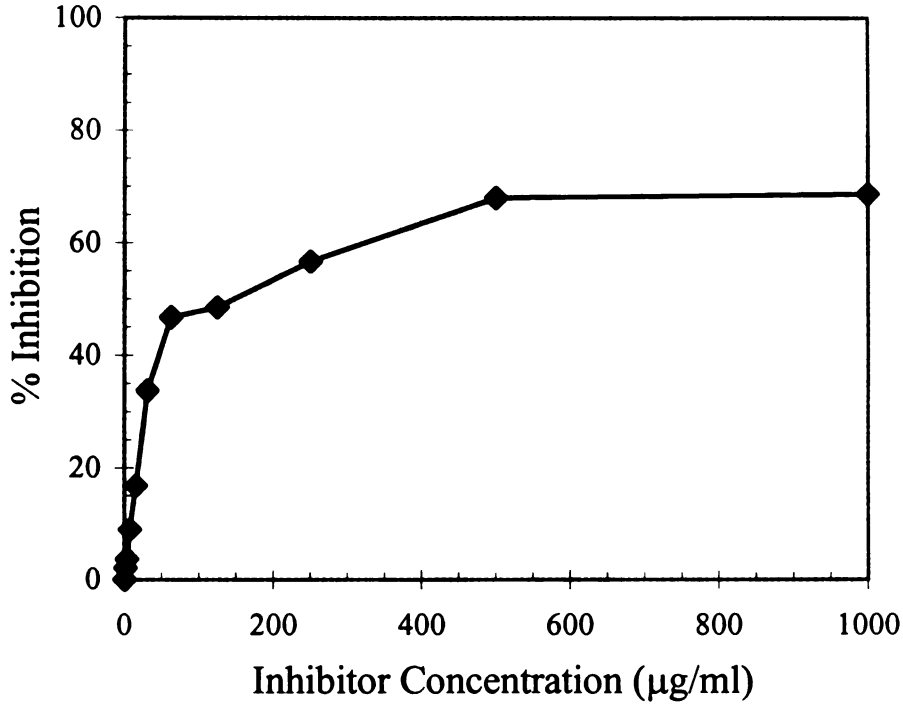

Fig. 5.8. Inhibition of antibody-antigen interaction by diclofenac-ovalbumin conjugate. The inhibition curve for Subject 14 is shown.

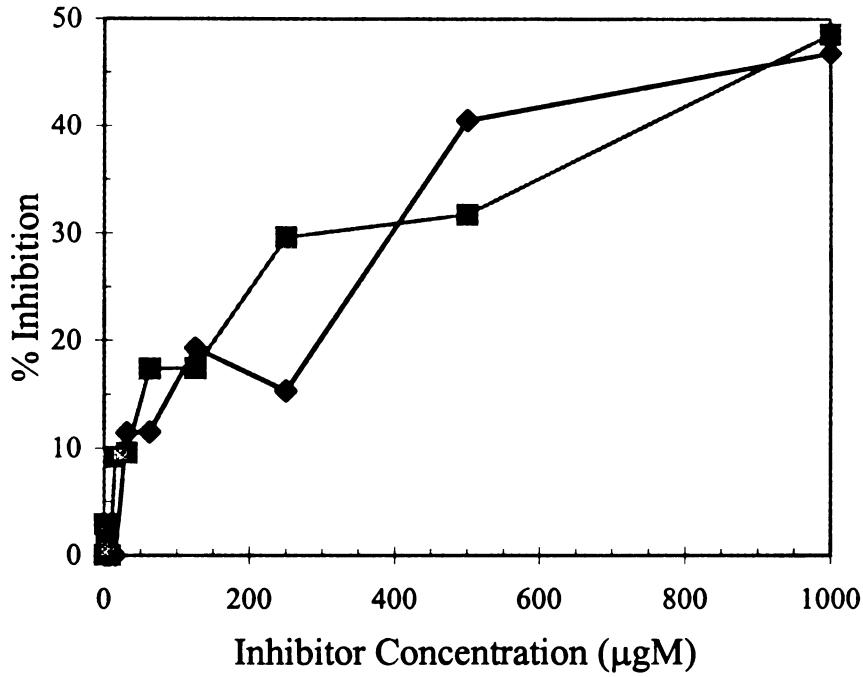

Fig. 5.9. Inhibition of antibody-antigen interaction by diclofenac (diamonds) and diclofenac glucuronide (squares). The inhibition curves for Subject 14 are shown.

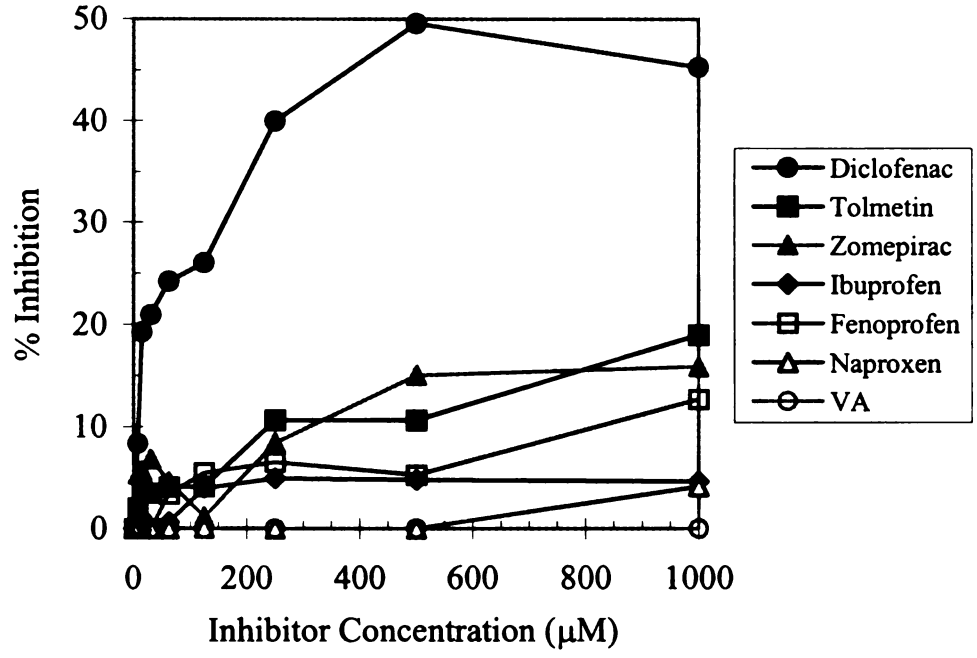

Fig. 5.10. Inhibition of antibody-antigen interaction by NSAIDs and valproic acid (VA). The inhibition curves for Subject 14 are shown.

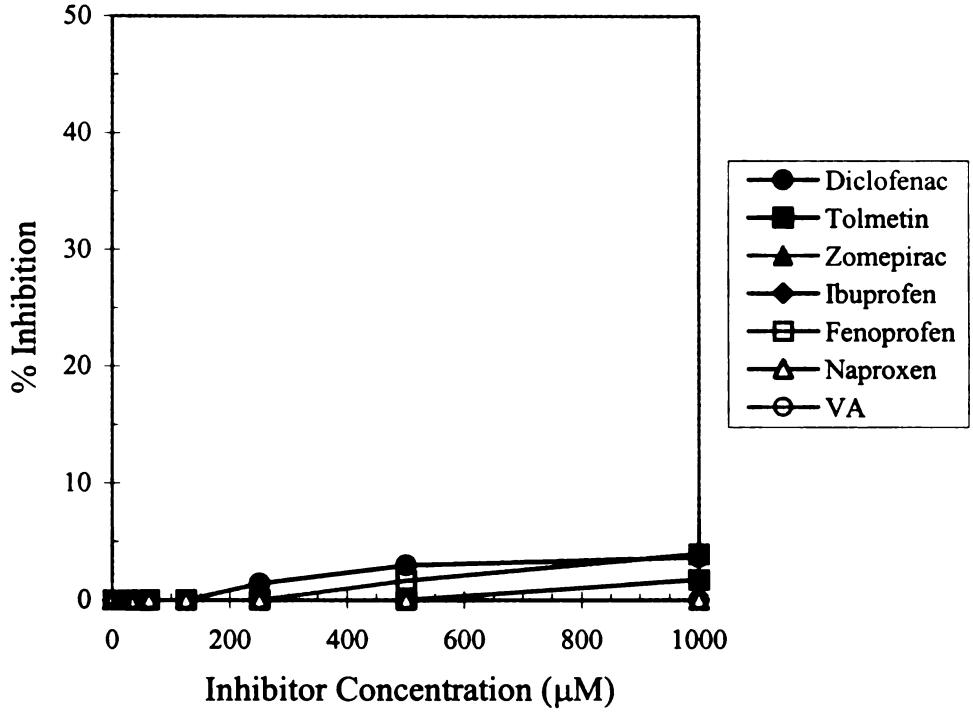

Fig. 5.11. Inhibition of antibody-antigen interaction by NSAIDs and valproic acid (VA). The inhibition curves for a control are shown.

5.4 DISCUSSION

The pathomechanisms of hypersensitivity to NSAIDs are largely unknown. Currently, the widely accepted hypothesis is that toxicities to aspirin and other NSAIDs are caused by inhibition of cyclooxygenase activity by these compounds, which results in an overproduction of cysteinyl leukotrienes from arachidonic acid (Szczeklik *et al.*, 1977; Szczeklik, 1987; Stevenson and Lewis, 1987). However, the prostaglandin inhibition hypothesis cannot explain all the adverse reactions to NSAIDs. Many patients with NSAID sensitivity did not show cross-reactivity with other NSAIDs that are not structurally related even though all of these NSAIDs are potent inhibitors of cyclooxygenase *in vitro* (Blanca *et al.*, 1989a; Fernandez-Rivas *et al.*, 1993; Katz *et al.*, 1993; Quiralte *et al.*, 1996). These observations indicated that cyclooxygenase inhibition was not involved in these adverse reactions to NSAIDs. Much evidence has suggested immunologic mechanisms may be implicated in the adverse reactions to NSAIDs in humans (Rake and Jacobs, 1983; Fernandez-Rivas *et al.*, 1993; Boelsterli *et al.*, 1995).

The metabolic conversion of drugs to chemically reactive products is known to be a prerequisite for many idiosyncratic drug reactions (Boelsterli, 1993; Pumford *et al.*, 1997). If not quickly excreted, the highly reactive products of xenobiotics may irreversibly interact with cellular macromolecules and mediate cellular necrosis, carcinogenicity or hypersensitivity reactions. Idiosyncratic hypersensitivity reactions may account for 3-25% of all adverse drug reactions (Pohl *et al.*, 1988), and are a constant problem for physicians and patients because of their unpredictable nature (with respect to both chemical structure and apparent dose), potentially fatal outcome and resemblance to other disease processes (Parker, 1982; Pohl *et al.*, 1988).

The clinical manifestations of drug 'allergic reactions' are quite diverse, and include fever, urticaria, anaphylaxis, serum sickness, and hematologic and tissue toxicities (Parker, 1982). Our current understanding of how drug allergy occurs is based largely on the hapten hypothesis. Applied to NSAIDs, it assumes that they are small molecules and chemically incapable of irreversibly modify a protein *in vivo*. Instead, they must be metabolized to reactive species, such as acyl glucuronide conjugates or acyl-CoA conjugates, which may covalently bind to plasma or cellular macromolecules. These drug-macromolecule complexes may then act as an immunogens and induce the production of specific antibodies (a humoral response) and /or the generation of specific T lymphocytes (a cellular response) toward the hapten (drug) and/or native macromolecule epitopes. The recognition of the native macromolecule would develop a loss of self-tolerance and induce the production of autoantibodies. Although these reactions are rare, they are of increasing concern due to the large number of individuals exposed to medication.

The nature of the allergic reactions depend on the nature of the immune responses. There are four types of immune-mediated tissue damage (Janeway and Travers, 1994). Types I-III are antibody-mediated and are disguished by the different types of antigens recognized and the different classes of antibodies involved. Type I responses are mediated by IgE. This is the classic immediate hypersensitivity reaction occurring within seconds or minutes of antigen contact. The IgE activates mast cells to release vasoactive amine, leukotrienes and cytokines. Types II and III are mediated by IgG, which can activate either complement-mediated or phagocytic effector mechanisms. These different effector mechanisms lead to markedly different tissue damage and pathology. Type II responses are directed against cell-surface or matrix-associated antigen, leading to tissue damage, whereas type III responses are directed against soluble antigens, and

the tissue damage involved is caused by responses triggered by an immune complex. Type II and type III reactions generally occur within a few hours of exposure. In type IV hypersensitivity reactions, the allergen may be a foreign protein or a chemical substance that reacts with self proteins. Once a person is sensitized to a modified self or a foreign protein, re-exposure leads to a T-cell response that evolves over several days, so these reactions are called delayed-type hypersensitivity. Type IV response can be subdivided into two classes; in the first class, tissue damage is caused by activation of inflammatory responses by T_H1 cells, mediated mainly by macrophages, and in the second damage is directly caused by cytotoxic T cells. All of the classes of hypersensitivity responses are seen in both autoimmunity and allergy to drugs. With the exception of type I hypersensitivity; IgE responses have not been observed in autoimmunity.

This study demonstrated the presence of low titer of drug-specific IgG antibodies in selective patients who have had adverse reactions, mainly respiratory reactions, to NSAIDs and aspirin, and that these antibodies show cross reactivity with other structurally similar NSAIDs. Drug-specific IgE antibodies were not detected in any patient's sample. This finding suggests reactions to NSAIDs in some patients may be due to an immune response to the drug and/or related drugs rather than interference with the arachidonic acid metabolism pathway. Other reports suggest that reactions to NSAIDs may be also partly due to an inflammatory reaction to an autoantigen or a chronic viral infection (Szczeklik, 1997). Elevated markers of autoimnunity (Lasalle *et al.*, 1993; Szczeklik *et al.*, 1995, 1997) and enhanced IgG4 synthesis (Szczeklik *et al.*, 1992) in patients with AIA were reported.

Factors that may contribute to the development of adverse drug reactions mediated by reactive drug metabolites include the chemical character of the drug, interindividual variation in drug metabolism (influence by both genetic and

environmental factors) and the properties of the targeted tissue or plasma macromolecules. The role of drug metabolism is critical to the hapten hypothesis. Genetic variations in drug metabolism of NSAIDs, such as drug oxidation or acyl glucuronide formation, detoxication, or biliary or urinary metabolite excretion may all contribute to variable susceptibility. Interindividual variations in NSAID acyl glucuronide conjugation reactions in humans should be better characterized, but only limited data is available (Pacifici *et al.*, 1990; Mulder, 1992), despite the increasing awareness of the reactivity of acyl glucuronides to form protein adducts. Similarly, the mechanism of canalicular excretion of NSAIDs needs further characterization, particularly since the importance of NSAID excretion *via* bile has been recognized (Boelsterli *et al.*, 1995).

The reason why drug-specific antibodies were found in selected patients is not clear. A similar observation was obtained in animal models. NSAIDs protein adducts could only induce an antibody response in some of the mice or rats after immunizations (Williams *et al.*, 1995; Zia-Amirhosseini *et al.*, 1995; Chapter 4). Oral administration of antigens can induce systemic hyporesponsiveness, called oral tolerance. It is known that oral tolerance can develop when an antigen interacts with the gut-associated lymphoid tissues (GALT), though the exact mechanism of oral tolerance has not been clearly defined (Strobel and Mowat, 1998). The most commonly studied antigens have been soluble proteins and peptides. However, the oral administration of metals such as nickel (van Hoogstraten *et al.*, 1993) and reactive haptens such as dinitrochlorobenzene (DNCB) or trinitrobenzenesulfonic acid has also been shown to produce tolerance against the sensitizing effects of these compounds (Reese and Cebra, 1975; Gautam and Battisto, 1983). Oral tolerance produced by these compounds is presumably mediated by covalent adducts formed in GALT. Recently, Ware *et al.* (1998) found that when rats were treated orally with diclofenac, the drug formed covalent

adducts to aminopeptidase N (CD13) and sucrase-isomaltase (SI) in enterocytes of the small intestine of rats. These results showed that covalent adducts of NSAIDs can be formed within GALT, where they may have a role in preventing drug allergies.

The selectivity of antibody detection could also be related to several other factors: 1) individual differences in the metabolic pathways of NSAIDs to reactive metabolites or the clearance of covalent bound protein adducts, or individual differences in the immune system among people; 2) the fact that ovalbumin is not the correct protein carrier. New antigenic determinants may be formed once NSAID metabolites react with human plasma or tissue proteins, thus making the detection of drug-specific antibodies difficult, as in occupational asthma caused by other low molecular weight compounds (Bernstein *et al.*, 1984); 3) the differences in intervals between the adverse reaction and the collection of the blood samples for this study. The longer the interval, the less likely the chance of finding positive results.

6.0 SUMMARY OF FINDINGS AND FUTURE STUDIES

6.1 Reactivity of Diclofenac Acyl Glucuronide and Irreversible Binding to Plasma Proteins In Vitro

As previously reported in studies of several acyl glucuronides of carboxylic acid-containing NSAIDs, diclofenac glucuronide (DG) is reactive under physiological conditions and appeared to follow first-order degradation kinetics, undergoing intramolecular acyl migration to other glucuronide isomers and hydrolysis to the parent compound. Lower pH increased DG stability. The stability of acyl glucuronides is also influenced by protein in the media. When DG was incubated in the presence of HSA, the degradation rate constant increased. The presence of HSA significantly decreased DG stability not only at pH 7.4 and 6.8 but also at pH 5.5, suggesting the contribution of both esterase-activity and the catalytic activity of HSA on DG stability.

In vitro irreversible binding of diclofenac to HSA occurred after incubation of DG with HSA in buffer. Maximum binding was observed after a 4 hr incubation and then gradually decreased after 24 hours, reflecting the lability of these adducts, presumably to hydrolysis. Our results show that DG, a carboxylic acid with an unsubstituted α-carbon, exhibits the fastest degradation rate and the most covalent binding *in vitro* of any NSAID investigated to date in the presence of HSA (see Fig. 2.6). These results are consistent with and added to the data obtained with other acyl glucuronides reported previously by our laboratory.

6.2 Disposition and Stability of Diclofenac Protein Conjugates in Humans

Irreversible binding of diclofenac to plasma proteins occurred in all subjects and was detectable in early samples even after a single 75 mg dose of diclofenac. The protein adducts accumulate upon repeated exposure to the drug. Much higher concentrations of drug-protein adduct (average 6.7 fold increase) were present in plasma after 10 days of multiple dosing of diclofenac. The diclofenac protein conjugates exhibited biphasic elimination, with an average terminal half-life of 10.3 days. Thus, the reactivity of diclofenac acyl glucuronide toward endogenous macromolecules *in vivo* may contribute to the high incidence of unexplained side-effects associated with this drug.

6.3 Immunogenicity of Diclofenac Protein Conjugates Formed *via* a Drug-Glucuronide Intermediate

In vivo covalent binding of NSAIDs to proteins *via* glucuronide may result in two different classes of protein adducts; in one the drug is linked directly to the amino acid of the protein (D-protein) and in the other the drug is bound to the protein via a glucuronic acid ring (DG-protein). Both diclofenac glucuronide-mouse serum albumin conjugate (DG-MSA) and diclofenac-mouse serum albumin conjugate (D-MSA) were immunogenic and could induce drug or acyl glucuronide-specific antibodies in mice. The titer of antibody produced against DG-MSA was similar to D-MSA indicating that the two classes of protein adducts could elicit an equally strong immune response *in vivo*. Interestingly, both immunogens produced a response in only a subset of animals immunized. This parallels the variable occurrence of NSAID reactivity in humans. The antibodies produced in mice could cross react with other NSAIDs and the cross-reactivity seems to depend on the

similarity of the chemical structure of acidic NSAIDs. Antibodies reactive to DG had higher cross-reactivity to glucuronide metabolites of other NSAIDs than to the drugs themselves. These data support the hypothesis that a hypersensitivity reaction to NSAIDs may be initiated by an immune response to drug or drug-glucuronide modified proteins and that cross-reactivity among NSAIDs may be caused by cross-reacting antibodies.

6.4 Immunologic Reactivity of Certain NSAIDs in Humans

Low titers of drug-specific IgG antibodies were detected in selective patients (4 of 18) who have had adverse reactions, mainly respiratory reactions, to NSAIDs and aspirin. These antibodies exhibit cross reactivity with other structurally similar NSAIDs. No conjugate specific antibodies were observed in any control sera. Drug protein conjugate specific antibodies were not found at measurable levels in the remaining 14 patients (Fig. 5.4). Drug-specific IgE antibodies were not detected in any patient's sample. This finding suggests reactions to NSAIDs in some patients may be due to an immune response to the drug and related drugs rather than interference with the arachidonic acid metabolism pathway.

6.5 Future Studies

Future studies should further test the hypothesis that reactive metabolite intermediates of drugs, *via* covalent binding with endogenous proteins, may play a role in the toxicities associated with acidic NSAIDs and address the risk factors that may contribute to the development of adverse drug reactions mediated by reactive drug metabolites. These studies should:

1) characterize metabolic pathways of NSAIDs that may be involved in the generation of reactive intermediates and identify reactive metabolites *in vivo*.

2) further assess the reactivity of reactive intermediates both *in vitro* and *in vivo* and elucidate the structure and mechanism of covalent binding to proteins *in vivo*.

3) identify the proteins covalently modified by reactive intermediates of NSAIDs.

4) investigate the relationship between the immunotoxicity and the interindividual variations in NSAID acyl glucuronide or acyl CoA conjugation reactions in humans.

5) develop an *in vitro* model to assess the potential of NSAIDs to cause organ toxicity *via* an immune based mechanism.

6) explore the possible role of human leukocyte antigen (HLA) in causing the immunologic toxicity of NSAIDs.

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