Investigation of a Metabolic Pathway Leading to an Idiosyncratic Drug Reaction: Is the Sulfate of 12-Hydroxynevirapine Responsible for the Skin Rash in Brown Norway rats?

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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University of Toronto

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An animal model of nevirapine (NVP)-induced skin rash was used to test the hypothesis that sulfonation of 12-OH NVP, a metabolite of NVP proven essential for rash development, is the link between 12-OH NVP and the skin rash. Female Brown Norway (BN) rats were co-treated with NVP or 12-OH NVP and sulfation inhibitors dehydroepiandrosterone (DHEA) and salicylamide. Co-treatment with salicylamide markedly decreased formation of the sulfate conjugate but did not prevent development of the rash suggesting that the sulfate is not involved. However, it is not known whether the sulfate formation in the skin was affected. Co-treatments with DHEA decreased the sulfate formation and prevented the rash but also had other effects on NVP metabolism. This implies that the sulfate metabolite is responsible for the rash. Additional studies will be required to resolve these conflicting results.
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12-OH NVP: 12-hydroxynevirapine
12-sulfoxy NVP: 12-sulfoxynevirapine
2-OH NVP: 2-hydroxynevirapine
3-OH NVP: 3-hydroxynevirapine
4-COOH NVP: 4-carboxynevirapine
ADR: adverse drug reaction
APC: antigen-presenting cell
DHEA: dehydroepiandrosterone
DILI: drug-induced liver injury
HLA: human leukocyte antigen
IDR: idiosyncratic drug reaction
MHC: major histocompatibility complex
NSAID: non-steroidal anti-inflammatory drug
NVP: nevirapine
P450: cytochrome P450
PAPS: 3’-phosphoadenosine 5’-phosphosulfate
SJS: Steven’s-Johnson syndrome
SLE: systemic lupus erythematosus
SSAO: semicarbazide-sensitive amine oxidase
SULT: sulfotransferase
TEN: toxic epidermal necrolysis
UDP - uridine dinucleotide phosphate
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Section 1: Introduction

1.1 Overview of adverse drug reactions

As with any xenobiotic introduced into a living organism, pharmaceutical agents – drugs – can not only treat a specific condition but can also cause harm. Adverse reactions to drugs occur in many patients, account for 6.7% of hospital admissions (1), and are ranked between 4th and 6th leading causes of death in UK and US (2-3). The increased morbidity and mortality associated with adverse drug reactions inflict great costs on society because of the longer and advanced care needed to treat them. An adverse drug reaction (ADR), even when occurring in a small number of people, can cause a drug to receive a black box warning or even be removed from the market (4). Thus, ADRs are also a major obstacle in drug development. Therefore, it is important to study the mechanisms of ADRs because a better mechanistic understanding may make it possible to prevent them.

ADRs can be classified according to an established system (5):

1.1.1 Type A (augmented):

These are reactions that are predictable from the known pharmacology, often representing an exaggeration of the pharmacological effect of the drug. There is often a clear dose-dependency and the reaction can be alleviated if the dose of the drug is reduced. Examples include hypotension associated with antihypertensives and bleeding associated with anticoagulants.
1.1.2 Type B (bizarre):

There are many terms that are used to describe the reactions that are unpredictable from a knowledge of the basic pharmacology of the drug and show no simple dose-response relationship. Apart from Type B, the term idiosyncratic drug reaction (IDR) is commonly used. Here, the term IDR will be used to describe reactions that do not occur in most people within the range of doses used clinically and do not involve known therapeutic effects of the drug (6). It is speculated that host-dependent factors are important in determining the predisposition to developing these adverse reactions. IDRs are usually classified as being immune-mediated and often involving reactive metabolites. These reactions tend to be more serious and can lead to death. Examples of IDRs include halothane-induced hepatitis and anticonvulsant hypersensitivity.

1.1.3 Type C (chemical):

This term is used to describe reactions whose biological effects can be predicted or deduced from the chemical structure of the drug or its metabolite. Acetaminophen-induced hepatotoxicity is an illustrative example of a Type C reaction.

1.1.4 Type D (delayed):

This is an adverse reaction that can occur many years after the exposure. These reactions include secondary tumors that develop many years after treatment with chemotherapeutics and teratogenic effects seen in children following drug intake by the mother during pregnancy, such as the fetal hydantoin syndrome caused by phenytoin.
1.1.5 Type E (end-of-treatment):

This type of reaction can occur when the drug is withdrawn, especially if the drug is stopped abruptly. Examples include withdrawal seizures that can occur when stopping phenytoin and the paroxetine withdrawal syndrome.

1.2 Idiosyncratic drug reactions

The proper definition of what constitutes an idiosyncratic drug reaction (IDR) is a subject for debate. Here, the term IDR will be used for a reaction that does not occur in most people within the range of doses used clinically and does not involve known pharmacological effects of the drug (6). While more then 6% of all hospital admissions in the UK are due to adverse drug reactions, only about 5% are caused by what are considered IDRs or Type B reactions (1). Because the incidence of IDRs is so low, they are rarely discovered during clinical trials and therefore cause a major problem in drug development. Just over 10% of drugs that entered the market between 1975 and 2000 in the United States had to be withdrawn or received a black box warning due to adverse effects that were not predicted in clinical trials (4). Presently, the most common causes for a drug candidate to be abandoned are toxicity and lack of efficacy. Having to withdraw a drug candidate can lead to failure of the company, and therefore a great effort is put into trying to predict the risk of IDRs and to find possible signals of an IDR during clinical trials (6).

ADRs and IDRs can affect almost any organ or system in the body. Below is a brief summary of adverse reactions arranged by organ:
**Liver:** Liver injury is one of the most common manifestations of IDRs (6). Hepatotoxicity can vary in severity from a mild and transient increase in serum transaminases to complete hepatic failure. One of the most well studied examples of drug-induced liver toxicity is halothane-induced hepatitis. Hepatotoxicity is one of the main reasons for drug withdrawal from the market; an example is tienilic acid, which was withdrawn in 1980.

**Skin:** Adverse cutaneous reactions affect 2 – 3% of hospitalized patients and are the most often occurring manifestation of IDRs (5). The two most severe skin reactions are Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Both reactions are associated with high incidence of death – 5% for SJS and up to 30% for TEN (7). Most drug-related rashes involve infiltration of leukocytes into different layers of the skin and are presumed to be immune-mediated (8).

**Multi-organ:** One of the most severe ADRs is anaphylaxis, which is an example of a Type 1 hypersensitivity reaction (5). The drugs most commonly associated with anaphylaxis are β-lactam antibiotics, non-steroid anti-inflammatory drugs (NSAID), sulfonamides, and high-molecular mass compounds such as protein and biotechnology-derived products (5, 9).

**Blood:** Another common type of reaction is blood dyscrasias which include agranulocytosis, hemolytic anemia, and thrombocytopenia (5). These conditions can be induced by many drugs in different classes.

There are a number of clinical characteristics that can be used to describe IDRs. The defining characteristic is the low incidence of these reactions as they do not occur in
most patients treated with a specific drug. Another very important characteristic of IDRs is a delay between starting the drug and the onset of the adverse reaction (10). In most cases, the delay between the first exposure and the onset is more then a week. The delay is different for different drugs and types of adverse reactions. Skin rashes typically occur after one or two weeks of treatment, the time until onset of agranulocytosis is one and up to three months, idiosyncratic drug-induced hepatitis typically occurs after one or two months, and drug-induced lupus usually occurs after several months of treatment (6). For some drugs, such as troglitazone, the delay can be more than one year of treatment (11). In contrast to the usual delay in onset of IDRs on first exposure to the drug, the delay on repeated exposure after a confirmed IDR is often very short and can occur in minutes, for example, with anaphylactic reactions.

Some IDRs can involve production of autoantibodies. The most common example of such a reaction is drug-induced systemic lupus erythematosus (SLE), which is a condition that can be induced by many drugs, including hydralazine and isoniazid. There are several examples in which autoantibodies to specific proteins have been associated with drug-induced hepatitis. For example, antibodies against cytochrome P450 1A2 have been detected in patients with hepatitis induced by dihydralazine (5).

While it is true that most patients exposed to drug would not develop an IDR at any dose achievable, it would be incorrect and misleading to call IDRs dose-independent. A clear dose-dependency might be missing within the narrow therapeutic range that most drugs are administered, but in all cases a dose can be found below which no patient will have an IDR. In fact, this approach is sometimes used for patients allergic to penicillin. If a life-threatening infection occurs to which there is no good treatment alternative to
penicillin, a patient can be started on an extremely low dose – about ten thousand times less than the therapeutic dose – and then the dose can be slowly increased until the therapeutic dose is achieved (12).

Another important characteristic of IDRs is adaptation or tolerance. While only a handful of patients will develop a full-blown IDR to a drug, it can cause asymptomatic events in a much greater number of patients. For example, a drug that causes idiosyncratic liver failure in a small number of patients can induce an increase in transaminases – an indicator of liver injury – with a much higher incidence. In most cases, the increase in transaminases is transient and they return to normal even if the treatment is continued (13).

Most patients that develop an IDR to a drug do not run a higher risk for a similar reaction towards other drugs. However, there are known examples of cross-reactivity, or cross-sensitivity, such as the aromatic anticonvulsant syndrome associated with phenytoin, carbamazepine, and phenobarbital. If a patient develops hypersensitivity to one of these drugs, the risk of developing an IDR to the other is 40 - 60% (14).

Many attempts have been made to find genetic and other associations for better prediction and possibly prevention of IDRs. Some associations between the risk of an IDR and a specific genotype have been found, for example, with genetically polymorphic drug metabolizing enzymes. It was determined that the risk for an IDR caused by isoniazid and sulfonamide antibiotics is greater for slow acetylators (15-16). If IDRs are immune-mediated, another risk factor could be a specific human leukocyte antigen (HLA), but only a few strong associations have been identified to date (6).
Apart from genetic associations, there are a number of other factors that appear to contribute to a higher risk of developing an IDR. These factors include sex, age, weight, and concomitant diseases; however, it is often impossible to isolate these factors from use of the drug (6). Women are at increased risk for some IDRs, such as nevirapine-induced skin rash (17), halothane-induced hepatitis (18), and clozapine-induced agranulocytosis (19). Obesity appears to be risk factor for developing halothane-induced liver injury (18). Pre-existing liver disease can complicate therapy with any drug, but there is no clear evidence that patients with pre-existing liver conditions have a higher risk for drug-induced liver injury (6). Infectious diseases, such as mononucleosis (20), HIV infections (21), and possibly herpes virus infection (22) appear to be risk factors associated with IDRs.

1.3 Mechanistic hypotheses of idiosyncratic drug reactions

There is circumstantial evidence that IDRs are caused by reactive metabolites and their characteristics suggest that most are immune-mediated. Several mechanistic hypotheses have been proposed to fit what is known about IDRs.

1.3.1 Hapten hypothesis

The hapten hypothesis posits that xenobiotics or their reactive metabolites induce an immune response by covalently binding to macromolecules, usually proteins. The hapten hypothesis is based on classic experiments by Landsteiner (23) that found a lack of immune response towards small molecules unless they were covalently bound to a protein.
According to the theory, the drug-modified proteins are viewed as foreign by the immune system. The drug-modified protein is taken up by antigen-presenting cells (APCs), hydrolyzed to peptide fragments, and the fragments are presented in the groove of the major histocompatibility complex (MHC) on antigen presenting cells to T cells. The recognition of the drug-modified protein fragment by T-cell receptors is regarded as signal one, which is needed to activate T-cells.

A good example consistent with the hapten hypothesis is the IgE-mediated IDR to penicillin, which is chemically reactive due to ring strain in its β-lactam ring. The ring strain can lead to attack by nitrogen or sulfur nucleophiles of proteins and modification of the proteins involved. There are antibodies directed against penicillin-modified proteins that are associated with penicillin allergies (24). When penicillin and antipenicillin antibodies are present, the antibodies can stimulate degranulation of mast cells, which release histamine, leukotrienes, and other inflammation-associated molecules. Anti-drug antibodies are also found in halothane and tienilic acid-induced hepatitis (6). IgE antibodies can mediate many allergic reactions such as anaphylaxis, but the relationship between pathogenicity and the presence of antidrug antibodies is clear only in the case of IgE-mediated immune reactions (6).

Another piece of evidence that supports the hapten hypothesis is that there are known reactive metabolites for most drugs that can cause IDRs that could act as haptens. However, not all drugs that can be metabolized to reactive species cause a significant incidence of IDRs, and it is not clear what determines if a reactive metabolite will cause an IDR or not (25-26). The relationship between the degree of covalent binding of a drug to a protein and the risk for developing an IDR is very vague. A better understanding of
the implications of covalent binding and review of the proteins that are typically affected is needed to provide more support for the hapten hypothesis.

1.3.2 Danger hypothesis

The danger hypothesis, proposed by Polly Matzinger in 1994, challenged the classical self-nonself hypothesis of immunology (27). It is known that in order to induce an immune response, costimulation of T-cells by activated APCs is needed, thus two signals are required for an immune response. Where covalent binding of a drug to a protein followed by presentation of the modified protein fragment on the MHC of an APC is considered signal one, in the context of the danger hypothesis, signal two is the costimulation of the T-cell by the APCs. The basis of the hypothesis is that, in general, foreign proteins do not generate an immune response unless a ‘danger signal’, which stimulates APCs, is present. The ‘danger signal’ is proposed to be a stress signal released from surrounding cells only when they are damaged. According to the danger hypothesis, the stress signal from damaged cells stimulates APCs that leads to upregulation of costimulatory molecules and ultimately allows the APCs to produce signal two that is necessary for the immune response. Without signal two, the result is tolerance. This also implies that the affected tissue is responsible for directing the nature of an immune response (28).

What are the implications of the danger hypothesis for IDR5? It is known that some reactive metabolites are able to cause cell damage, and that could generate a danger signal (29). This hypothesis could explain why some drugs that generate reactive
metabolites do not cause IDRs, because if the reactive species does not cause cell damage, no signal two would be generated and it would lead to tolerance.

If the danger hypothesis is correct, we should be able to identify molecules acting as danger signals, and according to Matzinger, those molecules should be endogenous and should be produced by damaged tissue. It is known that many drugs associated with a high incidence of IDRs cause oxidative stress. Therefore, it is likely that one type of danger signals involved in IDRs are molecules of the oxidative stress response, for example the Nrf2-mediated antioxidant signaling pathway (30). Other potential danger signals could be the stress-responsive intracellular molecules from damaged cells that are translocated from the nucleus to cytosol or extracellular space, such as high mobility group box 1 (HMGB1), heat shock proteins, and S100 proteins (30). A number of other endogenous proteins that fit the criteria of stress-association could also be potential danger signals.

1.3.4 Pharmacological interaction (p-i) hypothesis

The experiment that became the basis of the pharmacological interaction hypothesis proposed by Pichler found that T-cells isolated from patients with a history of an IDR proliferate in the presence of the drug that induced the IDR in the absence of drug metabolism (31). According to the pharmacological interaction hypothesis, many drugs can bind reversibly to the MHC-T-cell receptor complex and thus can stimulate an immune response, which in some cases can lead to an IDR (32).

There are examples where binding to MHC causes an allergic reaction. For example, metals such as nickel and beryllium are known both for their binding to MHC
and for the allergies associated with them. However, this binding is not typical irreversible covalent binding; it is none the less very strong. The reversible binding of most small molecules to protein is much weaker than the binding of metals, but there are several drugs that can stimulate T-cells via the T-cell receptor in their unmetabolized form (33). These drugs include lamotrigine, carbamazepine, sulfamethoxazole, mepivacaine, lidocaine, p-phenylenediamine, and radio-contrast media (33). Out of these drugs, sulfamethoxazole is the best characterized. Sulfamethoxazole is bioactivated to a hydroxylamine metabolite and then further to a reactive nitroso compound. However, when compared to the parent drug, it was found that the majority of the T-cell clones derived from patients that had an IDR towards sulfamethoxazole reacted to the parent drug (34). Even though the example of sulfamethoxazole supports the p-i hypothesis, and the isolated clones do respond better to the parent drug than to its metabolites, there is no good way of determining what form of the drug initiated the original immune response (6). Also, a recent study showed that lymphocytes derived from two patients with sulfamethoxazole allergy were positive to the nitroso metabolite in the lymphocyte transformation test and did not proliferate in response to the parent drug (35), thus suggesting that the immune response could be due to the nitroso metabolite and not the parent drug. This illustrates the problems associated with studying mechanistic aspects of IDR and indicates that mechanisms more complex than described by these hypotheses appear to exist.
1.4 Drug metabolism in ADRs and IDR s

As described above, with the exception of the p-i hypothesis, mechanistic hypotheses involve the concept of a drug or a metabolite of the drug binding to some endogenous protein as an important step in initiating an ADR. There are few examples of covalent binding of the parent drug but most xenobiotics require activation to a reactive species, and this usually occurs during the process of drug metabolism. That is why it is important to understand the mechanisms of drug metabolism and its implications for ADRs and IDR s.

Cytochromes P450 (P450s, or CYP) enzymes play a central role in the metabolism of drugs, other xenobiotics, and endogenous compounds. These enzymes are found throughout nature; many are involved in metabolism of sterols and vitamins A and D, while about 25% are generally considered to be involved in metabolism of xenobiotics (36). There are 57 different human P450s known to date and there is a great interindividual variation in the total amount of each (37). In fact, some people are completely devoid of a particular isoform, and this variability can have a strong effect on how a particular drug is handled in different people (38). This variability can also determine which individuals experience an ADR to a specific drug.

P450s oxidize drugs, carcinogens, and steroids and the reaction carried out by them can be summarized by the following equation: \( \text{NADPH} + \text{H}^+ + \text{RH} + \text{O}_2 \rightarrow \text{NADP}^+ + \text{H}_2\text{O} + \text{ROH} \) where R is the substrate and ROH is the product (37). All P450s are membrane-bound in mammals and most of them are found in the endoplasmic reticulum with the exception of five that are localized in mitochondria (37).
The P450s are the major contributors to metabolism of the 200 most commonly prescribed drugs in US (as of 2004) as they account for metabolism of approximately 75% of the drugs on that list (39). For the remaining drugs, roughly 10% are metabolized by uridine dinucleotide phosphatase (UDP) -glucuronosyltransferases, ~5% - by esterases and the rest are metabolized by flavin-containing monooxygenases, N-acetyltransferases and monoamine oxidases. CYP 3A4 is the major contributor to the P450-mediated metabolism as CYP 3A4 and 3A5 are responsible for metabolizing almost 50% of all P450-metabolized drugs (39) see Figure 1). CYP 2C9, 2D6 and 2C19 are next in significance and only a minor part of metabolism is carried out by CYP 2E1, 1A1 and 2B6.

As seen from the previous example, there are other enzymes apart from P450s that are capable of oxidizing drugs. For example, N- and S-oxidations can be carried out by flavin monooxygenase; oxidative deamination by monoamine oxidase and semicarbazide-sensitive amine oxidase (SSAO); dehydrogenation by monoamine oxidase; alcohol oxidation by alcohol dehydrogenase; aldehyde oxidation by aldehyde dehydrogenase; and C-oxidation by aldehyde oxidase and xanthine oxidase (40). Also, there is a separate group of enzymes that carry out conjugation reactions and further facilitate elimination of drugs. These enzymes include UDP-glucuronosyl transferases, esterases, and sulfotransferases.

The majority of changes to the structure of the drug make it more water-soluble and thus lead to attenuation of biological activity and ultimately to its excretion from the body. However, reactions catalyzed by P450 and other enzymes can lead to bioactivation as well. Bioactivation is the critical step in initiation of an immune response (41). The
hapten hypothesis provides the framework for investigation of the role reactive metabolites play in adverse reactions mediated by the immune system (23). According to the hapten hypothesis, a drug of a molecular weight less than 1000 Da needs to form a covalent bond to a protein in order to become immunogenic. The primary site for drug metabolism and bioactivation is the liver, but bioactivation can also occur in any other tissue capable of drug metabolism; for example, in keratinocytes in the skin. The main enzymes involved in bioactivation and the subsequent protein conjugation are the P450 enzymes such as CYP 1A1, CYP 1A2, CYP 1B1, CYP 2C9, CYP 2E1, CYP 2F1, and CYP 3A4 (41). Other enzymes located in white blood cells such as myeloperoxidase in macrophages, neutrophils and bone marrow can generate activated oxygen species that can activate drugs to radicals or electrophilic metabolites (10). In some cases conjugating enzymes such as sulfotransferases and glucuronosyltransferases that are normally involved in conjugation and detoxification of the drug, can produce chemically reactive intermediates (42).

A great number of chemicals can undergo bioactivation. The following articles present comprehensive lists of drugs (43-44), toxicants, and carcinogens that form reactive metabolites (45). A few examples of bioactivation by different enzymes include metabolism of the polycyclic hydrocarbons, benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene, by epoxide hydrolase (46), activation of the pesticide, ethylene dibromide, to an episulfonium ion by glutathione transferases (47), and the sulfate conjugation of aryl hydroxylamines and benzylic alcohols that generate a good leaving group and thus form reactive nitrenium and carbocations, respectively, following the loss of the sulfate (48). More examples are provided in Table 1.
We are interested in the covalent binding of reactive metabolites of drugs to protein because of its role in inducing immune-mediated adverse drug reactions. There are several basic principles that can describe the role of reactive species in toxicology. The basic reactions are either the reaction of a drug-derived electrophile with a nucleophile that results in covalent binding or the initiation of a free radical cascade (38). Covalent binding can serve as a surrogate marker of drug toxicity, but not all covalent binding leads to an immune response or an adverse drug reaction. For example, 3-hydroxyacetanilide, which does not cause liver toxicity in animals at equivalent doses, yields as much covalent binding as 4-hydroxyacetanilide, commonly known as acetaminophen and known for inducing liver toxicity (50-51).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Adverse reaction</th>
<th>Reactive metabolite</th>
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<tbody>
<tr>
<td>Benzo[a]pyrene</td>
<td>Carcinogenicity</td>
<td>Arene oxide</td>
</tr>
<tr>
<td>Paracetamol</td>
<td>Hepatotoxicity</td>
<td>Quinoneimine</td>
</tr>
<tr>
<td>Clozapine</td>
<td>Blood dyscrasias</td>
<td>Nitrenium ion</td>
</tr>
<tr>
<td>Phenytoin</td>
<td>Teratogenicity</td>
<td>Arene oxide</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>Cutaneous toxicity</td>
<td>Nitroso</td>
</tr>
<tr>
<td>Lamotrigine</td>
<td>Cutaneous toxicity</td>
<td>Arene oxide</td>
</tr>
<tr>
<td>Aminopyrine</td>
<td>Blood dyscrasias</td>
<td>Dication</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>Hepatotoxicity</td>
<td>Acyl glucuronide</td>
</tr>
</tbody>
</table>

**Table 1:** Examples of drugs that undergo bioactivation in humans and cause adverse reactions. Adapted from (49).
What is the relationship between drug metabolism/bioactivation and ADRs? This correlation is not absolute because many other events apart from covalent binding can occur (see figure 1) (38). A study conducted by Pfizer showed that out of 6 examined drugs that were withdrawn from the market, 5 (benoxaprofen, iproniazid, nefazodone, tienilic acid, and troglitazone) are known to have reactive products, and out of 15 drugs that received a black box warning, 8 (dacarbazine, dantrolene, felbamate, isoniazid,
ketoconazole, tolcapone and valproic acid) have reported reactive metabolites (26). That means that 62% of studied drugs are known to have reactive metabolites. In the same study, a number of drugs with known reactive metabolites were listed for warnings of hepatotoxicity (26). Those drugs are acetaminophen, carbamazepine, clozapine, diclofenac, disulfiram, halothane, leflunomide, methyldopa, rifampin, tacrine, tamoxifen, terbinafine, ticlopidine, and zileuton. In a system where reactive metabolites are produced, covalent binding may be a measure of reactivity of the products and can be used to estimate the potential toxicity of the drug. A different study assessed the relationship between the amount of covalent binding to liver proteins and hepatotoxicity in a number of drugs known to cause drug-induced liver injury (DILI) and drugs that do not cause DILI (52). The study was carried out in human liver microsomes and human hepatocytes, which include more metabolizing systems and thus better represent in vivo conditions. The study showed an overlap in the total amount of covalent binding between the drugs known to cause DILI and the ones that do not. However, when the results were corrected for the daily dose of each drug and thus better reflected the total liver exposure, there was a greater difference in covalent binding between the two groups of drugs, and the compounds that do not cause DILI had significantly lower values (52). These cases point towards the important role of reactive metabolites in inducing ADRs and IDRs but illustrate the complex relationship between formation of reactive metabolites and ADRs.
1.5 Sulfotransferases in metabolism and toxicity

Sulfotransferases are enzymes that catalyze the transfer of a sulfonate group from a donor molecule – usually 3’-phosphoadenosine 5’-phosphosulfate (PAPS) - to a great variety of hydroxyl and amine substrates in a process referred to as sulfonation or, more commonly, sulfation. There are two classes of sulfotransferases: cytosolic (53-54) and membrane-bound. The membrane-bound sulfotransferases play an important role in biological processes and sulfonate large biomolecules such as carbohydrates and proteins while the cytosolic sulfotransferases sulfonate small endogenous molecules such as hormones and bioamines as well as exogenous compounds such as various xenobiotic agents and they are also involved in drug metabolism (55).

While membrane-associated sulfotransferases are quite specific in the recognition of substrates, the cytosolic ones show a high degree of promiscuity (56). Examples of compounds sulfonated by human cytosolic sulfotransferases can be found in Table 2.

<table>
<thead>
<tr>
<th>SULT</th>
<th>Representative substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>2-naphthol</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
</tr>
<tr>
<td></td>
<td>tyramine</td>
</tr>
<tr>
<td></td>
<td>minoxidil</td>
</tr>
<tr>
<td>1A2</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
</tr>
<tr>
<td>1A3</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>tyramine</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
</tr>
<tr>
<td>1B1</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>T4</td>
</tr>
<tr>
<td></td>
<td>T3</td>
</tr>
<tr>
<td></td>
<td>rT3</td>
</tr>
<tr>
<td>1C1</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
</tr>
<tr>
<td></td>
<td>N-OH-2AAF</td>
</tr>
<tr>
<td>1C2</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td></td>
<td>dopamine</td>
</tr>
</tbody>
</table>
Table 2: Substrate specificity of human cytosolic sulfotransferases (adapted from (56)).

Sulfonation is generally considered to be a detoxification pathway because the products are more water-soluble and thus should be more easily eliminated from the body. In some cases, sulfonation can lead to activation of a pro-drug to its reactive form. That is the case with the hair growth stimulant minoxidil and the neuroendocrine peptide cholecystokinin because the sulfate conjugates are the active forms of these molecules (53).

For other compounds, sulfation can lead to bioactivation by enhancing the toxicity of a product. For example, sulfonation of N-hydroxy arylamines, N-hydroxy heterocyclic amines, and hydroxymethyl polycyclic aromatic hydrocarbons leads to very reactive electrophiles that are both carcinogenic and mutagenic (53). An increase in toxicity following sulfonation is not an uncommon scenario for benzylic and allylic alcohols and hydroxylamines (57). Sulfonation of these compounds leads to the formation of reactive species because the sulfate group is a good leaving group and can be cleaved off
heterolytically (42). The loss of HSO$_4^-$ from the product results in reactive carbocation or nitrenium ion intermediates that can covalently bind to proteins and DNA and lead to mutagenicity and carcinogenicity (42). The cleavage of the sulfate group is facilitated if the resulting cation can be resonance stabilized and that explains the formation of reactive species when the sulfate group is lost from sulfonated benzylic and allylic alcohols (Figure 2).

**Figure 2:** Resonance stabilization of cations formed by heterolytic cleavage from sulfonated benzylic alcohols and aromatic hydroxylamines (adapted from (42)).

Tamoxifen serves as a good example of bioactivation via sulfation. Tamoxifen is metabolized by P450 to hydroxylated metabolites that can undergo sulfation as a detoxification pathway. Two of metabolites of tamoxifen – 4-hydroxytamoxifen and α-hydroxytamoxifen are known to induce DNA adducts (58). The activation mechanism of 4-hydroxytamoxifen is through formation of a quinone methide (58). The α-hydroxytamoxifen is known to undergo sulfonation by rat liver hydroxysteroid sulfotransferases, which can be inhibited by sulfotransferase inhibitors (59). Although 4-hydroxytamoxifen can also undergo sulfation, this is a detoxification pathway (60),
unlike sulfation of α-hydroxy tamoxifen, which leads to DNA adduct formation. This example illustrates the site-dependency of the effect of the metabolic pathways and how sulfation can serve both as a road to detoxification and to bioactivation depending on the position of the sulfate group.

1.6 Nevirapine hypersensitivity

Nevirapine (NVP), marketed as Viramune, was approved by the US Food and Drug Administration in June 1996 for the treatment of human immunodeficiency virus (HIV) 1 infections (61). It is a nonnucleoside reverse transcriptase inhibitor that was the first in its class. Before NVP was marketed, the clinical trials showed that the drug could induce both liver toxicity and skin rashes (62). Initially the therapeutic dose was set at 400 mg/day, but that treatment schedule induced skin rashes in 32 – 48% of patients. When the dose recommendations were changed to 200 mg/day for the first two weeks followed by 400 mg/day, the incidence of skin rash decreased to 17%. A smaller number of patients (0.3% of all patients that develop a rash) develop severe rashes such as TEN or SJS (62). Now, more then ten years since NVP was marketed, the reported overall incidence of skin rash is decreased to 9%, but the incidence of liver toxicity appears greater than what was found in earlier studies and has increased from 1% to 3% (according to the nevirapine product insert).

There are a number of risk factors associated with development of the skin rash. Patients have the highest risk of developing the skin rash during the first six weeks of treatment, and most rashes develop during weeks 1 to 3 (62). The risk factors for developing the skin rash include female sex, ethnic background (skin rash is more
prevailing in Chinese), patients with a high CD4+ T cell count, and antihistamine and corticosteroid co-treatment (63). The therapeutic dose of NVP (400 mg/kg) appears to be a risk factor as well. This is supported by the findings that a lower lead-in dose during the first two weeks of treatment significantly decreases the incidence of skin rash. Therefore, patients who are naïve to NVP are started on a daily dose of 100 mg during the first week of treatment, and the dose is increased weekly until the therapeutic dose of 400 mg/day is reached (64).

The NVP-induced skin rash is an idiosyncratic reaction because it is unpredictable and does not occur in most patients at the therapeutic dose. IDRs can be very complex and the mechanism may differ from one drug to another and from one patient to another (6). This has made studying the mechanistic basis of these reactions extremely difficult. IDRs are likely to be immune-mediated, and there is circumstantial evidence that reactive metabolites are involved (6). The controversy in the case of nevirapine lies in the apparent lack of correlation between blood levels of NVP and its metabolites and the incidence of skin rash (65).

The ideal way to study the mechanisms of NVP-induced skin rash would be in patients previously sensitized with NVP; however, this approach in unrealistic as it is both unethical and potentially life-threatening. Therefore, the best way to study such complex reactions is by utilizing animal models (66). This lab has previously established an animal model of NVP-induced skin rash, which allows us to investigate the sequence of events that lead to induction of the skin rash in vivo and allows us to study the metabolic and immune-mediated mechanisms of the rash (67).
1.7 Animal model of nevirapine-induced skin rash

We have previously developed an animal model of NVP-induced skin rash in Brown Norway rats (67). This animal model has proven to be a good tool for studying the skin rash because the mechanism of the rash appears to be shared between patients and female Brown Norway rats, which is evident from the similarity of the characteristics of the rash.

It was shown that 100% of female Brown Norway rats develop red ears by day 7 and skin rash by day 21 of treatment with NVP when administered in food at a dose of 150 mg/kg/day, while only 20% of female Sprague-Dawley rats develop the rash by week three or later (67). The timing in Brown Norway rats is consistent with that of patients who run the highest risk of developing a rash during the first six weeks of treatment, and with the highest frequency during weeks one to three (62). There is also some similarity in the severity of the rash. In patients, rashes of different severity occur ranging from mild erythematous rashes to SJS and TEN, which are characterized by blistering skin eruptions (62). In rats, the rash starts with mild lesions that can progress over time to a more severe phenotype (67). Female patients are at higher risk of developing a rash, and the rash is often more severe compared to males (68). The incidence of the rash is also higher in female Brown Norway rats (100%) compared to the males (25%) (67). There is an infiltration of perivascular lymphocytic cells in the dermis in skin lesions of patients who developed the rash and mononuclear cells are found in dermis and epidermis of patients with SJS and TEN (69). In rats, a mononuclear infiltrate was observed in the dermis, and occasional apoptotic keratinocytes were found in epidermis, which is similar to the findings in SJS patients and patients with severe lesions (67). The sensitivity can be
transferred from sensitized to naïve rats with transplanted spleen cells, indicating a strong immune component (70). T cells have been observed in the skin of both patients and rats with nevirapine-induced skin rash, and a low CD4+ T-cell count decreases the risk of rash in humans while depletion of CD4+ T-cells in rats delays the onset of the rash in female Brown Norway rats (70). On the other hand, depletion of CD8+ cells appears to intensify the rash in rats (70). As most drug-induced skin rashes are hypothesized to be immune-mediated, finding CD4+ T-cells in both patients and in the animal model is supportive of this hypothesis. There is some dose-dependency shared between humans and our animal model. As discussed above, when patients received nevirapine at a dose of 400 mg/day, 32 – 48% develop a skin rash (17) while the incidence decreased to 17% when a lead-in dose of 200 mg/day for the first two weeks of treatment was introduced (62). All female Brown Norway rats develop a skin rash when administered nevirapine at a dose of 150 mg/kg/day (the drug was given in food), but no rat developed a rash at a dose of 75 mg/kg/day (67). Escalated dosing has proven to be protective against the rash in both humans and rats. The rash can be prevented in female Brown Norway rats when they are given a dose of nevirapine of 40 or 75 mg/kg/day for the first two weeks of treatment, followed by 150 mg/kg/day – the dose that induces a 100% incidence of the rash in rats given the high dose from the beginning (70). On primary challenge, female Brown Norway rats develop red ears around day 7 and a skin rash by day 14-21 of the treatment (67). When the rats that developed a rash on primary challenge were rechallenged with drug after a month and up to a year after the original treatment, the rats developed red ears within 24 hours of the dose and skin rash by day four to day seven of treatment (67). In rats, the rash is also accompanied by general malaise that is not seen on primary
exposure, and a severe systemic reaction can be induced by a dose as low as 5 mg/kg/day (71). Although no controlled studies on rechallenge have been conducted in patients that developed severe nevirapine-induced skin rash, there is evidence of accelerated onset and greater severity of the rash in patients exposed to nevirapine again. The rapid onset of skin rash on rechallenge in both humans and rats supports the involvement of the immune system in the progression of the rash. With all the similarities in the characteristics of nevirapine-induced skin rash between humans and Brown Norway rats, it is reasonable to conclude that this model provides a unique opportunity for dissecting the mechanisms of an idiosyncratic drug reaction.

1.8 Metabolic pathways and their significance for skin rash

A fundamental question in the study of IDRs is whether the parent drug or a reactive metabolite is responsible for the reaction. The major metabolic pathways in both species include 2-, 3- and 12-hydroxylation (72-73). There are many potential reactive metabolites of NVP (see Figure 3). Previous studies in our group were focused on finding whether the parent drug or one of its metabolites is responsible for the rash (71), and it was determined that the 12-hydroxy nevirapine (12-OH NVP) pathway is responsible for inducing the skin rash.
Figure 3: Biotransformation pathways and possible reactive metabolites of NVP.

In humans, nevirapine is primarily cleared through oxidation by P450 enzymes followed by conjugation with either glucuronide to water-soluble conjugates, or by further oxidation to a carboxylic acid (72). Studies of the biotransformation of NVP by human liver microsomes showed that CYP 2B6 is mainly responsible for oxidation to 3-hydroxynevirapine (3-OH NVP), CYP 2D6 mediates oxidation to 8- and 12-hydroxynevirapine (8- and 12-OH NVP), CYP 3A4 to 2- and 12-hydroxynevirapine (2- and 12-OH NVP) while CYP 3A5 is a minor contributor in the oxidation to 2- and 12-OH NVP (74). After oral administration of NVP, most of the drug is excreted as 2-, 3- or 12-OH NVP glucuronide conjugates, each accounting for about 20% of administered dose while the parent drug and non-glucuronidated metabolites account for about 2.5% each.
of the total recovered dose (72). The peak plasma level of NVP is $3.5 \mu g/ml$ and $0.25 \mu g/ml$ for 12-OH NVP (72).

Our lab has shown that when female Brown Norway rats received a dose of 150 mg/kg/day in food, the trough plasma levels of nevirapine are $40 \mu g/ml$, which is associated with a 100% incidence of skin rash (71). There is a dose-dependence for the induction of skin rash - the plasma concentration of nevirapine in male Brown Norway rats is less then $5 \mu g/ml$ at the same dose of the drug and the incidence of skin rash in those animals was 25% (71). Male Brown Norway rats metabolize nevirapine faster leading to lower plasma levels of the parent drug and higher excretion of 3-OH and 4-COOH NVP (71).

A number of studies focused on determining the metabolic pathway leading to the skin rash. During these studies it was discovered that inhibition of nevirapine metabolism by 1-aminobenzotriazole did not decrease the formation of 12-OH NVP and led to an increased incidence of rash (71). This appears to be because 1-aminobenzotriazole also inhibits further oxidation of 12-OH NVP by P450 because it markedly decreases the formation of the downstream carboxylic acid. Inhibition of alcohol dehydrogenase did not decrease the formation of the carboxylic acid. It was proven that the 12-hydroxylation pathway is responsible for the rash by replacing the methyl hydrogens with deuterium, which inhibited the formation of 12-OH NVP and decreased the incidence of rash, and also by inducing the skin rash by treating animals with a dose of 12-OH NVP that is lower than the dose of NVP needed to cause the rash (75 mg/kg/day for 12-OH NVP versus 150 mg/kg/day for NVP). Surprisingly, the blood levels of the parent drug when the deuterated analog was administered were much lower than when nevirapine
was administered (71). We propose that this is because the free radical intermediate partitions between oxygen rebound to form 12-OH NVP and loss of another hydrogen atom to form a reactive quinone methide (Figure 4). The quinone methide inhibits P450, and because there is less P450 inhibition with the deuterated analog, it is metabolized more rapidly.

**Figure 4:** Proposed mechanism for the formation of the reactive quinone methide that inhibits P450 and proposed mechanism for protein binding of the 12-sulfoxy NVP.

In female Brown Norway rats, the incidence of the rash appears to be correlated with plasma levels of nevirapine and 12-OH NVP and this observation supports the importance of the 12-hydroxylation pathway for the induction of the skin rash. A similar relationship between the plasma levels of the parent drug and its metabolites and the incidence of skin rash and liver toxicity was assessed in a clinical study that involved
patients receiving nevirapine (65). Plasma levels of NVP and the metabolites were measured and compared in case-control pairs. Among the 22% of patients who developed skin rashes there were no significant differences in plasma levels of NVP or 12-OH NVP or its proportion out of all metabolites measured in plasma and the rashes appeared to be idiosyncratic.

1.9 What do we not know about nevirapine-induced skin rash? – rationale for studies

Even though the metabolic pathway leading to a skin rash has been identified, the mechanism of induction of skin rash by 12-OH NVP is still unknown. The chemical structure of 12-OH NVP does not allow it to spontaneously rearrange into the reactive quinone methide. Therefore, further bioactivation is needed if the ultimate cause of the rash is a reactive metabolite.

The metabolism of NVP by the liver is well-described, at least for humans (72). There is little doubt that liver is the major site of biotransformation and bioactivation of this drug, and there is a significant incidence of hepatotoxicity observed in patients. However, nevirapine also causes a skin rash. According to Matzinger’s danger hypothesis, the affected organ determines the immune response. There is enough evidence for the immunological component in the NVP-induced skin rash. Therefore, if the danger hypothesis is correct, the trigger signal for the skin rash must be coming from the skin. We propose that the 12-OH NVP travels to skin and undergoes subsequent activation by sulfotransferases to form the 12-sulfoxy NVP. We hypothesize that 12-sulfoxy NVP can react with nucleophiles in the skin through an $S_N2$ reaction mechanism.
leading to elimination of $\text{HSO}_4^-$ and covalent binding of the remaining structure to the proteins in the skin, thus inducing an immunological response.

The skin is the largest organ of a vertebrate and it possesses several metabolizing enzymes (75). The levels of most of the phase I and II enzymes in human skin is low, but sulfotransferase activity is high (76). A number of P450s found in the liver are also present in the skin; however, there are some differences because not all hepatic P450s are expressed in the skin and there is also the addition of skin-specific P450s (Table 3). Human keratinocytes also express an array of transporters allowing the uptake of drugs (75).

<table>
<thead>
<tr>
<th>CYP isozyme</th>
<th>Liver cocktail (pmol)</th>
<th>Skin cocktail (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A1</td>
<td>-</td>
<td>3.6</td>
</tr>
<tr>
<td>1A2</td>
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<td>-</td>
</tr>
<tr>
<td>1B1</td>
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<tr>
<td>2D6</td>
<td>3</td>
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<td>2E1</td>
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</tr>
<tr>
<td>3A5</td>
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</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>104</strong></td>
<td><strong>22</strong></td>
</tr>
</tbody>
</table>

**Table 3:** Comparison of cytochrome P450 isozymes present in the liver versus skin P450 cocktail (adapted from (77))

Examples of drugs known to undergo sulfation in human skin include triclosan and minoxidil - a hair growth promoter that is sulfated in the skin to the active compound, minoxidil sulfate, by at least four different sulfotransferase isoforms. Rat skin also contains sulfotransferases (76), and it is also known to metabolize minoxidil to its sulfated form. Rat skin is believed to contain at least one form of phenol sulfotransferase,
also termed SULT1A1 (76). Therefore, it is reasonable to assume that some aspects of xenobiotic metabolism and bioactivation can occur in the skin of Brown Norway rats.

The present studies focused on determining the role of 12-sulfoxyl NVP in the nevirapine-induced skin rash. The goal was to manipulate the circulating levels of 12-sulfoxyl NVP by co-administering NVP or 12-OH NVP with sulfation modulators. Salicylamide and dehydroepiandrosterone (DHEA) were used in this work. Salicylamide is a compound known for having sulfation as a major route of biotransformation, and it is known that a single dose of 2 mmol/kg of salicylamide depletes PAPS in rats (78). DHEA is a specific substrate for SULT 2A which has higher expression in female rats. DHEA also functions as a competitive inhibitor of SULT 2A (6, 79). In this study the hypothesis was tested by co-administering NVP with either salicylamide or DHEA and measuring the circulating levels and excretion of NVP, 12-OH NVP, 4-COOH NVP, and 12-sulfoxyl NVP. It was then determined if levels of metabolites, especially 12-sulfoxyl NVP, correlated with the incidence of the skin rash.

In the scope of this project, further oxidation of NVP and 12-OH NVP to 4-COOH NVP was also studied using in vitro incubations of NVP and 12-OH NVP with rat liver microsomes. The purpose of these studies was to determine if an aldehyde intermediate, which might be involved in mediating the skin rash, could be detected.

1.10 Hypothesis

1) Sulfation of 12-OH NVP in the skin leads to the formation of 12-sulfoxyl NVP, which can undergo an S_{N}2 reaction with nucleophiles in the skin leading to elimination of the sulfate group and covalent binding of the remaining molecule
to skin proteins. This, in turn, activates the immune system and causes the skin rash. Inhibition of the sulfation of 12-OH NVP in skin should decrease the incidence of the skin rash.

2) P450 is responsible for oxidation of 12-OH NVP to 4-COOH NVP.
Section 2: Materials and Methods

2.1 Synthesis of 12-OH NVP

The synthesis of 12-OH NVP followed the method described in (71) with some modifications. To a flame-dried round bottomed flask equipped with a magnetic stirrer 6.1 g of NVP, oven-dried at 60°C overnight, was added. The flask was sealed and equipped with a nitrogen balloon. Anhydrous tetrahydrofuran was added, the solution was cooled to -78°C and 140 mmol lithium diisopropylamide (2M solution in tetrahydrofuran/heptane/ethylbenzene) was added over a period of 5 minutes. The solution was kept at -78°C for 2 hours with stirring. Then the reaction mixture was allowed to warm to -40°C and anhydrous oxygen was bubbled through the solution over 4 hours while the temperature was maintained at -40 to -20°C. The now clear solution was acidified with 2N hydrochloric acid over ice and the organic layer was extracted with 3 x 30 ml of 2N hydrochloric acid. The combined aqueous layers were brought to pH 8 using sodium carbonate and extracted with 4x100 ml of CH₂Cl₂. The combined organic layers were washed with brine and water, dried over anhydrous MgSO₄ and evaporated in vacuo to yield solid product. The crude product was purified using open column chromatography with silica gel (Sigma-Aldrich, pore size 60 Å, 70 – 230 mesh, column dimensions 40 x 400 mm). The solvent system used was hexanes:ethyl acetate in proportions 60:40 and increased to 100% ethyl acetate to yield fluffy pale yellow powder in 20% yield. The purity of the obtained product was analyzed using mass spectrometry, ESI-MS; mz (%) 283 MH⁺, (99%). ¹H NMR (CDCl₃) δ 0.4 – 0.6 (m, 2H), 0.92 – 1.08 (m, 2H), 3.78 – 3.80 (m, 1H), 4.67 (dd, J = 0.8, 0.8 Hz, 1H), 4.95 (m, 1H), 6.96 (d, J = 8 Hz,
1H), 7.07 (m, 1H), 8.14 (dd, J = 4, 2 Hz, 1H), 8.25 (d, J = 6 Hz, 1H), 8.54 (dd, J = 4, 4 Hz, 1H), 8.73 (bs, J = b Hz, 1H).

2.2 Synthesis of 12-sulfoxy NVP

Synthesis of the sulfate of 12-OH NVP was performed according to the method described in (71). The final product was purified with open column chromatography with silica gel (Sigma-Aldrich, pore size 60 Å, 70 – 230 mesh, column dimensions 10 x 70 mm) using 80% chloroform and 20% methanol. The purity of the product was analyzed using mass spectroscopy, ESI-MS; mz (%) 361 (M-H), (99%).

2.3 Animal care

Female Brown Norway rats were obtained from Charles River (Montreal, QC) and acclimatized for 1 week before being put in studies. During acclimatization and the experiments, the animals were housed in 12h:12h light:dark cycles at 22°C and received standard rodent chow and water. All animal studies were conducted in accordance with the guidelines of the Canadian Council on Animal Care.

2.4 Experimental design

When rats were treated with NVP or 12-OH NVP, the dose was scaled up to the final dose over 3 – 5 days to avoid toxic effects of high blood concentrations of NVP or 12-OH NVP. All substances apart from 1-aminobenzotriazole were suspended in 0.5% methyl cellulose in water and administered by oral gavage or by subcutaneous injections. 1-Aminobenzotriazole was dissolved in water and administered by oral gavage. The
blood samples for metabolite analysis were taken 4 hours after drug administration 2-3 times a week. The urine was collected over 24 hour periods 2-3 times a week using metabolic cages. Drug treatment was continued until the animals developed a skin rash or up to a maximum of 30 days.

2.4.1 Treatment with NVP, salicylamide, and DHEA

Female Brown Norway rats were treated with NVP together with either salicylamide or DHEA in order to study the effect of those sulfation inhibitors on the incidence of skin rash. These experiments were repeated several times to confirm the results. All drugs were ground to obtain particles that are easier to disperse, suspended in 0.5% methyl cellulose and administered by gavage with minimal time between NVP and the inhibitor. During one experiment, NVP and the inhibitors were given four hours apart, but it did not affect the outcome of the experiment and this practice was abolished. The dose of NVP was escalated during the first four to five days of the study from 50 mg/kg/day to the full dose of 100 mg/kg/day. The lower dose was given in the beginning of the study to avoid central nervous system toxicity associated with high peak plasma levels of NVP. DHEA was given at doses of 50 or 100 mg/kg/day. Salicylamide was given at a dose of 274 mg/kg/day (2 mmol/kg/day).

2.4.2 Treatment with NVP, 1-aminobenzotriazole, and DHEA

NVP was suspended in 0.5% methyl cellulose and administered by daily subcutaneous injections at a dose of 30 mg/kg/day for the first two days followed by 60 mg/kg/day for the duration of the study. 1-Aminobenzotriazole was dissolved in water at
a concentration of 25 mg/ml and administered by gavage at a dose of 50 mg/kg/day. DHEA was suspended in 0.5% methyl cellulose and administered by gavage at a dose of 50 mg/kg/day.

2.4.3 Treatment with 12-OH NVP and DHEA

12-OH NVP was administered at a dose of 100 or 150 mg/mg/day by oral gavage. DHEA was administered at doses of 25, 50 and 100 mg/kg/day by oral gavage. All drugs were suspended in 0.5% methyl cellulose and given with minimal time between administrations.

2.5 Quantification of NVP and its metabolites

Plasma concentrations and 24-hour urinary excretion were measured for NVP, 12-OH NVP, 4-COOH NVP, and 12-sulfoxyl NVP.

2.5.1 Quantification of NVP, 12-OH NVP, and 4-COOH NVP in plasma

Plasma (50 µl) was mixed with internal standard (ethyl-NVP, 5.4 µg/ml, 50 µl) and placed on a Strata® solid phase extraction column (C18-E, 100 mg, by Phenomenex) after preparing the column with methanol and water. The column was washed with 1 ml of water and the metabolites eluted with 1 ml of methanol. The methanol was collected, dried, and re-constituted with 50 µl mobile phase (16% acetonitrile and 84% water with 2 mM ammonium acetate and 1% acetic acid). The samples were separated on HPLC and analyzed by mass spectrometry. The separation was carried out on an Ultracarb C18 30 X 2.0 mm, 5 µm column (Phenomenex) under isocratic conditions with a mobile phase
consisting of 16% acetonitrile and 84% water with 2 mM ammonium acetate and 1% acetic acid and the flow rate of 0.2 ml/min.

2.5.2 **Quantification of 12-sulfoxy NVP in plasma**

Plasma (50 µl) was mixed with internal standard (naproxen, 2.5 µg/ml 50 µl) and concentrated on a solid phase extraction column as per above. The reconstituted samples were separated on HPLC and analyzed by mass spectrometry. The separation was carried out on an Ultracarb C18 100 X 2.0 mm, 5 µm, column with a gradient elution 20 → 80% acetonitrile and the second solvent was water with 2 mM ammonium acetate and 1% acetic acid. The flow rate was 0.2 ml/min.

2.5.3 **Quantification of NVP, 12-OH NVP, and 4-COOH NVP in urine**

For quantification of NVP, 12-OH NVP, and 4-COOH NVP in urine, 50 µl from a 24-hour sample was mixed with 100 µl internal standard (ethyl-NVP, 27 µg/ml in the mobile phase) and 10 µl of β-glucuronidase (approximately 10 000 U/ml in 100 mM KH₂PO₄ buffer) and incubated overnight at 37°C prior to concentrating on an Strata® solid phase extraction column as per above The samples were separated using the same HPLC conditions as described for plasma samples above.

2.5.4 **Quantification of 12-sulfoxy NVP in urine**

For quantification of 12-sulfoxy NVP, the internal standard was naproxen (250 µg/ml in water) and no pre-incubation with β-glucuronidase was performed. The samples were concentrated on a solid phase extraction column as per above in the presence of the
internal standard (naproxen, 250 µg/ml) and reconstituted in 50 µl mobile phase prior to analysis. The samples were separated by HPLC using same conditions as described for plasma samples above.

2.6 Mass spectrometry

Mass spectrometry was carried out using a PE Sciex API 3000 quadrupole system with an electrospray ionizing source. The ion pairs used for the analysis were: 267.0/226.1 for NVP, 283.1/161.0 for 2-OH NVP, 283.1/214.0 for 3-OH NVP, 283.1/223.1 for 12-OH NVP, 297.1/210.1 for 4-COOH NVP, 255.1/227.2 for ethyl-NVP (positive ionization mode), 361.0/96.0 for 12-sulfoxy NVP and 229.0/169.8 for naproxen (negative ionization mode). Standard curves prepared for 2-OH NVP (0.43 – 102.9 µg/ml), 3-OH NVP (0.36 – 86.8 µg/ml), 12-OH NVP (0.38 – 91.0 µg/ml), 4-COOH NVP (0.26 – 61.8 µg/ml), 12-sulfoxy NVP (0.28 – 14.0 µg/ml) and NVP (0.74 – 176.9 µg/ml) had $R^2$ values of $>0.99$.

2.7 Preparation of rat liver microsomes

Freshly isolated liver from male and female Brown Norway rats was used for preparing liver microsomes through homogenization using the Ultra-Turrax T25 homogenizer (Janke & Kunkel, IKA®-Labortechnik) and centrifugation of the homogenate at 9000 x g at 4°C. The supernatant was re-centrifuged at 100,000 x g at 4°C yielding the microsomes in the pellet that were re-suspended in glycerol-phosphate buffer containing 20% glycerol and 0.4% KCl in 50 mM KH$_2$PO$_4$ buffer pH7.4, homogenized
manually and stored at -78°C before use. Protein concentration was measured with a BCA Protein Assay Kit (Novagen).

2.8 Incubation with rat liver microsomes

Liver microsomes from female Brown Norway rats, 50 µl (0.900 mg protein) and from male Brown Norway rats, 50 µl (1.430 mg protein) were incubated with NVP (30 µg/ml) and 12-OH NVP (100 µg/ml) in a presence of a NAPDH-regenerating system in 100 mM phosphate buffer. Total incubation volume was 250 µl. To start the reaction, glucose-6-phosphate dehydrogenase was added to a final concentration of 0.4 U/ml to each tube except controls, and 250 µl ice-cold acetonitrile was used to quench the reaction. Internal standard (ethyl-NVP, 5.4 µg/ml, 50 µl) was added to each tube, the contents were centrifuged, separated by solid phase extraction as per above, evaporated in vacuo at 50°C, and re-constituted to 50 µl prior to analysis. The samples were analyzed using HPLC-MS/MS as per above.
Section 3: Results

3.1 Intravenous administration of 12-sulfoxy NVP

As a first step in studying the effects of sulfation inhibitors on the incidence of skin rash, a preliminary experiment was carried out to investigate the kinetics and determine the limits of detection of 12-sulfoxy NVP in vivo. The goals of the experiment were to observe how well 12-sulfoxy NVP can be detected in plasma, to roughly assess the elimination rate, and to investigate if the metabolite is stable or if it undergoes hydrolysis in plasma to form 12-OH NVP. 12-sulfoxy NVP was given as an intravenous injection in the tail vein of one female Brown Norway rat at a dose of 100 mg/kg. Following administration, plasma samples were collected for 24 hours and the concentrations of 12-OH NVP and NVP were measured. The samples were also analyzed for the presence of 12-sulfoxy NVP, but due to lack of a quantification method for this metabolite at the time the experiment was performed, only the peak area was measured and reported. It was found that 12-sulfoxy NVP is hydrolyzed rapidly to 12-OH NVP (Figure 5A). Thirty minutes after the administration, the concentration of the metabolite had decreased to a little more than half of the initial concentration; the metabolite could be detected in plasma for six hours after the injection (Figure 5B).
Figure 5: A female Brown Norway rat was injected intravenously with 12-sulfoxy NVP (100 mg/kg). The presence of NVP, 12-OH NVP, 4-COOH NVP (A) and 12-sulfoxy NVP (B) were monitored over 24 hours. Concentrations of NVP, 12-OH NVP, and 4-COOH NVP were measured in plasma and disappearance of 12-sulfoxy NVP is reported as area of the peak in the mass spectrometry spectra.
3.2 Treatment of female Brown Norway rats with NVP + salicylamide and NVP + DHEA

After the proper conditions for measuring 12-sulfoxy NVP in plasma and urine samples were determined, experiments on manipulating the sulfation pathway were begun. In the first set of experiments, female Brown Norway rats were treated with NVP 100 mg/kg/day, NVP 100 mg/kg/day + salicylamide 274 mg/kg/day (2 mmol/kg/day) or NVP 100 mg/kg/day + DHEA 50 or 100 mg/kg/day. Plasma samples were collected weekly and processed for the concentrations of NVP and its metabolites. All rats treated with NVP only or with NVP + salicylamide developed a skin rash, but none of those co-treated with DHEA did (Figure 6A).

The results showed that co-administration of DHEA decreases plasma concentrations of NVP, 12-OH NVP, and 12-sulfoxy NVP (figure 6). The results of salicylamide co-treatment were the opposite. The two rats receiving this co-treatment developed skin rash and had markedly decreased plasma concentrations of 12-sulfoxy NVP throughout the study. The rats also had decreased concentrations of NVP and 12-OH NVP at day 15 of the study. This time point is likely an outlier, but the experiment was repeated in order to increase confidence in the results and to verify the potential effect of salicylamide on the plasma levels of nevirapine.

To make sure that observed differences in blood concentrations of NVP and 12-OH NVP were not due to interference caused by simultaneous administration of NVP and inhibitors, the inhibitors were administered 4 hours after NVP during the above study. This strategy was abolished in the experiments that followed because this did not change the outcome or the plasma concentrations of NVP and metabolites. The results from this
experiment also showed that DHEA can prevent the skin rash and had the same effect on plasma concentrations and urinary excretions of nevirapine and metabolites at both higher (100 mg/kg/day) and lower (50 mg/kg/day) doses. Therefore, the lower DHEA dose of 50 mg/kg/day was used for some of the experiments described below.
Figure 6: Incidence of skin rash (A) and plasma concentrations of NVP (B), 12-OH NVP (C) and 12-sulfoxy NVP (D) in female Brown Norway rats treated with NVP only (100
mg/kg/day, n = 4), in combination with DHEA (50 and 100 mg/kg/day, n = 4) and in combination with salicylamide (274 mg/kg/day, n = 2). NVP and inhibitors were administered four hours apart.

The above experiment was repeated with a higher n. Female Brown Norway rats were treated with NVP 100 mg/kg/day, NVP 100 mg/kg/day + salicylamide 274 mg/kg/day (2 mmol/kg/day) or NVP 100 mg/kg/day + DHEA 100 mg/kg/day. All rats treated with NVP only or with NVP + salicylamide developed a skin rash but none of those treated co-treated with DHEA did (Figure 7A).

The blood levels of NVP were 20 - 40 µg/ml in NVP treated rats, ~ 15 µg/ml for NVP + salicylamide-treated rats and decreased to < 10 µg/ml for the DHEA co-treated rats (Figure 7). The same trend was found for 12-OH NVP plasma levels. NVP and 12-OH NVP plasma concentrations were significantly decreased in rats receiving NVP + DHEA (0 – 7 µg/ml). The plasma levels of 4-COOH NVP fell below the minimum level of quantification for all treatment groups. Plasma concentrations of 12-sulfoxy NVP were calculated to 1 – 7 µg/ml for rats receiving NVP only and were below the limit of quantification for salicylamide cotreated animals and below 1 µg/ml for DHEA cotreatment group. The treatment was repeated several times. Out of all rats treated in different experiments only one rat receiving NVP 100 mg/kg/day + DHEA 100 mg/kg/day got a mild skin rash.
Figure 7: Incidence of skin rash (A) and plasma concentrations of NVP (B), 12-OH NVP (C) and 12-sulfoxy NVP (D) in female Brown Norway rats treated with NVP (100
mg/kg/day) \((n = 4)\), NVP + DHEA \((100 \text{ mg/kg/day}) \ (n = 3)\) and NVP + salicylamide \((274 \text{ mg/kg/day}) \ (n = 3)\).

**Figure 8:** Urinary excretion of 12-OH NVP (A), 4-COOH NVP (B), 12-sulfoxy NVP (C), 2-OH NVP (D) and 3-OH NVP (E) in female Brown Norway rats \((\text{NVP} \ n = 4,\)
NVP + inhibitors \( n = 3 \) treated with NVP 100 mg/kg/day, NVP + salicylamide 100 mg/kg/day and 274 mg/kg/day, respectively, and NVP + DHEA 100 mg/kg/day each.

The urinary excretion of all 12-OH, 2-OH, 3-OH and 4-COOH was comparable for NVP and NVP + salicylamide treated rats (Figure 8). The animals receiving DHEA had an increased urinary excretion of 4-COOH NVP compared to the other groups, but excreted less of all other metabolites, except for 3-OH NVP, where no difference in excretion was observed (Figure 8). The urinary excretion of 12-sulfoxy NVP for NVP + DHEA and NVP + salicylamide treatment groups was decreased to ~ 200 \( \mu \text{g}/24 \) hours compared to ~ 600 \( \mu \text{g}/24 \) for NVP only treated group.

To gain confidence in the results of NVP + salicylamide co-treatment, the experiment was repeated. All the animals in both treatment groups developed a skin rash (Figure 9A). The plasma levels of NVP and 12-OH NVP were ~ 75 and 40 \( \mu \text{g}/\text{ml} \) respectively and did not differ between the two groups. Plasma concentrations of 12-sulfoxy NVP fell below the limit of quantification for the mass spectrometer and thus are not reported.
Figure 9: Incidence of skin rash (A) and plasma concentrations of NVP (B) and 12-OH NVP (C) for female Brown Norway rats treated with NVP 100 mg/kg/day or NVP + salicylamide 274 mg/kg/day (2 mmol/kg/day) (n = 4 each group).
In this experiment, no difference was observed in the urinary excretion of all the metabolites, except for 12-sulfoxy NVP. The excretion during 24 hours was 2000 – 4000 µg for 12-OH NVP and 2-OH NVP for both groups and ~ 2000 µg for 3-OH NVP (Figure 10). Rats treated with NVP excreted 2000 – 4000 µg 12-sulfoxy NVP per 24 hours, but the salicylamide co-treated animals excreted < 1000 µg/24 hours (figure 10).

Figure 10: Urinary excretion of 12-OH NVP (A), 4-COOH NVP (B), 12-sulfoxy NVP
(C), 2-OH NVP (D) and 3-OH NVP (E) for female Brown Norway rats treated with NVP 100 mg/kg/day or NVP + salicylamide 274 mg/kg/day (2 mmol/kg/day).

3.3 Treatment of female Brown Norway rats with NVP, 1-aminobenzotriazole, and DHEA

It was noticed from previous studies that when NVP is administered together with DHEA, the incidence of skin rash decreases drastically and the plasma concentrations and urinary excretion of 12-sulfoxy NVP was also lower compared to rats treated with NVP only. However, all rats receiving DHEA also have lower concentrations of the parent drug and of 12-OH NVP, the precursor of the 12-sulfoxy NVP. In an attempt to minimize the differences in plasma concentrations of the parent drug, NVP and DHEA were dosed together with 1-aminobenzotriazole, an inhibitor of CYP 450 that is oxidized to benzine which inhibits P450 in a non-specific manner (80). The route of administration of NVP was changed from gavage to subcutaneous injections, and the dose was decreased to 60 mg/kg/day in order to avoid the neurotoxicity associated with high peak plasma levels that can occur in the presence of a P450 inhibitor. The chosen dose regimen for NVP and 1-aminobenzotriazole was previously shown to induce the skin rash with a 100% incidence in this animal model (71).

All rats receiving NVP and 1-aminobenzotriazole and none of the rats co-treated with DHEA developed the skin rash (Figure 11A). The rash was milder during the course of this experiment compared to experiments where no 1-aminobenzotriazole was administered; however, the animals developed deep skin lesions after the drugs were discontinued. This delayed reaction is likely due to continued leaking of NVP into the
bloodstream from the reservoirs in the skin that formed during the subcutaneous treatment.

The strategy to make the levels of NVP the same with and without DHEA co-treatment by adding 1-aminobenzotriazole to block induction or prevention of inhibition by NVP was not successful. 1-Aminobenzotriazole is a mechanism-based inhibitor of P450, however, this compound can also have induction effects on P450, as it was shown that 1-aminobenzotriazole up-regulates mRNA expression of CYP 2B6 and CYP 3A5 (81). In this study, the plasma concentrations of the parent drug were still lower compared to those of rats receiving NVP and 1-aminobenzotriazole only – approximately 30 µg/ml for the NVP + 1-aminobenzotriazole + DHEA group compared to ~ 75 µg/ml for NVP + 1-aminobenzotriazole group. However, the plasma concentrations of 12-OH NVP were comparable at 2 – 4 µg/ml for the two groups (Figure 11) although it appeared to peak earlier in the DHEA co-treated group. The plasma concentrations of 4-COOH NVP and 12-sulfoxy NVP were below the limit of detection. Even though other effects of 1-aminobenzotriazole, apart form the mechanistic inhibition, should be taken in consideration, it is unlikely that any potential induction of P450 contributed to the results obtained here.

The urinary excretion of 12-sulfoxy NVP was decreased as well in the NVP + 1-aminobenzotriazole+ DHEA group at 50 – 150 µg/24 hours while it was 200 – 400 µg/24 hours for the animals receiving NVP + 1-aminobenzotriazole only. In contrast to the plasma levels, the urinary excretion of 12-OH NVP was increased in the NVP + 1-aminobenzotriazole + DHEA group at 2000 – 4000 µg/24 hours while it was 100 – 1000 µg/24 hours receiving NVP + 1-aminobenzotriazole only (Figure 12A). The urinary
excretion of the 2- and 3-OH NVP as well as 4-COOH NVP was higher in rats receiving DHEA co-treatment (Figure 12).

Figure 11: Incidence of skin rash (A) and plasma concentrations of NVP (B) and 12-OH NVP (C) in female Brown Norway rats treated with NVP (60 mg/kg/day) + 1-
aminobenzotriazole (50 mg/kg/day) \((n = 4)\) and NVP (60 mg/kg/day) + 1-aminobenzotriazole (50 mg/kg/day) + DHEA (50 mg/kg/day) \((n = 3)\).

Figure 12: Urinary excretion of 12-OH NVP (A), 4-COOH NVP (B) sulfate of 12-OH NVP (C), 2-OH NVP (D) and 3-OH NVP (E) in female Brown Norway rats treated with NVP (60 mg/kg/day) + 1-aminobenzotriazole (50 mg/kg/day; \(n = 4\)) and NVP (60 mg/kg/day) + 1-aminobenzotriazole (50 mg/kg/day) + DHEA (50 mg/kg/day; \(n = 3\)).
3.4 Treatment of female Brown Norway rats with 12-OH NVP + DHEA

During a preliminary experiment, several doses of DHEA (25, 50, and 100 mg/kg/day) were administered together with 12-OH NVP (100 mg/kg/day) to observe the dose-response relationship of the inhibitor. However, no such relationship was observed because all rats receiving 12-OH NVP only developed a skin rash, while the incidence was 0% for the rats co-treated with all doses of DHEA (Figure 13). The plasma concentrations of 12-OH NVP were decreased to 20 – 40 µg/ml in DHEA co-treated rats compared to 40 – 60 µg/ml in rats receiving 12-OH NVP only, and the 12-sulfoxy NVP plasma concentrations were decreased to ~ 2 µg/ml compared to ~ 7 µg/ml for the same treatment groups (Figure 13). The urinary excretions of 12-OH NVP and 12-sulfoxy NVP were lower compared to rats receiving 12-OH NVP only while the excretion of the carboxylic acid was comparable for all rats (Figure 14). The medium dose of 50 mg/kg/day for DHEA was chosen for the experiments that followed.
Figure 13: Incidence of skin rash (A) and plasma concentrations of 12-OH NVP (B) and 12-sulfoxy NVP (C) in female Brown Norway rats treated with 12-OH NVP only (100 mg/kg/day, $n = 2$) or 12-OH NVP in combination with DHEA (25, 50, and 100 mg/kg/day, $n = 2$ each group).
Figure 14: Urinary excretion of 12-OH NVP (A), 4-COOH NVP (B) and 12-sulfoxo NVP (C) in female Brown Norway rats treated with 12-OH NVP only (100 mg/kg/day; n
or 12-OH NVP in combination with DHEA (25, 50, and 100 mg/kg/day; \( n = 2 \) each group).

An attempt was made to match the plasma concentrations of 12-OH NVP in the animals treated with 12-OH NVP only and the DHEA co-treatment group. For this purpose the female Brown Norway rats were treated with 12-OH NVP (100 mg/kg/day), 12-OH NVP (100 mg/kg/day) + DHEA (50 mg/kg/day) and a higher dose of 12-OH NVP (150 mg/kg/day) + DHEA (50 mg/kg/day). All of the rats receiving 12-OH NVP only developed skin rash (Figure 15A). Out of the rats receiving a combination of 12-OH NVP and DHEA (50 mg/kg/day), only one rat receiving the lower dose (100 mg/kg/day) of 12-OH NVP developed a rash while 3 out of 4 rats treated with the higher dose of 12-OH NVP (150 mg/kg/day) developed a rash (Figure 15A).

The plasma levels of 12-OH NVP were decreased to 20 – 30 µg/ml in rats receiving both 12-OH NVP and DHEA compared to 12-OH NVP only where levels were 40 – 50 µg/ml (Figure 15). It was surprising to discover that even when rats were treated with a higher dose of 12-OH NVP together with DHEA, the plasma levels were still lower compared to the rats treated with a lower dose of 12-OH NVP only. The plasma levels of 12-sulfoxy NVP were decreased in rats receiving all doses of 12-OH NVP + DHEA to 1 - 2 µg/ml compared to 3 – 10 µg/ml for 12-OH NVP only-treated animals (Figure 15).

The urinary excretion of 12-OH NVP was comparable in rats receiving 12-OH NVP only and 12-OH NVP 150 mg/kg/day + DHEA (Figure 16). The excretion of the carboxylic acid of NVP was increased for all rats co-treated with DHEA while the excretion of the sulfate of 12-OH NVP was decreased (Figure 16).
Figure 15  Incidence skin rash in female Brown Norway rats treated with 12-OH NVP 100 mg/kg/day, 12-OH NVP 100 mg/kg/day + DHEA 50 mg/kg/day and 12-OH NVP 150 mg/kg/day + DHEA 50 mg/kg/day (n = 4 each group) (A). Plasma concentrations of 12-OH NVP (B) and 12-sulfoxy NVP (C) in the same rats (n = 4 for each group).
Figure 16: Urinary excretion over 24 hours of 12-OH NVP (A), 4-COOH NVP (B), and 12-sulfooxy NVP (C) in female Brown Norway rats treated with 12-OH NVP 100
mg/kg/day, 12-OH NVP 100 mg/kg/day + DHEA 50 mg/kg/day and 12-OH NVP 150 mg/kg/day + DHEA 50 mg/kg/day (n = 4 for each group).

3.5 Oxidation of 12-OH NVP by rat liver microsomes

12-OH NVP was the major metabolic product in the incubation of NVP with rat liver microsomes and no carboxylic acid was observed (Figure 17A). The carboxylic acid of NVP was detected in the incubation of 12-OH NVP (100 µg/ml) with hepatic microsomes from male and female rats and NADPH (Figure 17B). No aldehyde intermediate was observed in these reactions.
Figure 17: Oxidation of NVP and 12-OH NVP by rat hepatic microsomes. NVP metabolite concentrations from incubations with microsomes from female rats, n=3 (A). 4-COOH NVP concentrations from incubations of 12-OH NVP with microsomes from male and female rats, n=1 (female) and n = 3 (male) (B).
Section 4: Discussion, Conclusions, and Future Directions

4.1 Discussion

During the initial categorization of NVP’s metabolic pathways in different species it was also shown that 12-OH NVP is the major metabolite found in plasma and urine of female rats (73). Our results from the in vitro incubations of NVP with rat liver microsomes from female rats show that 12-OH NVP was the major metabolite produced during the incubations and thus are consistent with previous findings.

Previous results from our group suggest that P450 mediates further oxidation of 12-hydroxy NVP to the carboxylic acid. This hypothesis originated from the observation that when NVP is administered together with the P450 inhibitor, 1-aminobenzotriazole, the production of 2-OH NVP, 3-OH NVP, and 4-COOH NVP was inhibited, but the production of 12-OH NVP remained the same and the animals developed a rash (71). If the further oxidation of 12-OH NVP to the carboxylic acid is P450-mediated, 1-aminobenzotriazole could not only decrease oxidation of NVP to the hydroxylated metabolites but also further oxidation to the carboxylic acid. Because 1-aminobenzotriazole inhibits both the formation and elimination of 12-OH NVP, the overall effect is small and, in fact, in the presence of 1-aminobenzotriazole, the dose of NVP required to induce a rash is actually smaller than in its absence. It was also discovered that inhibition of alcohol dehydrogenase, an enzyme typically involved in oxidation of alcohols to aldehydes, did not decrease the production of 4-COOH NVP in vivo (71). This finding provided further support for P450-mediated oxidation of 12-OH NVP to the carboxylic acid.
To test whether P450 can carry out oxidation of 12-OH NVP to the carboxylic acid, 12-OH NVP was incubated with rat liver microsomes from male and female rats. Finding a time-dependent formation of 4-COOH NVP in incubations of rat liver microsomes and 12-OH NVP shows that P450 can catalyze a reaction commonly carried out by alcohol dehydrogenases. Although oxidation of alcohols to aldehydes and carboxylic acids is commonly catalyzed by alcohol and aldehyde dehydrogenases, respectively, cases of P450-mediated oxidation of alcohols have been described before. For example, it is known that CYP 2E1 can oxidize a number of small hydrophobic alcohols as well as ethanol. The oxidation of NVP to 12-OH NVP can proceed in two steps: either via an aldehyde intermediate or by 12-OH NVP undergoing a four electron oxidation without the aldehyde intermediate leaving the active site of the P450 resulting in direct oxidation to the 4-carboxy NVP. The fact that no 4-COOH NVP was found in incubations of P450s with NVP can be explained by slow formation of 12-OH NVP, the precursor of the carboxylic acid. The aldehyde was not observed in the incubation of NVP or 12-hydroxy NVP with rat liver microsomes and NADPH, suggesting the occurrence of direct oxidation. Direct oxidation has been described before for CYP 2E1-catalyzed oxidation of ethanol to acetic acid where it was shown that ~ 90% of the acetaldehyde intermediate that is formed during the reaction is directly converted to the acid without dissociation from the active site of the enzyme (82).

There are significant differences in NVP metabolism between male and female rats that can explain the overall higher formation of 4-COOH NVP in incubations with male rat liver microsomes compared to those from females. The incidence of skin rash is about four-fold lower in male Brown Norway rats compared to females, which is due to
higher metabolism rate of NVP that results in significantly lower plasma concentrations of the parent drug and 12-OH NVP (71), but higher concentrations of 4-COOH NVP, that was found to be the major metabolite found in 24 hour urine samples for both male and female Sprague-Dawley rats, with male rats having a higher overall production (73). These differences are likely due to sex differences in expression of P450s in male and female rats. For example, this could be explained if NVP is primarily metabolized by a male-predominant P450. In humans, CYP 3A4, 2D6, and 2B6 are responsible for metabolism of NVP (74). There are no data available on which rat P450 isozymes are involved in oxidations to the different metabolites, but if the reactions are carried out by male-predominant isozymes such as CYP 3A2 or 2C11 (83), this could explain the differences in metabolite production.

Present studies showed that 12-OH NVP undergoes sulfation in rats and yields measurable levels of the sulfated product. The goal of this work was to investigate the importance of sulfotransferases and 12-sulfoxy NVP as the link between the 12-OH NVP pathway and skin rash. Attempts were made to inhibit the in vivo sulfation of 12-OH NVP in rats and study the effects of sulfate formation on the incidence of the rash. Two sulfation inhibitors with two different mechanisms were used in these experiments: salicylamide, which is known to deplete the co-factor PAPS (78), and DHEA, a substrate and competitive inhibitor to SULT 2A enzymes (79). Humans have only one isoform of this enzyme – SULT 2A1 – while rats have multiple forms: SULT 2A1, A3, and A4. Since 12-OH NVP is a benzylic alcohol, it has some structural similarity with steroid hormones and thus is a potential substrate to SULT 2A enzymes (55).
In the initial experiments, DHEA and salicylamide were administered together with NVP. Co-treatment with NVP and salicylamide did not prevent the onset of the rash even though plasma concentrations and the urinary excretion of 12-sulfoxy NVP were decreased and the plasma concentrations and urinary excretion of the other metabolites remained unchanged. Taken together, co-treatment of female Brown Norway rats with NVP and salicylamide showed that the skin rash can be induced even if production of the 12-sulfoxy NVP is low, suggesting the sulfation pathway might not play an important role in the development of skin rash in this animal model.

The basis of this experiment is that when salicylamide is sulfated, it depletes the co-factor PAPS, which is necessary for sulfation, and the inorganic sulfate, that is needed for PAPS synthesis (78). Both sulfated salicylamide and 12-sulfoxy NVP have to be excreted in urine. It is possible that a renal transporter is required for excretion of both compounds, and if the substances are competing for the transporter, salicylamide sulfate could compete with 12-sulfoxy NVP for clearance. The sulfate of 12-OH NVP can undergo hydrolysis back to 12-OH NVP in plasma – this was determined when 12-sulfoxy NVP was administered to a female Brown Norway rat via an intravenous injection. If this happens, the urinary plasma levels and urinary excretion of the 12-sulfoxy NVP in the urine may be misleading because they would suggest a falsely low production of the sulfate. Also, sulfation is a low-capacity pathway, meaning that in the presence of a depleting agent, the metabolism pathway of 12-OH NVP would shift from sulfation to, for example, glucuronidation or further oxidation to the carboxylic acid. During preparation of urine samples the metabolites are de-glucuronidated using β-glucuronidase. There was no increase in urinary excretion of hydroxylated metabolites or
the carboxylic acid of NVP, and therefore there is no apparent evidence for compensation by other metabolic pathways. The results from this experiment suggest that 12-sulfoxy NVP is not involved in NVP-induced skin rash. However, it may be that sulfation in the skin is what induces the skin rash. Overall sulfation in the skin probably accounts for only a small fraction of the total sulfate formed that is measured in the blood and urine. If salicylamide does not deplete PAPS in the skin because the enzyme that sulfates salicylamide is not in the skin or the turnover is too slow to deplete PAPS, the sulfate in the blood and urine may not reflect sulfation in the skin.

Contrary to the results obtained with salicylamide, administration of DHEA together with NVP not only decreased the urinary excretion of the 12-sulfoxy NVP, it also prevented the skin rash. However, DHEA also decreased the plasma concentrations and urinary excretion of NVP and 12-OH NVP. It is known that the 12-OH NVP pathway is responsible for the skin rash because the rash can be induced by administrating 12-OH NVP at a lower dose than NVP. Adequate plasma levels of the parent drug (30 – 40 µg/ml for female Brown Norway rats) when NVP is administered are needed as well for the development of the skin rash because only then sufficient plasma concentration of 12-OH NVP, that is essential for the skin rash, can be obtained (71). Therefore it was impossible to draw conclusions on the role of 12-sulfoxy NVP from these experiments. The observed effect of DHEA on the plasma concentrations of 12-OH NVP could come from an interaction of DHEA with P450. In humans, NVP is converted to 12-OH NVP by CYP 3A4 and 2D6 (74). A study showed that DHEA increases 2- and 12-hydroxylation of NVP in incubations with human liver microsomes (84). We propose that NVP acts as a suicide inhibitor of P450 by forming a quinone
methide during the initial step in 12-hydroxylation (71). Inactivation of CYP 3A4 by nevirapine in human liver microsomes was confirmed recently (85). If DHEA interacts with P450 it may decrease the suicide inactivation of P450 causing an increased formation of 12-OH NVP, which in turn is oxidized to 4-COOH NVP and excreted in urine. In these experiments, marginally increased excretion of 4-COOH NVP was observed while excretion of 2- and 3-OH NVP was only marginally decreased. These findings are consistent with the above theory.

It was surprising to discover that during the co-treatment with DHEA, 1-aminobenzotriazole failed to decrease the difference in the plasma levels of NVP between the two groups of rats. Compared to the animals that received NVP + 1-aminobenzotriazole, the rats receiving NVP + DHEA + 1-aminobenzotriazole had decreased plasma concentrations of NVP and a decreased urinary excretion of the 12-sulfoxy NVP, but they also had a higher excretion of all the other metabolites (Figure 13). Taken together, i.e. the increased excretion of 12-OH NVP and the carboxylic acid suggests that the affinity of DHEA for P450 is stronger than that of NVP and that DHEA still interacts with P450-mediated metabolism of NVP. It is important to consider that even though the plasma concentrations NVP were lower in rats that did not develop the skin rash, these animals produced and excreted more of 12-OH NVP and yet did not develop skin rash. Therefore, it is reasonable to conclude that the rats receiving NVP + DHEA + 1-aminobenzotriazole did not develop skin rash due to lower production of 12-sulfoxy NVP and thus the results form this experiment are consistent with the hypothesis that 12-sulfoxy NVP is essential for development of the rash.
Addition of 1-aminobenzotriazole to DHEA co-treatment changed the metabolite profile of the co-treatment groups. In the absence of 1-aminobenzotriazole, the urinary excretion of 2-, 3-, and 12-hydroxy NVP was lower compared to the NVP control, but in the presence of 1-aminobenzotriazole the relationship is the opposite (figures 8 and 12). This effect is also likely due to interaction of DHEA with P450. In the absence of 1-aminobenzotriazole, the metabolic pathway is pushed towards oxidation of 12-OH NVP to the carboxylic acid by P450. If that is a favorable pathway, the prevention of inhibition of P450 by NVP leads to production of more of the most favored metabolite. 1-aminobenzotriazole is a non-specific inhibitor of P450 and was shown before to decrease production not only of the hydroxylated metabolites of NVP with the exception of 12-OH NVP, but also of the 4-COOH NVP (71). Therefore, if DHEA is interacting with P450, it could restore a part of the production of all metabolites, thus causing an increase in the urinary excretion.

There may be other effects of DHEA apart from compensating for the P450 inhibition by NVP which can be related the increased production of 2-OH NVP, as well as 12-OH NVP in the microsomal incubations with NVP and DHEA (84). For example, it is known that DHEA has effects on several nuclear receptors that regulate transcription of xenobiotic-metabolizing enzymes. Addition of DHEA to a culture of primary human hepatocytes increased the expression of several P450 genes such as CYP3A4, CYP2C9, and CYP2C19 by activating human pregnane X receptor (PXR) (86). The same study also demonstrated that DHEA was active in the induction of CYP2B6 via the constitutive androstane receptor (CAR) (86). A different study showed that DHEA induces expression of CYP 4A via the peroxisome proliferator-activated receptor α (PPARα) (87).
Peroxisome proliferator-activated receptor α is also involved in regulation of hepatic hydroxysteroid sulfotransferase expression, and studies in rats showed that DHEA induces expression of SULT 2A (88). It was also shown that when administered to rats, DHEA induces several hepatic enzymes including P450 4A, NADPH:P450 oxidoreductase, palmitoyl coenzyme A and others (89). Apart from up-regulating a number of P450, DHEA down-regulates CYP 2C11 in rats (90). These changes in gene expression may have contributed to the complex results obtained in our studies.

In order to avoid any complications of the effect of DHEA on P450 and focus on the inhibition of sulfation, 12-OH NVP was used in further experiments. The 12-OH NVP pathway is responsible for the induction of the skin rash and is directly upstream from 12-sulfoxy NVP. Co-administration of different doses of DHEA together with 100 mg/kg/day of 12-OH NVP prevented the onset of skin rash, but it also decreased the plasma levels of 12-OH NVP (Figure 14). Since there is evidence that further oxidation of 12-OH NVP is mediated by P450, there is a potential for induction of P450 by DHEA that could explain the decreased plasma concentration of 12-OH NVP. When the higher dose of 12-OH NVP was given together with DHEA in an attempt to match the blood levels of 12-OH NVP between 12-OH NVP group and the co-treatment group, the plasma concentrations of 12-OH NVP and 12-sulfoxy NVP for all rats receiving DHEA remained continuously lower compared to the rats not receiving DHEA and did not differ for the rats receiving the higher or the lower dose of 12-OH NVP in combination with DHEA (Figure 16). The same pattern was observed for the urinary 12-OH NVP and 12-sulfoxy NVP excretion. The only reasonable explanation for higher incidence of skin rash for the rats receiving a higher dose of 12-OH NVP together with DHEA lies in the higher
exposure to 12-OH NVP even though this was not confirmed by blood and urinary concentrations of 12-OH-NVP. It is possible to argue that a greater dose of 12-OH NVP would also generate more of the 12-sulfoxy NVP. Therefore, the higher incidence of skin rash in rats treated with 150 mg/kg/day of 12-OH NVP + DHEA compared to the ones treated with 100 mg/kg/day + DHEA could be due to different exposure to 12-sulfoxy NVP, presenting further support for the importance of 12-sulfoxy NVP as the skin rash inducing agent. The findings of an increased production of 4-COOH in all rats co-treated with DHEA further suggests that P450s are involved in the oxidation of 12-OH NVP to the carboxylic acid, and this is in line with the hypothesis that DHEA modifies the interaction of NVP with P450.

When investigating the metabolic pathway or the reactive metabolite responsible for an adverse drug reaction it is important to consider where the reaction occurs. In the case of NVP, we are interested in the mechanisms and the pathways that lead to the skin rash. It is known that 12-sulfoxy NVP is a stable compound in aqueous solutions and that it reacts slowly with nucleophiles where the product is observed only after 1.5 – 24 hours of reaction. 12-sulfoxy NVP reacts specifically with sulfur-containing nucleophiles such as N-acetylcysteine but not with amines (91). We propose that 12-sulfoxy NVP undergoes an S_N2 reaction that leads to binding to tissue nucleophiles with simultaneous elimination of the sulfate group. In the attempt to show reactivity of 12-sulfoxy NVP, the compound was incubated with homogenized liver tissue, but no covalent binding to liver proteins was detected (unpublished results). To date, we do not have data on the metabolic events that occur in the skin. Co-administration of NVP with salicylamide decreased the plasma levels of the 12-sulfoxy NVP and its urinary excretion, which
reflects the depletion of sulfate and inhibition of sulfation in the liver, but at this point it is unclear whether the sulfate was decreased in the skin as well. Until we can obtain evidence that the sulfation inhibitors used in these studies executed their effects on metabolism of NVP in the skin, and not only on hepatic metabolism, we will not be able to absolutely prove or rule out the importance of sulfation pathways for NVP-induced skin rash in the rat model.

A recent study by K. Park’s group investigated bioactivation of nevirapine in humans and different rat strains (92). The study confirmed formation of 12-sulfoxy NVP in rats: the metabolite was detected in rat urine and bile samples, but the metabolite was not found in human urine samples. However, the fact that 12-sulfoxy NVP was not detected in the human samples does not mean that this metabolite is not produced at all. The production of 12-sulfoxy NVP in humans can be lower than in rats due to overall lower production of 12-OH NVP in comparison to rats (71-72). 12-sulfoxy NVP was detected in bile of the treated rats both by our group (71) and K. Park’s group (92). A bile sample could also be a more fitting place to search for 12-sulfoxy NVP in humans.

Results published in (92) support the formation of the quinone methide in both humans and rats. It is proposed that the mercapturate conjugates detected in those experiments originate either from a reaction of an aryl epoxide intermediate (3-mercapto conjugate) or from the quinone methide (12-mercapto conjugate), which, in turn, can be a product of the substitution of the sulfate group or the result of P450-mediated oxidation of NVP directly to the reactive quinone methide. There is strong evidence that the 12-OH NVP pathway is essential to induce skin rash in female Brown Norway rats, but a recent clinical study did not find association between high plasma concentrations of 12-OH
NVP in patients and development of skin rash (65). However, the study population was heterogeneous and there were a number of confounding factors; therefore, if a significant range of concentrations of 12-OH NVP was not present, the detection of a relationship between plasma levels of 12-OH NVP and skin rash would be difficult. Also, it appeared that plasma concentrations of 12-OH NVP were higher in populations associated with higher risk for developing skin rash, such as women and patients receiving prednisone.

In summary, the experiments presented and discussed above did not ultimately prove or disprove that 12-sulfoxo NVP is responsible for inducing skin rash in the female Brown Norway rats. The experiment where salicylamide was used as the sulfation inhibitor showed that skin rash can develop even when overall detected production of 12-sulfoxo NVP is strongly reduced, adding to the argument that 12-sulfoxo NVP is not responsible. Administration of DHEA together with NVP lead to both decreased overall production of 12-sulfoxo NVP and inhibition of skin rash; however, DHEA had other effects that lead to decreased plasma levels and urinary excretion of NVP and 12-hydroxy NVP. Therefore, this experiment could not thoroughly test the hypothesis. Results from other experiments suggested the importance of 12-sulfoxo NVP for the induction of the skin rash. Specifically, in the experiment in which rats received NVP + DHEA + 1-aminobenzotriazole, there was an increase in the overall production of 12-OH NVP as shown by high urinary excretion in the rats compared to NVP + 1-aminobenzotriazole and yet the DHEA-treated animals did not develop a rash while those not receiving DHEA did. Also, the fact that rats developed skin rash when administered a higher dose of 12-OH NVP in combination with DHEA but did not develop the rash when the lower dose of 12-OH NVP was given with DHEA, indicates that 12-sulfoxo NVP is important
because the animals receiving more 12-OH NVP were at some point exposed to higher levels of 12-sulfoxy NVP. In conclusion, these experiments showed how metabolism of NVP can be affected in unexpected ways when metabolic inhibitors are introduced, but the presented approaches did not produce conclusive evidence to prove or deny the crucial role of 12-sulfoxy NVP in NVP-induced skin rash. In order to thoroughly investigate involvement of 12-sulfoxy NVP, different methods have to be used, and the alternative strategies can be found in the Future directions section.

4.2 Future Directions

The above experiments demonstrate the importance of having a valid animal model to test hypotheses. The results also show that caution should be used when working with inhibitors of a metabolic pathway because inhibitors can have a broad spectrum of other effects that might affect the outcome of the experiment.

There is much that remains to be determined to provide a conclusive answer to the question of how the 12-OH NVP pathway induces a skin rash in this animal model. For example, we need to test if 12-sulfoxy NVP is reactive and can covalently bind to skin proteins. Many drugs that cause IDRs produce reactive metabolites and some degree of covalent binding to the tissue. Covalent binding to proteins is the basis of the Hapten hypothesis, and binding to MHC II receptor is proposed to be the initial step in activation of the immune system according to the PI hypothesis. To test the reactivity of 12-sulfoxy NVP, future studies should include synthesis of the compound and \textit{in vitro} incubations with liver and skin tissue followed by a sensitive method to detect protein binding. Also, the Danger hypothesis suggests that the affected tissue directs the immune response, and
since skin is the site of the adverse reaction, the initial activation should happen there. Therefore, bioactivation by the skin needs to be proven.

Also, we need to confirm that the 12-OH NVP can be bioactivated in vivo. One way of doing so is by testing for covalent binding of the drug in vivo. Female Brown Norway rats should be treated with NVP until development of skin rash, sacrificed, and then the skin tissue should be collected and examined for covalent binding. Covalent binding of NVP has been detected before by our group in liver proteins; however, that binding is likely due to the formation of quinone methide during the initial step in the oxidation of NVP to 12-OH NVP (see Figure 5) because the liver is rich in P450s and is the major site for NVP metabolism. Since we are interested in the events that take place in the skin, we should look for the presence of covalent binding in the site of interest. As mentioned earlier, keratinocytes in the skin have limited P450 activity but are rich in sulfotransferases. We speculate that 12-OH NVP reaches skin carried by the blood from the liver and is sulfated in the skin. If covalent binding is detected in the skin, attempts can be made to characterize the modified protein. Also, the amount of the covalent binding can be assayed for different doses of the drug and in the presence or absence of sulfation inhibitors, and examined for the correlation with the plasma levels of the 12-sulfoxy NVP and the incidence of skin rash.

All the previous studies carried out in our lab point towards the importance of 12-OH NVP pathway as the metabolic pathway responsible for the skin rash: the rash can be induced by 12-OH NVP administered at a lower dose than NVP; inhibition of P450 by 1-aminobenzotriazole led to decreased plasma levels and urinary excretion of all metabolites, except for 12-OH NVP and actually led to a rash at lower doses of NVP.
Ultimately, administration of 12-trideutereonevirapine decreased formation of 12-OH NVP in rats and prevented the onset of the rash.

Even though bioactivation through sulfation of 12-OH NVP appears as the most logical mechanism for the induction of the rash, future studies should also examine involvement of other possible downstream metabolites of 12-OH NVP in the induction of the rash, such as the aldehyde of 12-OH NVP. When 12-OH NVP is oxidized to the carboxylic acid, an intermediate aldehyde might be produced. To date, we have not observed the aldehyde in the *in vitro* incubations of 12-OH NVP with rat liver microsomes, but the production of this compound can not be ruled out. We can attempt to trap the aldehyde formed in *in vitro* incubations using mild trapping agents such as compounds containing a hydrazine group or the Aldehyde Reactive Probe (O-(Biotinylcarbazoylmethyl) Hydroxylamine, manufactured by Cayman chemical). Also, the binding of the aldehyde of NVP to lymphocytes could be studied *in vivo* because that is known to activate lymphocytes (93).

It is clear from the experiments presented above that different approaches have to be tried in order to determine the role of 12-sulfoxo NVP in the NVP-induced skin rash. Here, DHEA and salicylamide were used to inhibit the sulfation pathway in female Brown Norway rats. It is obvious that at least DHEA executed a number of other effects apart from inhibiting SULT 2A, such as modulation of P450 metabolism and potentially affected the function of the immune system. Therefore, other sulfation inhibitors should be tested to determine if they produce effects that are consistent with the results observed here. A number of non-steroidal anti-inflammatory drugs such as salicylic acid are known to inhibit SULT 1A1 among which mfenamic acid is the most potent inhibitor of the
human SULT 1A1 (94). These substances can be administered to rats, but caution should be observed as they can cause duodenal bleeding. There is a great number of dietary and environmental chemicals that are proven to have effects on different human and rat sulfotransferases isoforms (see (95) for review). For example, human SULT 1A1 that mainly sulfonates phenols is inhibited by the flavanones, hesperitin (IC\text{50} 23.4 \mu M), and eriodictyol (IC\text{50} 15.2 \mu M) as well as the flavonols, catechin (IC\text{50} 8.4 \mu M) and epicatechin (IC\text{50} 42.5 \mu M) against 100 \mu M for 2-naphthol (96). Human SULT 2A1 (the DHEA sulfotransferase) is also inhibited by phthalates (substances used as plasticizers) (95) as well as the flavanones, hesperidin (IC(50) 91.0 \mu M) and eriodictyol (IC(50) 66.1 \mu M) as compared to 2 \mu M for DHEA (96). The flavonoids are naturally occurring in diet and should therefore be safe for administration to rats; however the bioavailability can be low. It is also well-established that endocrine disruptors can act as sulfotransferase inhibitors. Compounds such as 4-\text{n}-nonyphenol, 4-\text{tert}-octyphenol, and benzyl butyl phthalate have \text{Ki} values of \leq 10 \mu M against DHEA sulfation by human liver cytosol (97). Even though these substances are efficient inhibitors of sulfotransferases, their use is less feasible in \textit{in vivo} studies due to toxicity and effects on the endocrine system. An alternative route of exposure can also be adapted, possibly allowing for use of substances that are toxic if ingested. Local inhibition of skin sulfotransferases can be attempted by applying the sulfation inhibitors as a topical solution. Female Brown Norway rats can be administered NVP in the food and the sulfation inhibitor can be applied daily on the skin; the skin can be monitored for development of the rash which can potentially be induced on all parts of the skin, except the part treated with the inhibitor.
As an alternative approach to study the effects of sulfation of 12-OH NVP on the skin rash, knockout animals lacking a particular sulfotransferase isoform could be used. Knockout techniques have been restricted to mice for a long time, but with new developments customized rat knockout models can be created as well (98). Prior to creating a knock-out rat model, the different rat sulfotransferases would need to be tested for their ability to sulfonate 12-OH NVP, and the most potent can be chosen as a candidate for knockout. Using these approaches we will be able to finally conclude if the 12-sulfoxy NVP is responsible for skin rash induction in female Brown Norway rats.

4.3 Conclusions

Using the animal model of NVP-induced skin rash, we were able to test the importance of 12-sulfoxy NVP for the development of the skin rash. The results of experiments do not provide conclusive evidence for the connection between 12-sulfoxy NVP and nevirapine-induced skin rash. The results of some experiments suggest that 12-sulfoxy NVP is likely to be the link between the 12-OH NVP pathway and skin rash, but a different experiment design needs to be used to provide a conclusive answer.

This work also showed that P450s are involved in further oxidation of 12-OH NVP to the carboxylic acid, a reaction typically carried out by alcohol and aldehyde dehydrogenases. The results observed in the in vitro and in vivo experiments provide insight in the sex differences of metabolic activation of NVP.
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