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Human Sulfotransferase 1A1-Dependent Mutagenicity of 12-Hydroxy-nevirapine: The Missing Link?

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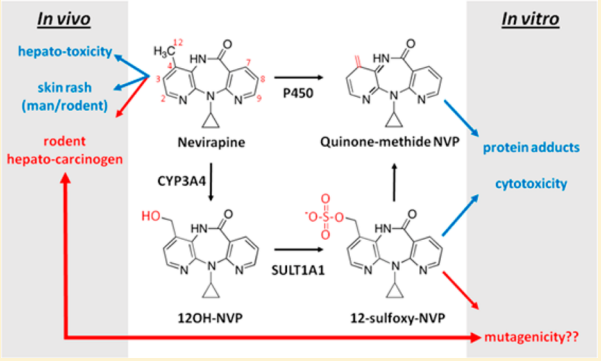
Supporting Information

ABSTRACT: Nevirapine (NVP) is a frequently used anti-HIV drug. Despite its efficacy, NVP has been associated with serious skin and liver injuries in exposed patients and with increased incidences of hepatoneoplasias in rodents. Current evidence supports the involvement of reactive metabolites in the skin and liver toxicities of NVP, formed by cytochrome P450-mediated oxidations and/or subsequent phase II sulfonation. However, to date, standard *in vitro* genotoxicity tests have provided no evidence that NVP is either mutagenic or clastogenic. The human sulfotransferase 1A1-dependent mutagenicity of 12-hydroxy-NVP, one of the major metabolites of NVP, is demonstrated here.



Nevirapine (NVP) is a widely prescribed non-nucleoside inhibitor of HIV-1 reverse transcriptase (NNRTI), the first one approved by the FDA in 1996. It is effective when used as a part of combination therapy to treat HIV-1-infected individuals and as monotherapy for prevention of mother-to-child HIV-1 transmission.[1] Despite its therapeutic efficacy, in particular against vertical HIV transmission, severe skin and liver injury has been reported, although individual susceptibilities for NVP adverse effects differ among patients.[2,3] NVP has also been associated with increased incidences of hepatoneoplasias in rodents.[4] Additionally, epidemiological data suggest that NNRTI use, in general, is a risk factor for non-AIDS-defining cancers in HIV-positive patients.[5]

Current evidence indicates generation of reactive NVP metabolites as the culprit of its associated liver and skin toxicity. NVP is metabolized at first instance through oxidative metabolism mediated by cytochrome P450s, by hydroxylation at the C3, C8 (both mainly by CYP2B6), C2, or C12 position (latter two mainly by CYP3A4) (Figure 1).[6] In particular, oxidative metabolism at the C12 position has been implicated in NVP induced toxicity. The reactive C4−C12 quinone methide was previously suggested to be the reactive species, formed either directly by cytochrome P450-mediated dehydrogenation or by C12 hydroxylation, sulfonation, and subsequent sulfate loss.[7] Protein adducts could be detected using *in vitro* approaches for 12-sulfoxy-NVP in serum albumin and glutathione S-transferase π and for the synthetic surrogate 12-mesyloxy NVP with hemoglobin.[8−10] Several of these protein adducts were detected in blood of patients receiving NVP, leaving strong indications of NVP haptenation and its causal role in these associated adverse drug reactions.[8,11] Currently, evidence suggests the involvement of the NVP quinone methide in the induction of hepatotoxicity, whereas the 12-sulfoxy-NVP metabolite is responsible for skin rash, with a possible prominent role for sulfotransferase 1A1 (SULT1A1).[12−14]

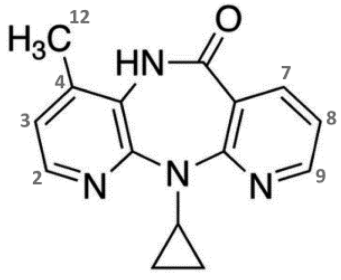


Figure 1. Chemical structure of NVP.

The synthetic surrogate for 12-sulfoxy-NVP, 12-mesyloxy-NVP, has been demonstrated to be reactive with DNA.[15,16] The induction of hepatoadenomas and carcinomas in rodents as well as the increased risk for non-AIDS-defining cancers with long-term NNRTI use raises concerns of possible genotoxicity of NVP.[4,5] Still, standard *in vitro* mutagenicity tests have provided no evidence that NVP is either mutagenic or clastogenic.[4] These standard assays use exogenous liver-derived metabolic systems, and reactive sulfoxy ester intermediates are thus generated externally, demonstrating an often limited penetration capacity of the indicator cells due to their increased hydrophilicity.[17] As such, sulfotransferase-mediated bioactiva-

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tion is not detected in standard mutagenicity assays, a possible explanation for the discrepancy between the *in vitro* and *in vivo* outcomes of NVP genotoxity testing.[17] The objective of this current study was to determine whether 12OH-NVP is mutagenic in a SULT-dependent manner.

Several human sulfotransferase-competent Ames tester strains have been developed previously by the group of Dr. Hansruedi Glatt (Germany), including those expressing human SULT1A1.[17−21] Although these have been demonstrated to be quite successful in detecting mutagenicity of specific sulfoxyesters, some drawbacks were noted by these authors (see Supporting Information).[20] To overcome these issues, new human SULT1A1 expressing Ames tester bacteria were developed, namely, MA98_SULT1A1 and MA100_SULT1A1, both demonstrating controllable and stable SULT1A1 expression (see Supporting Information).

The SULT1A1-dependent mutagens FFA and 1-HMP were tested with these new human SULT1A1-competent Ames strains to verify their sensitivity in the detection of SULT1A1-dependent mutagens (Figure 2A,B).[18,21] FFA induced 198 ± 19

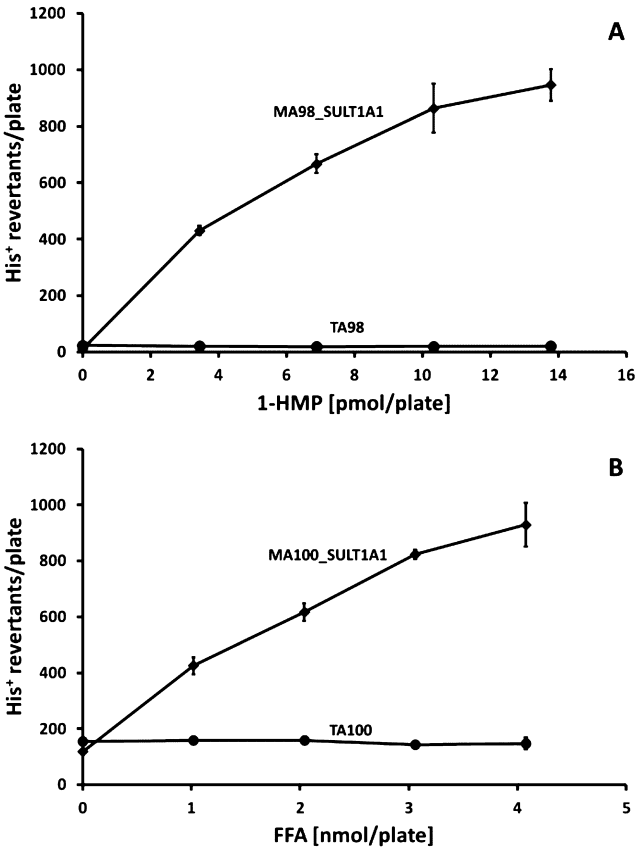

Figure 2. Dose−response curves of 1-HMP (A) with strains MA98_SULT1A1 and TA98 and of FFA (B) with strains MA100_SULT1A1 and TA100.

His+ revertant colonies per nmol. This was 81 ± 10 revertant per pmol for 1-HMP with strain MA98_SULT1A1. These mutagenic activities for FFA and 1-HMP are, respectively, 22- and 6-fold higher than those reported previously for these compounds with SULT1A1-expressing Ames strains.[18,21] Both compounds did not demonstrate any mutagenicity for the same dose gradients with the comparable SULT1A1-empty strains TA98 and TA100 (Figure 2A,B). The new SULT1A1 tester strains were further characterized with respect to their SULT1A1 bioactivation capacity, using pentachlorophenol (PCP), a potent inhibitor of SULT1A1.[22] The PCP-inhibition study with strain MA98_SULT1A1 (Figure 3) demonstrated a very efficient inhibition of 1-HMP-induced mutagenicity, with an IC_{50} value of 0.17 μM (r^2 = 0.974).

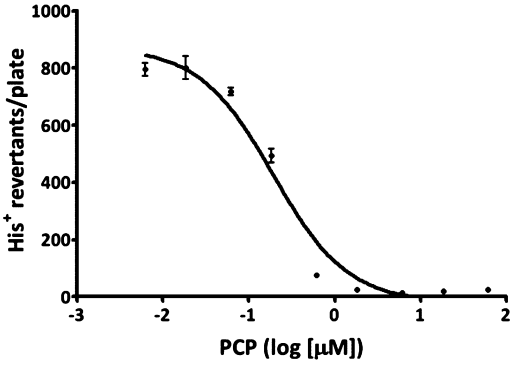

Figure 3. PCP inhibition plot of 1-HMP (8.6 pmol) induced mutagenicity in strain MA98_SULT1A1.

The metabolite 12OH-NVP was tested with the two new SULT1A1 strains, first applying a logarithmic dose gradient (Figure 4A), testing over 4 orders of magnitude in dose levels.

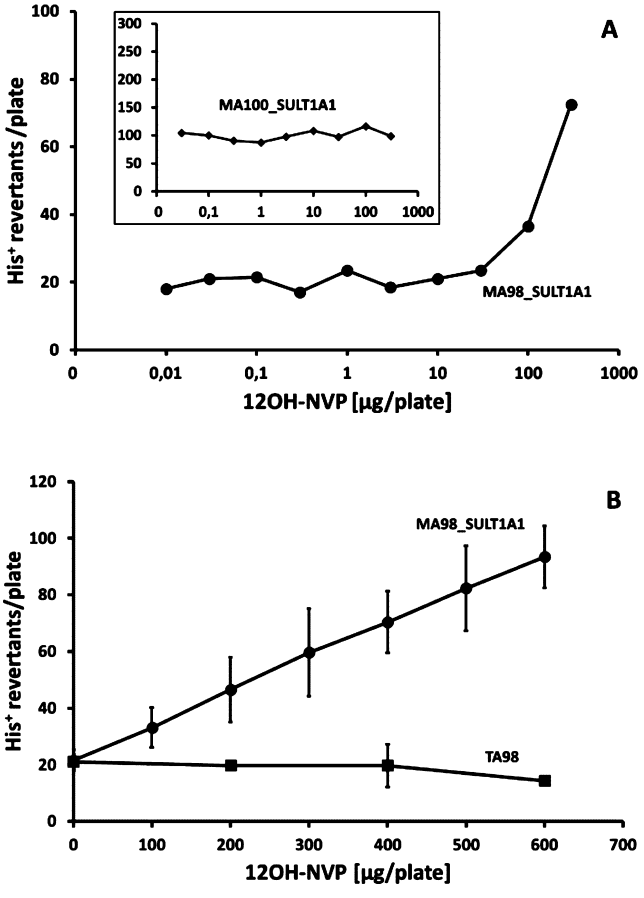

Figure 4. Dose−response curves of 12OH-NVP with a (A) logarithmic or (B) linear dose gradient (dose−response curve of MA98_SULT1A1 in panel B represents the average of four independent experiments).

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MA98_SULT1A1 demonstrated a positive response, which was not the case for MA100_SULT1A1, indicating a preference for inducing frameshift mutations. Of note, both strains, which are derivatives of strains TA98 and TA100, contain G/C stretches in their DNA, part of their L-His-reversion targets, corroborating the reactivity of the synthetic 12-mesyloxy-NVP with deoxyguanosine and deoxycytidine.[16,23] When tested with a linear dose gradient, 12OH-NVP demonstrated a mutagenic activity of 34 ± 1 revertant colonies per μmol with strain MA98_SULT1A1 (Figure 4B). 12OH-NVP demonstrated no mutagenicity with the comparable SULT1A1-empty strain TA98 at the same dose-levels (Figure 4B), indicating the bioactivation role of human SULT1A1 in the mutagenicity of 12OH-NVP. The SULT1A1 inhibitor PCP was used to further confirm the SULT1A1 bioactivation role in 12OH-NVP mutagenicity.[22] When applying 1.8 μM PCP (approximately 10-fold of the IC_{50} concentration, *vide supra*), a complete abolishment of 12OH-NVP's mutagenicity was observed ($p < 0.001$) (Figure 5A). This abolishment is not due to PCP-induced cytotoxicity in *Salmonella typhimurium* LT2, as can be deduced from the mutagenic response of 2-nitrofluorene (2NF), a direct mutagen, in combination with the same dose level of PCP with strain TA98 (Figure 5B).

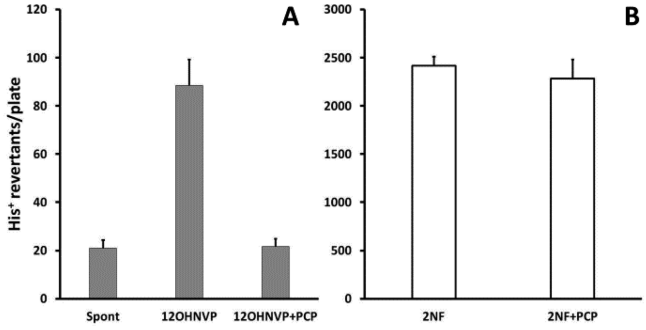

Figure 5. Effect of PCP (1.8 μM) on (A) 12OH-NVP (500 μg) induced mutagenicity of strain MA98_SULT1A1 and (B) on 2NF (0.5 μg) induced mutagenicity of strain TA98 (spont, number of spontaneous revertants).

In a very recent pharmacokinetic study of 180 HIV-infected patients taking the daily recommended dose, NVP plasma concentrations ranged from 1 to 26 mg/L.[24] On the basis of findings in an earlier study by Riska et al., approximately one-third of the daily dose is hydroxylated at the C12 position, the majority of which ends up as a glucuronidated metabolite.[25] 12OH-NVP demonstrated mutagenicity with strain MA98_SULT1A1 in the preincubation assay, using 12OH-NVP concentrations that are an order of a magnitude higher than those found for NVP in the patient group mentioned above. However, the intracellular 12OH-NVP concentrations reaching SULT1A1 in the SULT1A1-competent Ames strain MA98_SULT1A1 will be substantially lower compared with the extracellular concentration due to cell wall penetration restrictions. Moreover, due to the first pass effect, hepatic NVP plasma concentrations in patients will be substantially higher compared to those in peripheral blood samples reported in the patient study mentioned above.[24] As such, data presented in the current study implicates the reasonable assumption of the occurrence of at least some SULT-dependent NVP-mutagenicity in the liver, the major expression site of both CYP3A4 (C12 hydroxylation) and SULT1A1, when taking the recommended daily oral NVP dose.[26,27] This becomes even more likely when the 12OH-NVP detoxificating glucuronidation pathway (UGT) is hampered by genetic polymorphism and/or when CYP3A4-mediated 12-hydroxylation is favored.[28] The later is expected to occur when the detoxifying 8-hydroxylation route (CYP2B6) is less effective, resulting in NVP becoming more available for CYP3A4-mediated metabolism. The CYP2B6 gene is highly polymorphic, with several haplotypes (CYP2B6*6, *11, *15, and *18) associated with reduced catalytic activity and protein stability.[3] Increased NVP plasma levels have been associated with these haplotypes, in particular for the CYP2B6*6 allele, occurring with frequencies of 15−40% in Asians, 25% in Caucasians, and more than 50% in African Americans and black Africans.[3]

Data from this current study is of particular concern for the administration of NVP as a first line choice among initial therapy regimes for children younger than 3 years of age and infants as well as in perinatal settings.[29] These concerns are based first on the outcomes of the very recent draft of the human proteome, indicating CYP3A4 as the main cytochrome P450 in human fetal liver, together with a lack of CYP2B6 (http://www.humanproteomemap.org).[30] This condition favors the formation of 12OH-NVP over 8OH-NVP. Second, fetal liver contains very low levels of xenobiotic-metabolizing UGTs, as demonstrated in an earlier study, with an expected reduction in detoxification of 12OH-NVP through glucuronidation.[31] One may thus expect a tendency for augmented hepatic 12OH-NVP levels in infants and neonates. Third, sulfotransferases of the SULT1A subfamily, including SULT1A1, are highly expressed in neonate/fetal hepatic tissue.[30] Of note, SULT1C4, a sulfotransferase able to bioactivate a large spectrum of different procarcinogens, is also present in high concentrations in the fetal liver, further increasing the likelihood of the formation of 12-sulfoxy-NVP.[30,32] Although NVP dosage regimes are attenuated in infants and young children based on their hepatic drug biotransformation characteristics, data from the current study indicates an increased possibility of NVP-induced hepatic mutagenicity in perinatal and pediatric settings.[1]

In conclusion, we demonstrated here the SULT1A1-dependent mutagenicity of one of the major metabolites of NVP, 12OH-NVP, at dose levels that are physiologically relevant. This is the first time that the mutagenicity of a major NVP metabolite has been demonstrated in an *in vitro* assay, suggesting a link between it and the observed NVP hepatocarcinogenicity in rodents.[4] Although convincing evidence of NVP carcinogenicity in humans has yet to be presented, an epidemiological study of Powles et al. indicated an association of long-term treatment with non-nucleoside reverse transcriptase inhibitors (like NVP) and a higher incidence of non-AIDS-defining cancers.[5] In particular, both the high frequency of genetic polymorphisms in genes encoding detoxification pathways (CYP2B6, UGTs) as well as the presence of efficient hepatic bioactivation routes mediated by CYP3A4 and SULT1A1 are relevant in adults with long-term NVP dosing regimes, an inherent characteristic in the treatment of chronic diseases. Of particular concern is the likelihood of increased NVP hepatomutagenicity in perinatal settings as well as in infants and young children due to their specific biotransformation capabilities. The data presented here might be of relevance in the cost−benefit estimations of NVP treatment regimes, particularly in the design of dosage protocols for infants.

■ ASSOCIATED CONTENT

Supporting Information

Detailed descriptions of experimental materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

2NF, 2-nitrofluorene; 8OH-NVP, 8-hydroxy-nevirapine; 12OH-NVP, 12-hydroxy-nevirapine; CYP, cytochrome P450; NNRTI, non-nucleoside reverse transcriptase inhibitor; NVP, nevirapine; PCP, pentachlorophenol; SULT, sulfotransferase; UGT, UDP-glucuronyl-transferase

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