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Perspective

The Impact of N-nitrosamine Impurities on Clinical Drug Development

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ABSTRACT

Over the past few years, an increasing number of commercially available drugs have been reported to contain N-nitrosamine impurities above acceptable intake limits. Consequent interruption or discontinuation of the manufacturing and distribution of several marketed drugs has culminated into shortages of marketed drugs, including the antidiabetic drug metformin and the potentially life-saving drug rifampin for the treatment of tuberculosis. Alarmingly, the clinical development of new investigational products has been complicated as well by the presence of N-nitrosamine impurities in batches of marketed drug. In particular, rifampin is a key clinical index drug employed in drug-drug interaction (DDI) studies, and as a result of nitrosamine impurities regulatory bodies no longer accept the administration of rifampin in DDI studies involving healthy subjects. Drug developers are now forced to look at alternative approaches for commonly employed perpetrators, which will be discussed in this review.

Background

Ever since the first report of N-nitrosamine impurities in drug products containing the angiotensin II receptor blocker valsartan in 2018,[1] various batches of marketed metformin, ranitidine and varenicline have followed suit.[1] N-nitrosamines are chemical compounds with a functional N-nitroso group (>N—N=O), typically derived from secondary or tertiary amines in the presence of nitrosating agents. They are considered a “cohort of concern” by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M7,[2] due to their probable or possible human carcinogenic potential. Upon exposure, N-nitrosamines undergo metabolism by cytochrome P450 (CYP) for bioactivation to alkydiazonium ions, precursors of reactive electrophilic ions that can form DNA adducts, which can exert genotoxic effects (reviewed in Chakarov et al.[3]). Depending on the DNA adduct formed, their half-life can be from a few hours to several days, and single point or frameshift mutations may arise.[4] These mutations can impact the cellular fitness of conserved pathways such as DNA damage repair, protein sorting systems and mitochondrial integrity, as demonstrated in yeast models.[5] Moreover, rodent bioassay modelling reveals that N-nitrosamine dose–responses for mutagenicity and carcinogenicity are not linear.[6]

Further compounding the impurity issue is the detection of nitrosamine drug substance related impurity (NDSRI), which arise from the active pharmaceutical ingredient (API) itself being nitrosated, and may pose a mutagenic risk. Examples of drugs containing NDSRIs include nitroso-varenicline, nitroso-orphenadrine, nitroso-propranolol and nitroso-quinapril (reviewed in Schlingemann et al.[7]). Overall, due to their mutagenic and carcinogenic risk, N-nitrosamines in marketed medications not only have a potential impact on patients, but on drug developers as well, since many of the same drugs are applied as inhibitors, substrates or inducers in drug-drug interaction (DDI) trials for new investigational products.

N-nitrosamines Impacting Drug Interaction Studies

In a clinical setting, the concomitant intake of two or more drugs by patients can lead to DDIs that may compromise patient safety or interfere with drug effects. For instance, drugs inhibiting or inducing CYP liver enzymes or inhibiting drug transporter molecules may alter the uptake or clearance of other drugs and potentially lead to supra- or sub-therapeutic levels of victim drugs. To avoid unanticipated safety risks, the ICH- issued a guideline requiring that clinically relevant DDIs between investigational drugs and other drugs be assessed during the development of such new drugs.[8] Development of investigational drugs, especially small molecules, typically includes studies

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to assess the potential susceptibility of a new drug to pharmacokinetic (PK) interactions that are based on the metabolic pathway of an investigational drug or its suspected influence on key enzyme and transporters.

An example of a DDI drug impacted by N-nitrosamine impurity is ranitidine, a histamine-2 (H-2) blocker used to suppress gastric acid secretions, it was removed from the market in 2019 due to N-nitrosodimethylamine (NDMA) impurities (Table 1). Before this, it was commonly applied as an acid reducing agent in DDI studies for drugs in development at risk of pH-dependant PK changes. Another H-2 blocker, nizatidine, was also recalled due to similar impurity concerns. Therefore, alternative acid reducing agents should be used in DDI studies, for instance the H-2 blocker famotidine or, as a worst-case scenario design, a proton pump inhibitor such as esomeprazole or rabeprazole can be administered.[9,10]

In late 2019, the FDA was made aware that batches of metformin, a type 2 diabetes medication, manufactured outside of the US were found to contain NDMA. Upon further testing, it was determined that the extended-release (ER) formulation of the drug was affected, which was subsequently recalled from the American market due to this impurity.[11] However, the immediate-release (IR) formulation remains free of nitrosamines and is still available for patients. This version of the drug is also used as a substrate of drug transporters (e.g. OCT2 and MATE1/2K) in DDI studies and, therefore, while the observation of N-nitrosamines in ER-metformin has impacted patients, there has been no change in this respect for drug developers (Table 1).

Recent N-nitrosamine impurity findings can be attributed to closer monitoring of drug products after EMA and FDA issued guidance for industry to control the presence of N-nitrosamine impurities in human drugs in 2020 and 2021, respectively.[12,13] For instance, N-nitrosamine impurities have been identified in rifampin and rifapentine, which are key antibacterial drugs for the treatment and prevention of tuberculosis (TB), a life-threatening infectious disease. Specifically, 1-methyl-4-nitrospiperazine (MNP) and 1-cyclopentyl-4-nitrosopiperazine (CPNP) were found to be above the acceptable intake (AI) limit in rifampin and rifapentine batches, respectively. Consequently, in addition to negatively impacting the supply of TB treatment, the presence of N-nitrosamines in rifampin batches has also greatly affected clinical drug development. Rifampin is one of the perpetrator drugs commonly employed in clinical DDI studies as a strong clinical index inducer of several key liver enzymes, such as CYP3A4, a potent inducer of the efflux transporter P-glycoprotein (P-gp) as well as an inhibitor of the organic anion transporting polypeptide (OATP)1B1/3[14] (see Table 1). Prior to contamination concerns, rifampin had an excellent safety profile and was generally well tolerated, even at regimens higher than the common daily dose of 600 mg.[15] The only exception is that elevations in liver function tests have occasionally been reported in DDI studies, yet these can primarily be attributed to increased concentrations of victim drug metabolites as a result of CYP enzyme induction[16] and are assumedly not unique for rifampin as an inducer. Due to MNP exceeding the AI limit, regulatory agencies have halted the use of rifampin in healthy volunteer DDI studies[17] and encourage drug developers to engage alternative perpetrators. A full review of the application of rifampin in DDIs studies, the root cause of MNP in rifampin and alternative CYP3A4 inducers for DDI studies is described hereafter.

With such emphasis on nitrosamine control in drug products, a recent *in silico* analysis revealed that several DDI inhibitors and substrates are potential N-nitrosamine precursors due to their pharmacophore structure and thus likely candidates for NDSRI formation.[7] While impurities have not been found or confirmed in these compounds, Table 1 provides some examples of these potential NDSRIs related to drugs utilized in DDI studies along with alternative substrates/inhibitors. Of note, the potential nitrosamine risk from secondary amines is greater than that from tertiary amines, as the reaction rate of tertiary amines to form nitrosamines (nitrosative cleavage) is orders of magnitude lower.[63]

N-nitrosamines and Cancer Risk

N-nitrosamines are not restricted to drug products and can be found in drinking water, processed foods, cured meats, fish and tobacco smoke.[18] On average, daily dietary and environmental exposure to N-nitrosamine is estimated at 1.9 – 25.0 μg/day. This exposure is thought to contribute to approximately 6,000 cancer cases per 1 million people.[19] However, N-nitrosamine impurities in medications can further exacerbate this risk. For instance, valsartan prescriptions in the US exceeded 5,000,000 per year between 2013–2018,[20] and the estimated cancer risk associated with valsartan drug products contaminated by N-nitrosamine impurities ranges from 12–126 additional cancer cases per 100,000 exposed individuals.[21] Specifically, valsartan N-nitrosamine impurities were found to be associated with a slightly increased incidence of hepatic, colorectal and uterine cancer.[22,23] Although rifampin prescriptions are substantially less, with an estimated 6,000 patients in the US treated with rifampin as part of a 4- or 6-month TB regimen therapy in 2020,[24] the putative risk of exposure due to N-nitrosamines impurities following rifampin intake is still considerable.

The Root Cause and Consequences of MNP in Rifampin

Rifampin is a semi-synthetic drug derived from *Amycolatopsis mediterranie*, which naturally produces the antibiotic rifamycin B (reviewed in Wohlfart et al.[25]). During the last step of the synthesis process, conversion with 1-amino-4 methylpiperazine (AMP) results in the formation of rifampin. However, it should be noted that AMP is a precursor for MNP (Fig. 1) and it is thought that AMP reacts with free nitrites (hydrolysed from alkyl nitrites during the reaction) or can be oxidized by aerial oxygen to form MNP,[25] thus contaminating batches of rifampin. Another potential source of MNP in rifampin is through thermal degradation. Tao and colleagues demonstrated that MNP levels can increase by 25% when stored at 40 °C, and levels even double when stored at 60 °C.[26]

The carcinogenic risk of MNP in humans has not been evaluated, and animal studies have yielded mixed results. Chronic MNP inhalation in rats (340 mg/kg) for 15 h/day over 7.5 months resulted in nasal tumours in all exposed rodents. In addition, acute (1 h) inhalation (5 mg/kg) nearly doubled DNA damage observed in rat nasal mucosal cells. However, there are discrepant carcinogenic results for chronic exposure when administered orally (in drinking water), with only two of four rodent bioassays implicating tumour induction and reduced lifespan (reviewed in Klein et al.[27]). This inconsistency also continues into *in vitro* models, with both negative[28] and positive[29] mutagenesis results reported for MNP. Therefore, as precaution, regulatory agencies have indicated that MNP impurity in rifampin capsules above the AI limit should not be administered to healthy volunteers.[17]

Determining MNP Acceptable Intake Limits

The Acceptable Intake (AI) limit refers to the daily exposure to a compound that approximates a 1 in 100,000 cancer risk after 70 years of daily exposure.[12] Mathematically, the AI limit of a given drug in parts per million (ppm) is the function of 50% tumour incidence (TD_{50}) divided by the administered dose.[30] Noteworthy, while regulatory agencies agree that MNP exposure above the AI limit in healthy volunteers poses a considerable risk to study participants, the distinct agencies have set different cutoffs. The reason for this discrepancy stems from the limited data specifically with regard to MNP

Table 1
DDI Substrates and Inhibitors Impacted by or at Risk of N-nitrosamine Contaminations.

Perpetrator, *Drug Class*	Role in DDI Studies[14]	N-nitrosamine Impurity	Impact to DDI Studies	Alternatives for DDI Studies[14]
Perpetrator drugs with confirmed N-nitrosamine Impurities[1,58]				
Ranitidine *Histamine-2 blocker*	Acid reducing agent	NDMA	Product removed from the market in 2019, use alternatives	Famotidine or study with proton pump inhibitor (esomeprazole or rabeprazole)[9]
Nizatidine *Histamine-2 blocker*	Acid reducing agent	NDMA	Product recalled in 2020, use alternatives	Famotidine or study with proton pump inhibitor (esomeprazole or rabeprazole)[9]
Metformin *Anti-hyperglycemic agent*	OCT2, MATE1/2K substrate	NDMA (ER-metformin only)	No impact	IR-metformin is available for DDI studies and does not contain impurity[11]
Rifampin *Anti-infective agent*	1. Strong CYP3A4 2. OATP1B1/3 inhibitor (single dose)	MNP	Batches available for patients only,[33] use alternatives	1. Carbamazepine, efavirenz, lumacaftor, phenytoin 2. Atazanavir & ritonavir, clarithromycin, cyclosporin, gemfibrozil, lopinavir, ritonavir,
Propranolol *Beta-blocker*	Moderate CYP2D6 sensitive substrate	Nitroso-propranolol	Product recalled by Health Canada in 20,22[7], consider alternatives	Encainide, propafenone
Examples of perpetrator drugs at potential risk of NDSRI[7]				
Clopidogrel *Antiplatelet*	Moderate CYP2C8 index inhibitor	Risk due to tertiary amine	Minimal, alternative use optional	Gemfibrozil (strong index inhibitor)
Metoprolol *Beta-blocker*	Moderate CYP2D6 sensitive substrate	Risk due to secondary amine	Minimal, alternative use optional	Encainide, Propafenone
Fluoxetine, Paroxetine *Antidepressant*	1. Strong CYP2D6 index inhibitors 2. Strong CYP2C19 inhibitor (fluoxetine only)	Risk due to secondary amine	Minimal, alternative use optional	1. Mirabegron (moderate inhibitor) 2. Fluconazole
Duloxetine *Antidepressant*	1. Sensitive CYP1A2 substrate 2. Moderate CYP2D6 inhibitor	Risk due to secondary amine	Minimal, alternative use optional	1. Alosetron, Caffeine, Melatonin, Ramelteon, Tasimelteon, Tizanidine 2. Mirabegron
Desipramine *Antidepressant*	Sensitive CYP2D6 index substrate	Risk due to tertiary amine	Minimal, alternative use optional	Dextromethorphan, Nebivolol
Imipramine *Antidepressant*	Moderate CYP2D6 sensitive substrate	Risk due to tertiary amine	Minimal, alternative use optional	Encainide, Propafenone
Venlafaxine *Antidepressant*	R-venlafaxine sensitive CYP2D6 substrate S-venlafaxine moderate sensitive CYP2D6 substrate	Risk due to tertiary amine	Minimal, alternative use optional	Dextromethorphan
Ticlopidine *Antiplatelet*	Strong CYP2C19 inhibitor	Risk due to tertiary amine	Minimal, alternative use optional	Fluconazole

CYP, cytochrome P450; **DDI**, drug-drug interaction; **ER**, extended release; **IR**, immediate release; **MATE1/2K**, multidrug and toxin extrusion proteins 1/2K; **MNP**, 1-methyl-4-nitrospiperazine; **NDSRI**, nitrosamine drug substance related impurity; **NMDA**, N-nitrosodimethylamine; **OATP1B1/3**, organic anion transporting polypeptides 1B1/3; **OCT2**, organic cation transporter 2.

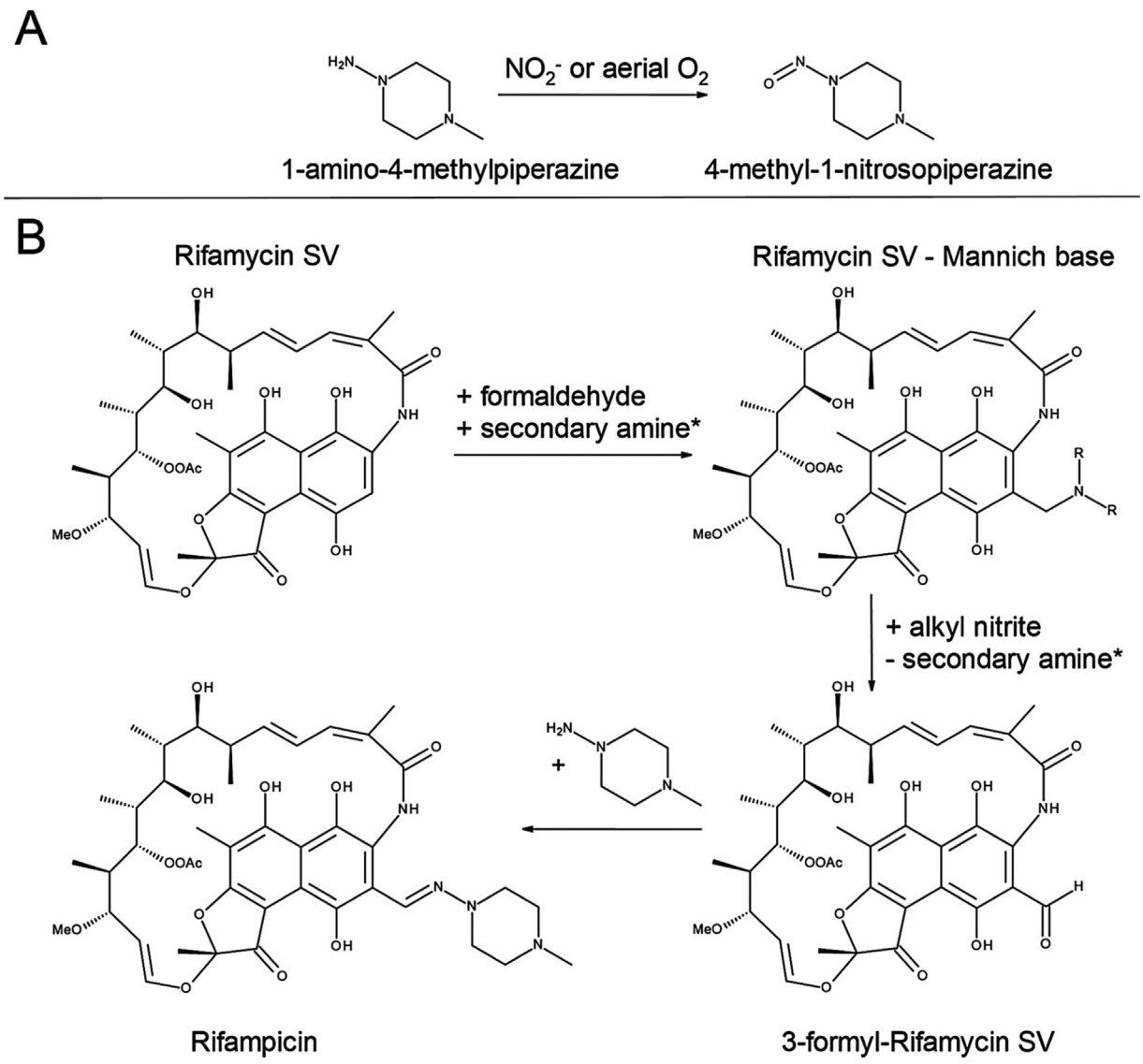

Figure 1. Formation of MNP during the synthesis of rifampicin. (A) In the presences of free nitrites or aerial oxygen, 1-amino-4-methylpiperazine (AMP) is converted to MNP. (B) Rifampin (a.k.a. rifampicin) synthesis steps include the fermentation of rifamycin B to rifamycin SV (not shown), followed by a Mannich reaction of rifamycin SV, subsequent oxidation (e.g. alkyl nitrite) and reaction with AMP to yield rifampin. Reprinted from J Pharm Biomed Anal. 2021 Sep 5;203:114,205, Wohlfart J, Scherf-Clavel O, Kinzig M, Sörgel F, Holzgrabe U., The nitrosamine contamination of drugs, part 3: Quantification of 4-Methyl-1-nitrosopiperazine in rifampicin capsules by LC-MS/HRMS, Copyright (2021), with permission from Elsevier.

carcinogenicity, the lack of direct studies evaluating its carcinogenicity in humans and inconsistent animal findings, therefore surrogate values are applied to determine the AI limit. The FDA extrapolates rodent TD_{50} from a known carcinogenic nitrosamine, NDMA (96 ng/day) to calculate MNP AI limit of 0.16 ppm for 600 mg rifampin dose.[30] However, when (consistent) animal data is not available, the EMA guideline references two approaches to determine AI limit.[31] The more conservative approach is to use the threshold of toxicological concern (TTC) value, i.e. 18 ng/day based on an estimated value for the entire nitrosamine class. The other option is a read-across method applying TD_{50} of a structurally similar compound to the nitrosamine in question. For MNP, a read-across approach applying NDEA (26.5 ng/day)[31] converts to an MNP AI limit of 0.04 ppm for rifampin at a dosing regimen of 600 mg rifampin per day. Interestingly, neither NDMA nor NDEA contain a piperazine ring, thus causing debate about how “structurally similar” they are to MNP (Fig. 2). A recent review by Dobo et al. summarized the TD_{50} from eight distinct N-nitrosopiperazines, and proposed 153 ng/day, a value derived from 1,2,6-trimethyl-4-nitrosopiperazine, which represents the lowest, reproducible TD_{50} for the structural class.[32] Nonetheless, whether the AI limit is set to 0.16 ppm, 0.04 ppm, or 0.255 ppm based on FDA, EMA, or Dobo et al. recommendations, respectively, current batches of rifampin contain MNP far exceeding these cutoffs (1.49–3.47 ppm),[33] and remain prohibited for healthy volunteer DDI studies. However, to avoid drug shortages and allow for continued TB treatment in the short run, the FDA have raised the AI limit of MNP in rifampin to 5 ppm to ensure adequate supplies for patient use only,[33] considering that the risk of not treating TB outweighs the theoretical risk of cancer.

Impact of Nitrosamine Risk Assessment Guidance

While only a small proportion (18%) of N-nitrosamines are non-carcinogenic,[34] this analysis focused on simple dialkyl nitrosamine molecules and this estimate could change when newly identified NSDRIs are considered as these compounds tend to be less

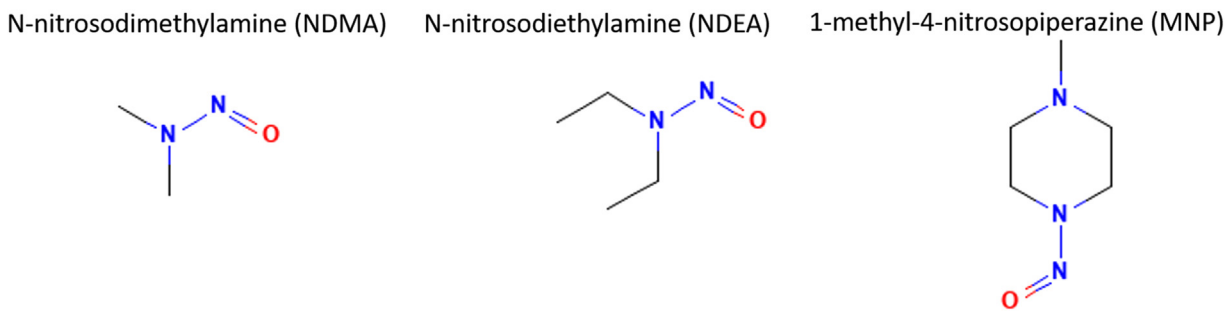

Figure 2. N-nitrosamine Chemical Structures, Adapted from PubChem Compound Summary.[60-62]

mutagenic.[7] An *in silico* structural analysis of pharmaceutical drugs suggests that up to 40% of API and 30% of the API impurities are potential N-nitrosamine precursors due to their presences of secondary or tertiary amines at risk of nitrosation.[7] These 'at risk' precursors should be evaluated for NSDRI or small N-nitrosamine degradation products such as NDMA and NDEA. Therefore, to address current N-nitrosamine concerns, regulatory agencies issued guidance to control N-nitrosamine impurities in existing medications as well as new drugs in development,[12,31,35,36] which will have a lasting impact on drug development and manufacturing. The 3-step process entails a risk assessment phase, confirmatory testing if risks are identified, and mitigation plans to prevent or reduce impurities. According to the EMA, a possible risk of N-nitrosamines impurities was identified in approximately 15% of small molecules, and as of February 2022, 2% of this subgroup with a risk of impurities were found to contain N-nitrosamines above the AI limit.[37] The FDA recommends potential drug synthesis or manufacturing remediation strategies that include using bases other than secondary, tertiary or quaternary amines; replacing nitrous acid with other quenching agents for azide decomposition; the addition of antioxidants to formulations to sequester oxidizing agents; and/or modification of the reaction micro-environment to neutral or basic pH so as to avoid an acidic pH condition, since a low pH environment tends to promote nitrosamine formation.[12] Altogether, it is anticipated that this guidance will help prevent future drug contaminations from reaching the market, which eventually should also pave the way for re-commencement of DDI studies using rifampin as a perpetrator. However, the temporary increase in AI limit for MNP in rifampin batches may remove any incentive manufacturers have to incur the cost of revising the manufacturing process and, hence, the issue of N-nitrosamine impurities may persist. Therefore, alternative CYP3A4 inducers for DDI studies will need to be considered.

Clinical DDI Studies Post-Rifampin Impurities

While the FDA (per Sponsor communication) and EMA[17] were amongst the first agencies to halt the use of rifampin in DDI studies enroling healthy subjects, the MHRA permitted its administration until late 2021, as reported on ClinicalTrials.gov (NCT05098041). A plausible approach for the continued use of rifampin would involve the implementation of a diet low in nitrosamines during a DDI study to reduce overall exposure, as described in a recent paper from our group.[38] In brief, because N-nitrosamines are common constituents in various foods, beverages, cosmetics, water and tobacco products,[18,19] limiting intake or use of these products has been postulated to offset the exposure risk. However, this simple mitigation step may not fully account for the potential toxicological risk of MNP exposure. Moreover, while most DDI studies require multiple doses of rifampin to establish a drug interaction, only a

Table 2
Summary of Rifampin and Alternative CYP3A4 Inducer Characteristics.

CYP3A4 Inducer	Induction Pathway	Other CYP Induction	Typical DDI Dose Regimen	Safety Profile	Overall Experience in DDI Studies[1]
Rifampin	PXR	Strong CYP2C19; moderate CYP1A2, CYP2B6, CYP2C8, CYP2C9 and P-gP clinical inducer	600 mg QD for 14 days	Excellent safety record	++++
Carbamazepine	CAR; minor PXR	Strong CYP2B6; moderate CYP2C8 and CYP2C9; weak CYP1A2, CYP2C19; P-gp clinical inducer	Dose titration: from 100 mg BID to 300 mg BID, for a total of 14–24 days	• Label contains black box warning • Risk of severe cutaneous adverse reactions • Hypersensitivity reactions risk increases with positive HLAB*1502 allele	+++
Phenytoin	CAR; minor PXR	Moderate CYP1A2, CYP2C9, and CYP2C19; P-gp inducer	100 mg TID for 14-21 days	• Narrow therapeutic window • Risk of seizures & neurological events	++
Lumacaftor	PXR	None	Combination drug: 200 mg lumacaftor / 250 mg ivacaftor BID for 21 days	Favourable safety profile	+
Efavirenz	PXR moderate induction	Moderate CYP2B6; weak CYP1A2 and CYP2C19; P-gp clinical inducer	600 mg QD for 8-21 days	Generally well tolerated	+++

1. Per ClinicalTrials.gov search. **BID**, twice daily; **CAR**, constitutive androstane receptor; **PXR**, pregnane X receptor; **QD**, once daily; **TID**, three times daily.

Table 3
Carbamazepine vs Phenytoin DDI Studies.

	Carbamazepine DDI Studies				Phenytoin DDI Studies				CBZ vs PHT
Substrate, *Drug Class* Metabolizing CYPs	Substrate Dose	CBZ Dose	Population	AUC Change	Substrate Dose	PHT Dose	Population	AUC Change	
Quetiapine *Anti-psychotic* Sulfoxidation and CYP3A4	Dose titration: 25 mg BID, 1 day → 300 mg BID, 29 days → 300 mg QD, 1 day	Dose titration: 200 mg QD, 1 day → 200 mg BID,3 days → 200 mg TID, 21 days → 200 mg QD, 1 day	18 M/F with schizophrenia, schizoaffective disorder, or bipolar disorder	−87%[45]	25-250 mg TID, 8 days → 250 mg TID, 12 days → 250 QD, 1 day	100 mg TID, 10 days	17 M with schizophrenia, schizoaffective disorder, or bipolar disorder	−80%[43]	Both induced a strong reduction
Ivabradine *HCN-gated channel blocker* CYP3A4	10 mg SD	400 mg QD, 16 days	18 M HV	−80%[46]	10 mg SD	150 mg BID, 5 days	18 M HV	−69%[47]	CBZ induced strong reduction. Moderate reduction with PHT over 5 days of dosing
Mirtazapine *Anti-depressant drug* CYP2D6, CYP1A2 and CYP3A4	Dose titration: 15 mg BID, 2 days → 30 mg BID, 5 days → 30 mg QD, 1 day 8 → 30 mg BID, 23 days	Dose titration: 200 mg QD, 1 day → 200 mg BID, 4 days → 400 mg QD, 14 days	24 M HV	−61%[48]	Dose titration: 15 mg QD, 2 days → 30 mg QD, 15 days	200 mg, 10 days	8 M HV	−47%[49]	Moderate reduction with CBZ and mild reduction with PHT co-administration
Quinidine *Anti-malarial medication* CYP3A4	200 mg SD	200 mg BID, 2 days → 400 mg BID, 14 days	8 M HV	−61%[51]	300 mg	Dose adjust to maintain plasma conc. 10-20 μg/ml	2 HV	−56%[51]	Similar mild reduction
Albendazole *Anthelmintic drug* CYP3A4	7.5 mg/kg BID	10-20 mg/kg, 8 days	9 M/F		7.5 mg/kg BID	3-4 mg/kg, 8 days	9 M/F		Moderate reduction
Metabolites: (+)-sulfoxide (-)-sulfoxide				−49%[50] −67%[50]				−66%[50] −78%[50]	
Pregabalin* *Pain medication* Negligible metabolism	200 mg TID, 8 days (fasting)	Stable therapy 300-1000 mg/day	14 M/F with epilepsy	−8%*,[59]	200 mg TID, 8 days (fed)	Stable therapy 100-500 mg/day	11 M/F with epilepsy	−14%*,[59]	No effect*

* Co-administration of pregabalin in patients on maintenance antiepileptic drug therapy (CBZ or PHT), results compared to published HV studies. **BID**, twice daily; **CBZ**, carbamazepine **F**, female; **HCN**, hyperpolarization-activated cyclic nucleotide; **HV**, healthy volunteer; **M**, male; **PHT**, phenytoin; **QD**, daily dosing; **SD**, single dose.

single dose of 600 mg rifampin to healthy subjects, administered as two doses of 300 mg, would be sufficient in DDI studies using rifampin as an inhibitor of drug transporter proteins like OATP1B1/3. However, the potential MNP carcinogenicity cannot simply be quantified in terms of a linear dose-response as also mentioned above and, therefore, even a single dose still represents a possible risk. Taken altogether, irrespective of whether the mitigation strategies outlined above could offer any solace, the administration of rifampin to healthy subjects is no longer approved by any of the major regulatory authorities.

Therefore, to ensure that the conduct of DDI studies can proceed as part of new drug development, there are several alternative CYP3A4 inducers that can be considered for healthy subjects. We previously reviewed candidate CYP3A4 inducers recommended by the FDA for DDI studies elsewhere,[38] where we championed phenytoin as a safe and effective inducer, however, carbamazepine has also been applied effectively in DDI studies by others (reviewed in Bolleddula et al.[39]). Moreover, the FDA has recently listed lumacaftor (in combination with ivacaftor) as a strong inducer of CYP3A4 for concomitant use clinical DDI studies and/or drug labelling, while efavirenz conceivably may have a role in DDI studies too.[8,40] A brief summary will be provided hereafter.

Phenytoin is an anticonvulsive drug and, like rifampin, induces CYP3A4 and CYP2C19 (though the latter to a lesser degree) (Table 2). It has been applied as a CYP3A4 inducer in over a dozen of DDI studies[41] and demonstrated a comparable reduction in substrate concentration to that of rifampin (reviewed in Van Haarst[38]). Moreover, phenytoin induced a strong reduction (≥80%) in substrate exposure of nisoldipine[42] and quetiapine.[43] In contrast to the good tolerability profile of rifampin, a disadvantage of phenytoin is that it has the potential to induce seizures and neurological events (Table 2). Phenytoin is metabolized by CYP2C9 and CYP2C19, has a long half-life (14–22 h) and a narrow therapeutic window. Therefore, for DDI studies it is important to genotype and exclude healthy subjects that are CYP2C9 or CYP2C19 poor metabolizers. In addition, subjects with a history of seizures, neurological conditions and suicide ideation should be excluded. Moreover, the common daily dose of 300 mg phenytoin prescribed to patients can be divided into 2–3 smaller doses spread over the day (e.g. 100 mg TID), when applied in DDI studies to healthy volunteers, so as to avoid plasma levels exceeding the therapeutic window.[40] With these risk mitigation measures in place, application of phenytoin in DDI studies at a dose of 300 mg/day, administered as 100 mg TID, was found to be safe and well tolerated in healthy volunteers.[38,41] Owing to the long half-life, 14–21 days are required before the CYP3A4 substrate is co-administered in order that a maximum induction of CYP enzymes is accomplished.

Carbamazepine is a commonly used alternative candidate.[39,40] Like phenytoin, carbamazepine is also indicated for seizures. However, it holds a black box warning due to risk of serious or fatal dermatologic reactions. Severe cutaneous adverse reactions have been associated with *HLAB*1502* allele, a gene commonly expressed in Asian populations.[44] Therefore, exclusion of this group for DDI studies or genotyping is recommended before administration. To further mitigate AEs, a dose titration regimen is also required. Carbamazepine is metabolised by CYP3A4 and is considered an auto-inducer. It also has a moderate induction effect on CYP2C8, CYP2C9 and is a weak inducer of CYP1A2, CYP2C19, and P-gP clinical inducer.[39] While there is far more clinical experience with carbamazepine for DDI studies than with phenytoin, their impact on substrate exposure tends to be similar (Table 3). In patients with a diagnosis of psychiatric disorders, quetiapine concomitant administration with carbamazepine drastically reduced quetiapine exposure by 87%.[45] Likewise, phenytoin induced an 80% reduction in quetiapine AUC.[43] Moreover, in healthy volunteers, 15 days of carbamazepine administration reduced ivabradine bioavailability by approximately 80%.[46] When a DDI study examining the impact on ivabraine was conducted with phenytoin as the inducer, ivabraine exposure decreased by 69%.[47] Of note, in the latter study, phenytoin was only administered for 5 days, and may not have reached maximum induction of CYP3A4 and, therefore, if given a longer run-in period, the decrease in ivabraine exposure could have been closer to that caused by carbamazepine. In addition, mirtazapine,[48,49] albendazole[50] and quinidine[51] also demonstrated fairly similar reductions in exposure when co-administered with carbamazepine or phenytoin (Table 3). Altogether, carbamazepine and phenytoin have a comparable effect on drug PKs, and either are suitable alternatives for rifampin CYP3A4 inducer for DDI studies.

Lumacaftor can treat the underlying defect in the F508del cystic fibrosis transmembrane conductance regulator (CFTR) protein in cystic fibrosis. It is marketed in combination with ivacaftor under the brand name Orkambi. Both drugs are so-called CFTR modulating agents; lumacaftor is an F508del CFTR protein stabilizer, whereas ivacaftor is a CFTR potentiator. Recent updates to the FDA recommendation for DDI studies in late August 2020 now list lumacaftor as a strong inducer of CYP3A4 that decreases the AUC of sensitive index substrates by ≥80%.[14] For instance, co-administration of lumacaftor with ivacaftor reduces the exposure to ivacaftor with more than 80%. A similar high effect on itraconazole exposure has been reported based on historical data.[52] The safety profile of lumacaftor (in the combination with ivacaftor) is favourable compared to phenytoin and carbamazepine, which would make it a potentially ideal candidate to replace rifampin in DDI studies. Moreover, ivacaftor has a negligible effect as a perpetrator and would therefore not be anticipated to meaningfully impact DDI results.[39] Another potential advantage for lumacaftor is that it shares the same CYP3A4 induction pathway as rifampin[53] by activating the pregnane X receptor (PXR).[54] While being a strong CYP3A4 inducer, however, lumacaftor currently lacks robust experience in DDI studies, in particular in combination with an index CYP3A4 substrate like midazolam.

Efavirenz is listed as a moderate index inducer of CYP3A4 in ICH M12 DDI Guidance document,[8] suggesting it is a potential candidate for use in CYP3A4 induction DDI studies. However, the ICH guidance dictates that strong index inducers be used in DDI studies, while moderate index inducers would be useful if strong index inducers are not available. Against the background of the abovementioned alternatives of phenytoin and carbamazepine, efavirenz would therefore not be the preferred option to replace rifampin. Moreover, there is conflicting data concerning potential selectivity of efavirenz towards induction of hepatic and intestinal CYP3A4, on the one hand suggesting a lack of intestinal CYP3A4 induction after treatment with efavirenz (up to 400 mg/day for 9–10 days)[55,56] and on the other hand demonstrating induction of both hepatic and intestinal CYP3A4 following administration of efavirenz (at 600 mg/day for 2 weeks) .[57] Uncertainty concerning efavirenz' induction potential towards intestinal CYP3A4 would leave this inducer less appropriate for use in DDI studies involving orally administered drugs.

Conclusion

A unified approach to detect, report and control N-nitrosamines in medications taken by all major regulatory authorities has led to an uptick in contaminated products, recalls and the need to seek alternatives or replacement drugs for both patients and drug developers. With regard to clinical development of new candidate drugs, a variety of perpetrator and victim drugs employed in DDI studies are at risk of containing N-nitrosamine impurities, and in several cases N-nitrosamine impurities have effectively been reported in commercial batches. Thus far, however, N-nitrosamine impurities have only critically impacted the supplies of rifampin and, as a consequence, drug

development in general, since rifampin is the primary clinical index drug employed in CYP3A4 induction DDI studies.

The presence of MNP in rifampin batches has shifted the risk-benefit ratio for both patients and clinical DDI study participants. While a temporary elevation of the AI limit has been justified to provide patients access to life-saving rifampin treatment, its administration remains prohibited for healthy volunteers in DDI studies that are conducted as part of clinical drug development. To this end, phenytoin and carbamazepine have emerged as suitable replacements, as both are strong CYP3A4 inducers and result in a similar reduction in substrate exposure and, therefore, the continuation of safe development of new investigational products can proceed. In our view, phenytoin holds an advantage over carbamazepine because of a better safety profile and no need for dose titration.

Resolving the N-nitrosamine impurity issues will take time and manufacturer investments, yet with the application of alternative drugs administered as perpetrator in DDI studies there remains a forward path for drug development.

Author Contributions

S.P.: Writing – original draft. A.v.H.: Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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