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Article

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The metabolic fate of the isocyanide moiety. Are isocyanides pharmacophore groups neglected by medicinal chemists?

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Abstract

Despite the isolation of hundreds of bioactive isocyanides from terrestrial fungi and bacteria as well as marine organisms, the isocyanide functionality has so far received scanty attention from a medicinal chemistry standpoint. The widespread tenet that isocyanides are chemically and metabolically unstable has restricted bioactivity studies to their antifouling properties and technical applications. In order to confirm or confute this idea, the hepatic metabolism of six model isocyanides was investigated. Aromatic and primary isocyanides turned out to be unstable and metabolically labile, but secondary and tertiary isocyanides resisted metabolization, showing, in some cases, cytochrome P450 inhibitory properties. The potential therefore exists for the secondary and the tertiary isocyanides to qualify them as pharmacophore groups, in particular as war-heads for metalloenzyme inhibition because of their potent metal-coordinating properties.

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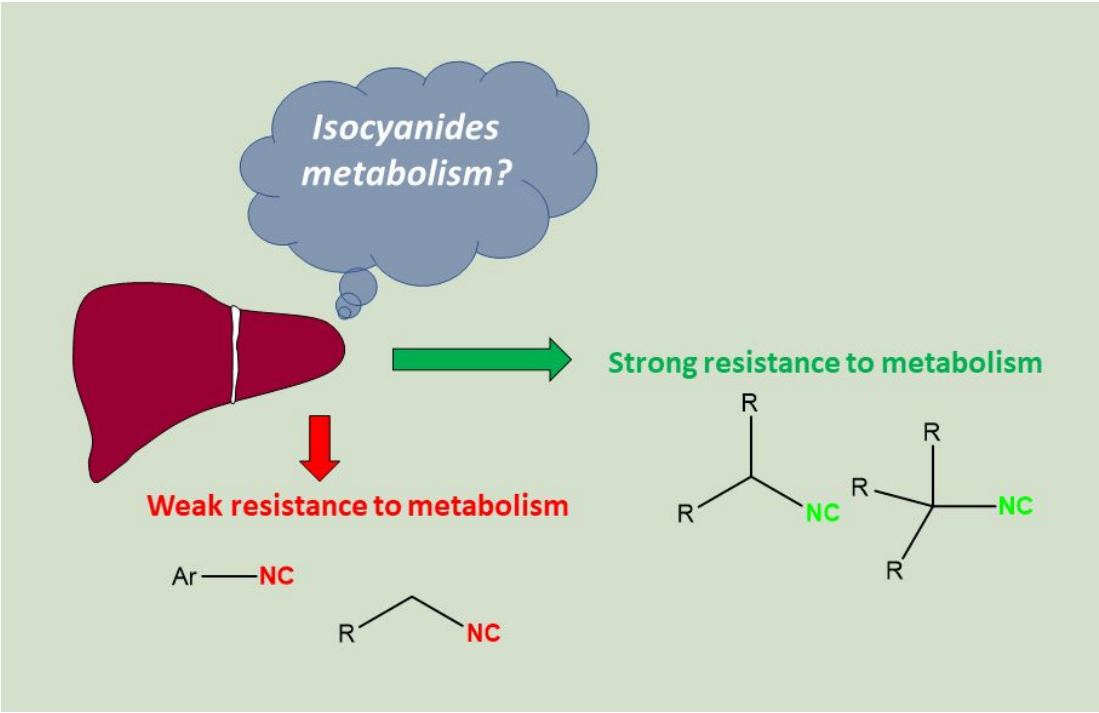


Introduction

Isocyanides (also known as isonitriles or carbylamines) represent a uniqueness in the study of functional groups in organic chemistry.[1] Indeed, due to their resonance form they can behave both as carbenes and nucleophiles and can easily undergo the so-called α-addition. The passage from a divalent to a tetravalent status is a highly exothermic and favorable process and it lies at the bottom of the isocyanide mediated multicomponent reactions.[2]

Furthermore, the isocyanide group is a coordinating agent for several metals thanks to its ability to act as strong σ-type donor.[3] At this point, two different scenarios are possible. In the first case, the complexes are usually stable with the isocyanide group inert to chemical reactions. For example, they are well-known the strong coordination properties of methylisocyanide with the heme group of hemoglobin,[4] the interaction with both ferric and ferrous iron of the cytochrome P450 (CYP) with alkylisocyanides,[5] the irreversible inhibition of xanthine oxidase by means of ethylisocyanide,[6] and the properties of technetium based contrast agents containing isocyanides which reached the market with the commercial myocardial perfusion agent Cardiolite® [Tc-MIBI]. In the second case, the interaction with the metal (*e.g.* silver and copper), increases the acidity of the hydrogen at the α-position of the isocyano group toward nucleophiles triggering a plethora of novel transformations which are regularly reviewed.[7] Probably, due to the ease of hydrolysis to formamide under slightly acidic conditions, chemists were distrustful of isocyanides as potential pharmacophore group to be used in medicinal chemistry. This idea is also supported by the fact that a leading book as "*The practice of medicinal chemistry*" third edition edited by Wertmuth in Chapter 7 lists isocyanides as metabolic labile pharmacophore groups which should not be used in medicinal chemistry.[8] Unfortunately, this statement is not supported by literature references and our search on Sci-Finder database did not result in any systematic study on the metabolic fate of isocyanides. On the other hand, in nature hundreds of isocyanides have been isolated and many have demonstrated to possess biological activity. The intricate structure of some of them, spurred synthetic chemists to attempt their total synthesis, with a minor interest on the effective biological role played by the isocyanide

group. Some authors speculated that the isocyanides can be used as a masked war-head which can be oxidized to isocyanates, strong carbamoylating groups of proteins, responsible for the antifouling, antibiotic, antifungal and antitumor properties of some of these isocyanide containing natural products. To this point, some nudibranchs do not synthesize isocyanoterpene as protective compounds by their own but obtain them from specific sponges used as food source. Anyway, the picture seems more complex and it is nowadays clear that the isocyanide group plays a role as a real pharmacophore group. Over the last decades, both natural and synthetic isocyanide containing molecules have emerged from literature as potent biologically active compounds.[9] For example, xantocillins (**A**) isolated both from soil and marine fungi, apart their well-recognized antibiotic properties, showed to be thrombopoietin receptor agonists,[10] autophagy inducer with potent anti-proliferative activity on a panel of human solid tumors,[11] and prostaglandin synthesis inhibitors.[12] Natural product axisonitrile-3 (**B**) as many others, showed strong antiplasmodial activity due to the ability of the isocyanide group to complex the iron atom of heme preventing the crystallization of hematin to hemozoin.[13] Hapalindoles such as **C** were active as blockers on sodium channels,[14] while kalihinol B (**D**) was a very potent antimicrobial agent against chloroquine-resistant FCR-3 *Plasmodium falciparum*.[15]

Recently, synthetic isocyanides such as **E** appeared in medical literature as agents against multidrug-resistant *Staphylococcus aureus*,[16] and inhibitors of M2 channels in influenza A virus H5N1 such as **F**,[17] while the antifouling agent **G** was demonstrated to be selective, able to perturb only two important enzymes in the aquatic organism *B. amphitrite* namely a NADH-ubiquinone oxidoreductase, and cytochrome P450 [18] (Figure 1).

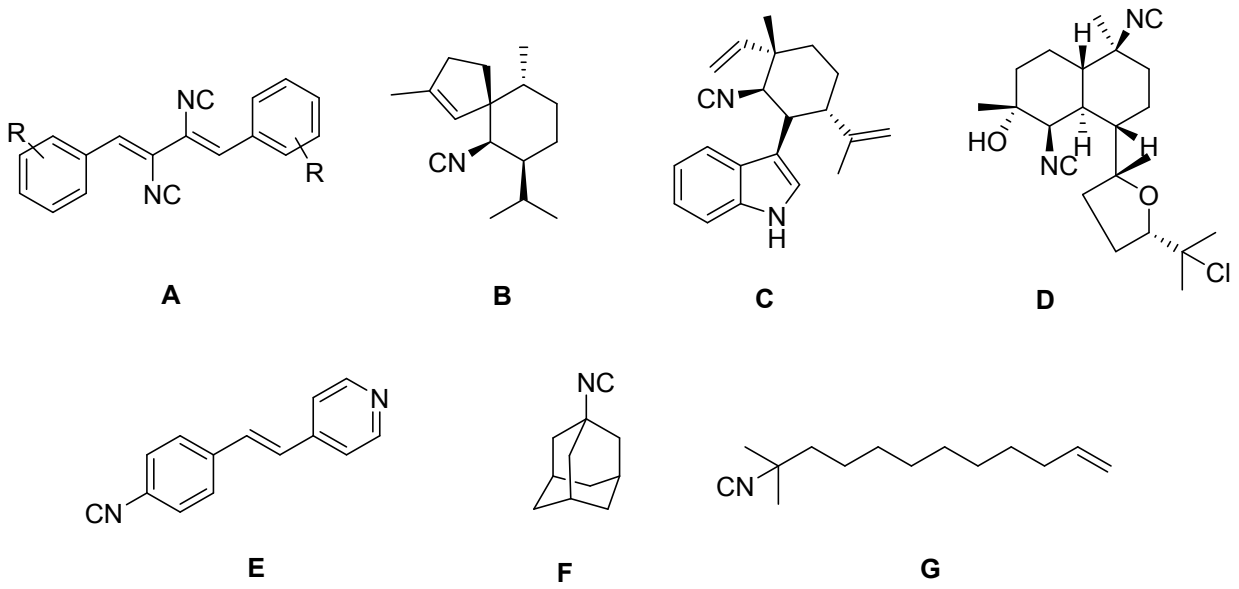


Figure 1. Selected examples of biologically active isocyanides.

All these results might support the idea of using the isocyanide functional group as a unique pharmacophore group useful for the discovery and development of novel biologically active compounds. For this reason, with the intent to evaluate the metabolic stability of the isocyanide group in the bloodstream, and the occurrence of hepatic metabolism, we studied the *in vitro* metabolic transformation of six different isocyanide models (primary, secondary, tertiary aliphatic isocyanides, aryl and benzylic isocyanides **1-6**), (Figure 2).

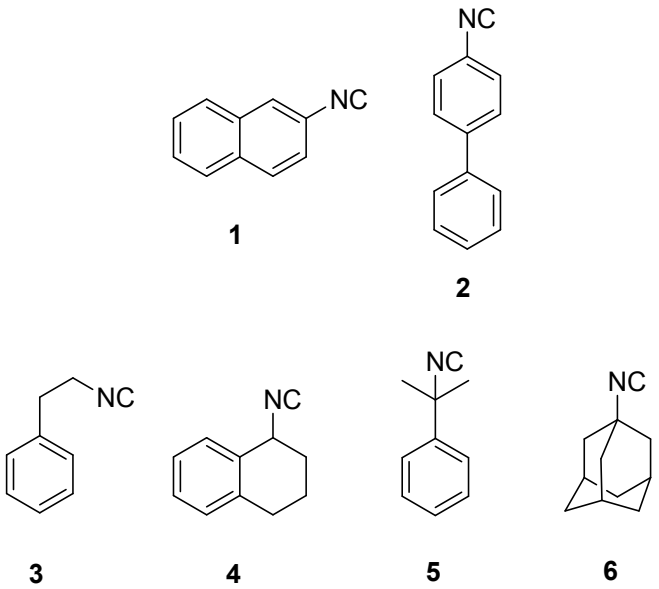


Figure 2. Models used to study the isocyanides metabolism.

Experimental procedures

Isocyanides and formamides were synthesized according to known procedures (Scheme S1, Supporting Information). The purity of compounds was determined by high performance liquid chromatography coupled with ultraviolet-visible detector (LC-UV) using the instrumentation and methods reported in Supporting Information. Purity of all final compounds was ≥ 95%.

General. Commercially available reagents and solvents were purchased from Sigma-Aldrich (Merck) or Alfa Aesar and were used without further purification. Tetrahydrofuran (THF) was distilled immediately before use from Na/benzophenone under a slight positive atmosphere of dry nitrogen. Dichloromethane was dried by distillation from P_2O_5 and stored on activated molecular sieves (4 Å). When needed the reactions were performed in flame or oven-dried glassware under a positive pressure of dry nitrogen. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a JEOL ECP 300 MHz spectrometer. Chemical shifts are reported in parts per million (ppm). Mass spectra were acquired with a Thermo Finningan LCQ-deca XP-plus equipped with an ESI source and an ion trap detector. High resolution mass spectrometry data were acquired with a Thermo Scientific Q-Exactive™ Plus Hybrid Quadrupole-Orbitrap™ mass spectrometer. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM). Thin layer chromatography (TLC) was carried out on 5 × 20 cm plates with a layer thickness of 0.25 mm (Merck Silica gel 60 F_{254}). When necessary they were developed with $KMnO_4$ reagent or Dragendorff reagent.

Compounds **1A–6A** (Scheme S1) were commercially available, while 2-isothiocyanato naphthalene, and 1-isothiocyanato-1,2,3,4-tetrahydro-napthalene were synthesized as described previously.[19]

General Procedure A for the synthesis of formamides (1FA-6FA): Formic acid (103.6 mmol, 7.0 equiv) and acetic anhydride (88.9 mmol 6.0 equiv) were allowed to mix for 1 h at 25 °C, after

which the appropriate amine (14.8 mmol 1.0 equiv) was added portionwise to the mixture at 0 °C. The ice bath was removed, and the reaction was stirred at 25 °C until the amine was consumed completely as determined by TLC (1-3 h). The reaction mixture was diluted with water (100 mL), the aqueous phase was extracted with CH_2Cl_2 (2×100 mL). The combined organic phases were washed with water (1×70 mL), brine (1×70 mL), dried over Na_2SO_4, filtered and concentrated to give the desired product without purification.

N-(naphthalen-2-yl)formamide (**1FA**). The title compound was prepared from naphthalen-2-amine (**1A**): off-white solid, yield 75%, m.p 132-134 °C; IR (KBr) 3055, 2919, 1728, 1529, 1299, 1279, 849, 827 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$, mixture of rotamers 1:1): δ 8.85 (d, J = 11.0 Hz, 1-H), 8.58 (br s, 1-H), 8.46 (s, 1-H), 8.22 (s, 1-H), 7.85-7.77 (m, 6-H), 7.67 (br s, 1-H), 7.53-7.39 (m, 6-H), 7.23 (s, 1-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$ mixture of rotamers 1:1): δ 162.9, 161.4, 134.4, 134.3, 133.8, 133.7, 131.2, 130.8, 130.1, 129.0, 127.9, 127.8, 127.7, 127.3, 127.2, 126.8, 125.6, 125.4, 119.7, 118.9, 117.2, 115.3 ppm. MS (ESI): *m/z* 172 $[M + H]^+$.

N-((1,1'-biphenyl)-4-yl)formamide (**2FA**). The title compound was prepared from 4-aminobiphenyl (**2A**): yellowish solid, yield 90%; ^{1}H-NMR (300 MHz, $CDCl_3$, major rotamer): δ 8.71 (br s, 1-H), 8.45 (s, 1-H), 7.65-7.21 (m, 9-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$, major rotamer): δ 159.3, 140.1, 137.8, 136.3, 128.9, 127.8, 127.4, 127.0, 120.5 ppm. MS (ESI): *m/z* 198 $[M + H]^+$.

N-(2-phenylethyl)formamide (**3FA**). The title compound was prepared from 2-phenylethanamine (**3A**): pale yellow oil, yield quantitative; ^{1}H-NMR (300 MHz, $CDCl_3$, major rotamer): δ 8.03 (s, 1-H), 7.27-7.16 (m, 5-H), 6.38 (br s, 1-H), 3.51-3.42 (m, 2-H), 2.79-2.77 (m, 2-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$, major rotamer): δ 161.7, 138.7, 128.8, 128.7, 126.7, 39.4, 35.5 ppm. MS (ESI): *m/z* 150 $[M + H]^+$.

N-(1,2,3,4-tetrahydronaphthalen-1-yl)formamide (**4FA**). The title compound was prepared from 1,2,3,4-tetrahydronaphthalen-1-amine (**4A**): off-white solid, yield quantitative; ^{1}H-NMR (300 MHz, $CDCl_3$, major rotamer): δ 7.94 (s, 1-H), 7.12-6.97 (m, 5-H), 5.0 (s, 1-H), 2.67 (s, 2-H), 1.94-1.67 (m 4-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 160.8, 137.1, 135.8, 128.8, 128.2, 126.9, 125.8, 45.8, 29.7, 28.8, 19.7 ppm. MS (ESI): *m/z* 176 $[M + H]^+$.

N-(2-phenylpropan-2-yl)formamide (**5FA**). The title compound was prepared from 2-phenylpropan-2-amine (**5A**): pale yellow oil, yield quantitative; ^{1}H-NMR (300 MHz, $CDCl_3$, major rotamer): δ 7.92 (m, 1-H), 7.80 (br s, 1-H), 7.37-7.24 (m. 5-H), 1.60 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$, major rotamer): δ 166.2, 165.1, 146.3, 128.4, 126.9, 125.0, 30.6 ppm. MS (ESI): *m/z* 164 $[M + H]^+$.

N-((3S,5S,7S)-Adamantan-1-yl)formamide (**6FA**). The title compound was prepared from (3S,5S,7S)-adamantan-1-amine (**6A**): white solid, yield 90%, m.p. 140-141 °C; IR (KBr) 3439, 2900, 2851, 1696, 1134, 1097, 798, 673 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO, major rotamer): δ 7.82 (d, J = 2.2 Hz, 1-H), 7.59 (br s, 1-H), 2.00 (br s, 6-H), 1.91 (d, J = 2.8 Hz, 6-H), 1.75 (d, J = 2.5 Hz, 3-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$, major rotamer): δ 162.6, 50.6, 43.8, 35.8, 29.1 ppm. MS (ESI): *m/z* 180 $[M + H]^+$.

General Procedure B for the synthesis of isocyanides (1-6): Under a nitrogen atmosphere, a stirred solution of the appropriate formamide (3.06 mmol, 1.0 equiv) in dry CH_2Cl_2 (10 mL) at 0 °C was treated with triethylamine (15.3 mmol 5.0 equiv), followed by $POCl_3$ (3.06 mmol 1.0 equiv) dropwise. The reaction was allowed to warm to 25 °C while stirring. After 20 min, the reaction was stopped by the addition of water (30 mL), and the organic layer was extracted with CH_2Cl_2 (2×30 mL). The combined organic extracts were washed with water (2×30 mL), dried over Na_2SO_4, filtered and concentrated to give the crude product. Purification by flash column chromatography PE/EtOAc 95:5 gave the desired isocyanide.

2-Isocyanonaphthalene (**1**). The title compound was prepared from *N*-(naphthalen-2-yl)formamide (**1FA**): pale yellow solid, yield 59%, m.p. 63-65 °C dec; IR (KBr) 3064, 2125, 1507, 1438, 1207, 1152, 894, 821, 754 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.83-7.74 (m, 4-H), 7.56-7.53 (m, 2-H) 7.53 (d, J = 8.9 Hz, 1-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 164.4, 132.7, 132.5, 129.5, 127.8, 127.7, 127.6, 127.4, 125.6, 123.7 (t, J = 12.9 Hz), 123.1 ppm.

4-Isocyano-1,1'-biphenyl (**2**). The title compound was prepared from *N*-((1,1'-biphenyl)-4-yl)formamide (**2FA**): yellowish-green solid, yield 40%, m.p. 77-78 °C; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.62-7.56 (m, 4-H), 7.47-7.43 (m, 5-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 164.7, 142.5, 139.4, 129.1, 128.3, 128.1, 127.2, 126.9 ppm.

(2-Isocyanoethyl)benzene (**3**). The title compound was prepared from *N*-(2-phenylethyl)formamide (**3FA**): yellow-orange oil, yield 51%; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.40-7.24 (m, 5-H), 3.58 (t, J = 6.7 Hz, 2-H), 2.97 (t, J = 6.7 Hz, 2-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 156.4 (t, J = 5.2 Hz), 136.6, 128.6, 128.5, 127.0, 42.8 (t, J = 6.3 Hz), 35.4 ppm.

1-Isocyano-1,2,3,4-tetrahydronaphthalene (**4**). The title compound was prepared from *N*-(1,2,3,4-tetrahydronaphthalen-1-yl)formamide (**4FA**): yellow oil, yield 50%; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.47-7.44 (m, 1-H), 7.30-7.23 (m, 2-H), 7.16-7.14 (m, 1-H), 4.83 (s, 1-H), 2.93-2.72 (m, 2-H), 2.16-2.04 (m, 3-H), 1.89-1.79 (m, 1-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.1, 136.2, 131.9, 129.3, 128.3 (2-C), 126.4, 52.4 (d, J = 5.7 Hz), 30.5, 28.4, 19.1 ppm.

(2-Isocyanopropan-2-yl)benzene (**5**). The title compound was prepared from *N*-(2-phenylpropan-2-yl)formamide (**5FA**): pale yellow oil, yield 60%; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.51-7.32 (m, 5-

H), 1.79 (s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 155.3 (t, J = 5.2 Hz), 142.2, 128.7, 127.8, 124.1, 60.6 (t, J = 5.7 Hz), 31.9 ppm.

1-Adamantyl isocyanide (**6**). The title compound was prepared from *N*-((3S,5S,7S)-adamantan-1-yl)formamide (**6FA**): white solid, yield 76 %, m.p. 185-187 °C (the substance tends to sublimate); IR (KBr) 2914, 2858, 2123, 1454, 1354, 1309, 1103, 1076, 890, 813 cm^{-1}; ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.07 (br s, 3-H), 2.02 (br s, 6-H), 1.65 (br s, 6-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 151.6, 54.4, 43.7, 35.6, 28.8 ppm.

Synthesis of *N*-(naphthalen-2-yl)-5,6-dihydro-1,2,4-dithiazin-3-amine (**1TNMr**)

2-isothiocyanato naphthalene was prepared from naphthalen-2-amine (**1A)** according to the literature method (Scheme S2).[19]

To a stirred solution of 2-mercaptoethylamine hydrochloride (0.061 g, 0.54 mmol, 1 equiv) and triethylamine (0.075 mL, 0.54 mmol, 1 equiv) in dry THF (1.0 mL), 2-isothiocyanato naphthalene (0.10 g, 0.54 mmol, 1 equiv) dissolved in dry THF (1.0 mL) was added dropwise, under nitrogen atmosphere. The reaction mixture was stirred overnight at room temperature, then it was concentrated under reduced pressure and the crude material was purified by column chromatography using PE/EtOAc 8:2, EtOAc and EtOAc/MeOH 9:1 as eluents to give the disulfide intermediate as an off-white solid, yield 76%, ^{1}H-NMR (300 MHz, DMSO): δ 9.8 (br s, 1-H), 8.04 (br s, 1-H), 7.93-7.82 (m, 4-H), 7.51-7.44 (m, 3-H), 3.83 (br d, 2-H), 3.01 (t, J = 6.2 Hz, 2-H) ppm; ^{13}C-NMR (75 MHz, DMSO): δ 180.6, 136.6, 133.3, 130.4, 128.3, 127.5, 127.4, 126.3, 125.2, 123.6, 119.8, 43.1, 36.5 ppm.

A solution of disulfide intermediate (0.043 g, 0.081 mmol, 1 equiv), DL-dithiothreitol (DTT) (0.0375 g, 0.243 mmol, 3 equiv) and two drops of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) as catalyst in 3.0 mL CH_2Cl_2 was stirred at reflux for 2.5 h. Then, the solution was washed with water (×2), brine (×1), dried over Na_2SO_4 and concentrated *in vacuo* to afford product as a pale yellow

solid, yield 54%, ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.34, (br s, 1-H), 7.92-7.72 (m, 4-H), 7.54-7.47 (m, 2-H), 7.33 (d, J = 8.5 Hz, 1-H), 6.66 (br s, 1-H), 3.82 (q, J = 5.8 Hz, 2-H), 2.80 (q, J = 5.8 Hz, 2-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 180.8, 133.9, 133.4, 132.1, 130.6, 128.0, 127.9, 127.4, 126.8, 123.5, 123.2, 47.9, 24.3 ppm. MS (ESI): *m/z* 180 $[M + H]^+$. MS (ESI): *m/z* 263 $[M + H]^+$.

Synthesis of 2-aminoethyl-(1,2,3,4-tetrahydronaphthalen-1-yl)carbamodithioate (**4TSM**)

1-Isothiocyanato-1,2,3,4-tetrahydro-napthalene was prepared from 1,2,3,4-tetrahydronaphthalen-1-amine **(4A)** according to the literature method (Scheme S3).[19]

To a stirred solution of 2-mercaptoethylamine hydrochloride (0.060 g, 0.53 mmol, 1 equiv) in CH_2Cl_2 (2.0 mL), 1-isothiocyanato-1,2,3,4-tetrahydro-napthalene (0.10 g, 0.53 mmol, 1 equiv) dissolved in CH_2Cl_2 (1.0 mL) was added dropwise, under nitrogen atmosphere, followed by 5 drops of *N,N*-dimethylformamide The reaction mixture was stirred overnight at room temperature, then it was concentrated under reduced pressure and the crude material was purified by column chromatography using EtOAc/MeOH 9:1 as eluent to give the desired product as an off-white solid, yield 67%, ^{1}H-NMR (300 MHz, $CDCl_3$): δ 8.48 (br s, 1-H), 7.26-7.07 (m, 4-H), 5.82, (s, 1-H), 3.65 (br s, 2-H), 3.26 (br s, 2-H), 2.86-2.68 (m, 2-H), 2.04-1.81 (m, 4-H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ 194.5, 137.8, 135.1, 129.4, 128.9, 127.7, 126.4, 55.7, 39.9, 32.5, 29.2, 28.7, 20.5 ppm. MS (ESI): *m/z* 267 $[M + H]^+$.

LC-UV analyses

A Shimadzu HPLC system (Shimadzu, Kyoto, Japan), consisting of two LC-10AD Vp module pumps, a SLC-10A Vp system controller, a SIL-10AD Vp autosampler and a DGU-14-A on-line degasser, was used for the analysis. All the chromatographic separations were performed on a Kinetex C18 XB 150 × 4.6 mm (5 μm d.p.) column as a stationary phase protected by a C18-Security Guard™ (Phenomenex, Torrance, CA). The SPD-M10Avp photodiode array detector was used to detect the analytes at 254 and 280 nm. LC Solution 1.24 software was used to process the

chromatograms. Aliquots (20 µL) of the supernatants obtained from the incubations were injected onto the HPLC system. The mobile phase (flow rate 1 mL/min) consisted of solvent A 0.5% formic acid aqueous solution and solvent B acetonitrile. The following gradient elution programs were used: *a)* 0 min B=20%, 12.5 min B=70%, 14.5 min B=70%, 15 min B=20%, 20 min B=20% - for incubations of compounds **1**, **3-5**; *b)* 0 min B=20%, 12.0 min B=80%, 14.5 min B=80%, 15 min B=20%, 20 min B=20% - for incubations of compound **2**. The eluents were filtered through a 0.45-µm pore size nylon membrane filter before use.

LC-MS^n analyses

A Thermo Finningan LCQ Deca XP plus system equipped with a quaternary pump, a Surveyor AS autosampler, a Surveyor photodiode array detector and a vacuum degasser was used for LC-MS analyses (Thermo Electron Corporation, Waltham, MA). All the chromatographic separations were performed on a Sinergy Polar 150 × 2 mm (4 µm d.p.) column protected by a Polar RP-Security Guard™ (Phenomenex, Torrance, CA) kept at a 35 °C. Aliquots (5 µL) of supernatants obtained from incubations were injected onto the system and eluted with a mobile phase (flow rate 250 µL/min) consisting of solvent A 0.2% formic acid aqueous solution and solvent B 0.2% formic acid in methanol. The following gradient elution programs were used: 0 min B=20%, 6 min B=70%, 10 min B=80%, 10.5 min B=20%, 15.5 min B=20% for **1** and **2**; 0 min B=15%, 7 min B=70%, 10 min B=80%, 10.5 min B=15%, 15.5 min B=15% for **3** and **4**.

The eluents were filtered through a 0.45-µm pore size nylon membrane filter before use. The eluate was injected into the electrospray ion source (ESI) and MS spectra were acquired and processed using Xcalibur® software. The operating conditions of the ion trap mass spectrometer were as follows: positive mode, spray voltage, 5.20 kV; source current, 80 µA; capillary temperature, 300 °C; capillary voltage, 7.00 V; tube lens offset, 5.00 V; multipole 1 offset, -5.00 V; multipole 2 offset, -11.50 V; sheath gas flow (N_2), 40 Auxiliary Units; Auxiliary sheath gas flow, 4 Auxiliary

Units. Data were acquired in full-scan and product ion scan modes (MS^n) using mass scan range *m/z* 105-800. The collision energy was optimized at 28-32%.

Rat and human liver preparations

Male Wistar Han rat liver microsomes (RLM), (protein concentration: 20 mg/mL, total CYP: 650 pmol/mg protein), human liver microsomes (HLM), (pooled mixed sex, fifty individual donors, protein concentration: 20 mg/mL, total CYP: 360 pmol/mg protein) were purchased from Corning B.V. Life Sciences (Amsterdam, The Netherlands). All the incubations were performed using a horizontal DUBNOFF shaking thermostatic bath (Dese Lab Research, Padova, Italy) while protecting from light.

Incubations with RLM, HLM, recombinant CYP3A4

The standard incubation mixture (250 µL final volume), was carried out in a 50 mM tris[hydroxymethyl]aminomethane (TRIS)/HCl buffer (pH 7.4) containing 3.3 mM $MgCl_2$, 1.3 mM $NADPNa_2$, 3.3 mM glucose 6-phosphate, 0.4 Units/mL glucose 6-phosphate dehydrogenase, acetonitrile as cosolvent (1% of total volume), and the substrate (100 µM). After pre-equilibration of the mixture, an appropriate volume of RLM or HLM suspension was added to give a final protein concentration of 1.0 mg/mL. The mixture was shaken for 60 minutes at 37 °C. Control incubations were carried out without the presence of NADPH-regenerating system or microsomes. Each incubation was stopped by the addition of 250 µL ice-cold acetonitrile, vortexed and centrifuged at 13000 rpm for 10 min. The supernatants were analyzed by LC-UV or LC-MS. Alternatively, when the chemical reactivity of the metabolites was studied, the following trapping agents (at 3 mM concentration) were added to the incubation mixture: glutathione in its reduced (GSH) or oxidised (GSSG) forms, *N*-acetylcysteine (NAC), and mercaptoethylamine (MEA).

Incubations with human hemoglobin

The standard incubation mixture (500 µL final volume), was carried out in a 50 mM tris[hydroxymethyl]aminomethane (TRIS)/HCl buffer (pH 7.4) containing acetonitrile as cosolvent (1% of total volume), human hemoglobin (lyophilized powder from Sigma Aldrich, final protein concentration 1 mg/mL), GSH (3 mM), and the substrate (100 µM). The mixture was shaken for 60 minutes at 37 °C. Control incubations were carried out without the presence of hemoglobin or GSH. Each incubation was stopped by the addition of 500 µL ice-cold acetonitrile, vortexed and centrifuged at 13000 rpm for 10 min.

Incubations with rhodanese

The standard incubation mixture (250 µL final volume), was carried out in a 50 mM borate buffer (pH 8.6) containing 0.25 mM sodium thiosulfate, acetonitrile as cosolvent (1% of total volume), and the substrate (200 µM). After pre-equilibration of the mixture, an appropriate volume of rhodanese from bovine liver (Type II, lyophilized powder from Sigma Aldrich) was added to give a final protein concentration of 1.0 mg/mL. The mixture was shaken for 60 minutes at 37°C. Control incubations were carried out without the presence of sodium thiosulfate or rhodanese or trapping agent (GSH or MEA). Each incubation was stopped by the addition of 250 µL ice-cold acetonitrile, vortexed and centrifuged at 13000 rpm for 10 min. The supernatants were analyzed by LC-UV or LC-MS.

CYP inhibition: aminopyrine N-demethylase assay

Aminopyrine *N*-demethylase activity was determined by detecting the amount of formaldehyde produced by RLM. The incubation was carried out in Tris/HCl buffer (50 mM, pH 7.4) supplemented with 150 mM KCI and 10 mM $MgCl_2$. The incubation contained 1 mM aminopyrine, acetonitrile (1% final volume), and 1.0 mg/mL of RLM in a total volume of 180 µL. Increasing concentrations (0.01, 1, 10, 25, 50, 75, 100 µM) of the tested isocyanides or known CYP inhibitors (ketoconazole and quinidine) were added to the incubation mixture. After pre-incubation of the

assay media for 3 min at 37 °C, the reaction was initiated by adding NADPH (final concentration 1mM) and carried out at 37 °C for 15 min with moderate shaking. The reaction was then terminated by addition of 90 µL of 20% (w/v) TCA solution. After centrifugation at 13000 rpm for 10 min, a 240 µL aliquot of the protein-free supernatant was treated with 120 µL of Nash reagent and incubated in a water bath at 37 °C for 40 min. The absorbance of the resultant solution was determined at 412 nm, subtracting the blank sample absorbance (without NADPH); the concentration of formaldehyde was quantified (nmol/min/mg) by comparison with a standard curve prepared from commercially available formaldehyde solution freshly standardized by iodometric titration. All incubations were performed in triplicate.

Nash reagent:[20] 3.75 g ammonium acetate, 50 mL glacial acetic acid, and 75 mL acetylacetone were dissolved in 25 mL deionized water. The solution was freshly prepared and used for one day.

Determination of the isocyanides reactivity toward protein sulphydryl groups (Ellman's assay)

To a 2140 µL of 100 mM phosphate buffer (pH 8.0) were added 60 µL of methanol and 750 µL of bovine serum albumin (BSA) stock solution (lyophilized powder from Sigma Aldrich, 10.1 mg/mL - 151.2 µM, final concentration 37.8 µM). Ellman's reagent (50 µL), (15 mM DTNB dissolved in 50 mM ammonium acetate) was added and the solution was incubated at room temperature for 5 minutes before measuring the development of absorbance at 412 nm. When reactivity of **1** and **2** toward BSA sulphydryl groups was assessed, 60 µL of the methanolic solutions of the isocyanides (final concentration 200 µM) were added in the mixture 10 min before adding the Ellman's reagent. Positive control assays were performed by adding NEM (final concentration: 200 µM) instead of the isocyanides.

The thiol molar concentration [SH] was calculated according to the Eq. 1 where *Ai* is the absorbance of the isocyanides incubations, *Ab* is the absorbance of isocyanides (measured in the absence of DTNB), and 14150 M^{-1} cm^{-1} is the extinction coefficient of the reagent.[21]

$$[\mathrm{SH}] = \frac{A_i - A_b}{14150_{\times} 1\mathrm{cm}}$$

Results

Isocyanides incubations in liver microsomes.

The hepatic metabolism of the selected isocyanides was investigated through *in vitro* incubations in rat (RLM) and human (HLM) liver microsomes supplied by a NADPH-regenerating system. Reactivity toward nucleophiles was then investigated by supplying the following trapping agents: reduced (GSH) and oxidised (GSSG) glutathione, *N*-acetylcysteine (NAC), and mercaptoethylamine (MEA).

The incubations of **1** and **2** were analysed by both LC-UV and LC-MS^n systems; since their poor (**3-5**) or absent (**6**) UV-Vis absorption property, these isocyanides were investigated by LC-MS^n only.

Microsomal metabolism of 2-isocyanonaphthalene (1) and 4-isocyano-1,1'-biphenyl (2).

Incubations of aryl isocyanides **1** and **2**, performed in RLM supplied with GSH without NADPH, gave the formation of three main metabolites (**1A**, **1FA**, **1NG**, and **2A**, **2FA**, **2NG** respectively) observed in LC-UV chromatograms with the substrates disappearing up to 75% after one hour (Figure 3a). When the aryl isocyanides were incubated in RLM supplied with GSH and in the presence of NADPH, an additional metabolite (**1SG** or **2SG**) was detected (Figure 3b). Control incubations of **1** and **2**, in the presence of GSH only, did not give any transformation.

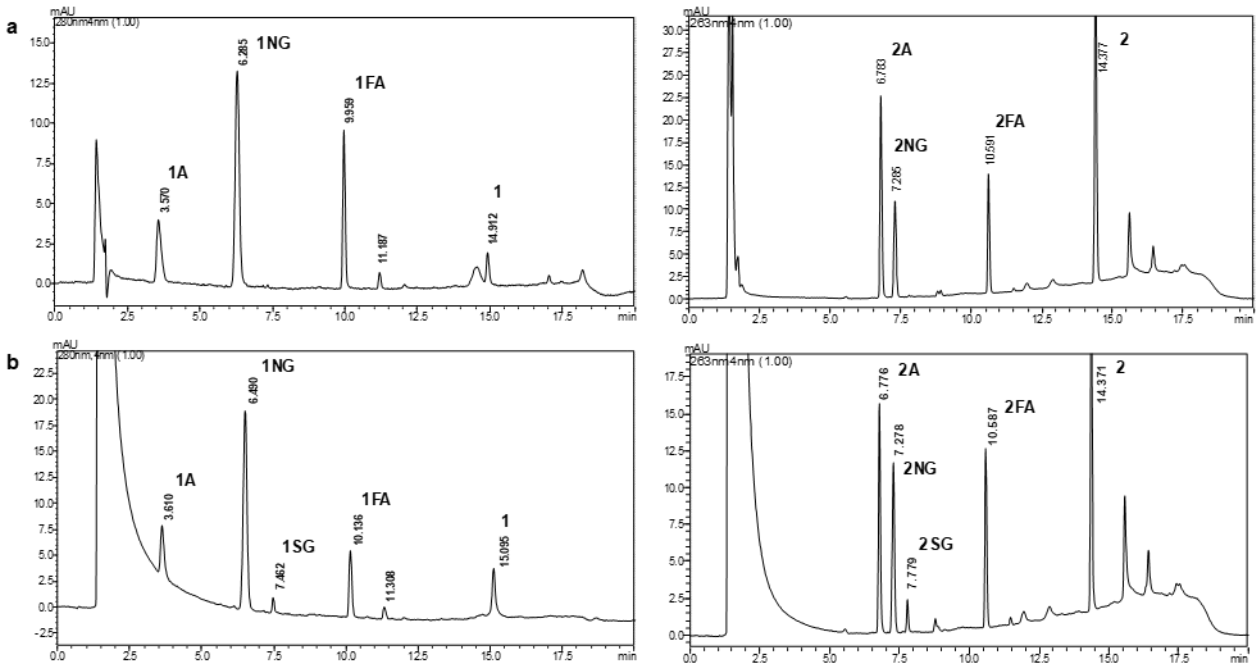


Figure 3. LC-UV chromatograms of isocyanides **1** and **2** incubations in RLM supplied by GSH: without NADPH (a), with NADPH (b).

The structures of metabolites from **1** and **2** were investigated by LC-tandem mass spectrometry. The samples were analysed in positive full scan and product ion scan (MS^n) modes revealing the formation of the arylamines **1A** (*m/z* 144), **2A** (*m/z* 170), and the formamides **1FA** (*m/z* 172), **2FA** (*m/z* 198) respectively; their structures were confirmed by chromatographic comparison with the synthetized reference compounds (Figure 4). In addition, formation of the formamidine adducts **1NG** (*m/z* 461) and **2NG** (*m/z* 483) deriving from α-addition between the amino group of GSH on isocyanide were observed (Figure 4). For both the adducts, **1NG** and **2NG**, the protonated molecules at *m/z* 461 and 487 corresponding to the addition of 307 Da, alongside the MS^n data suggested the equimolar reaction between the isocyanide and the amino group of GSH. Indeed, their MS^2 product ion spectra showed a main fragment at *m/z* 283 or 309 respectively, corresponding to the neutral loss of the Cys-Gly dipeptide (178 Da). Further fragmentation (MS^3) of these ions formed the main fragments at *m/z* 154 and 130 and 180 and 130 respectively which corroborated the proposed structures of metabolites (Figure 4), (See figures S2-S5 for spectral data).

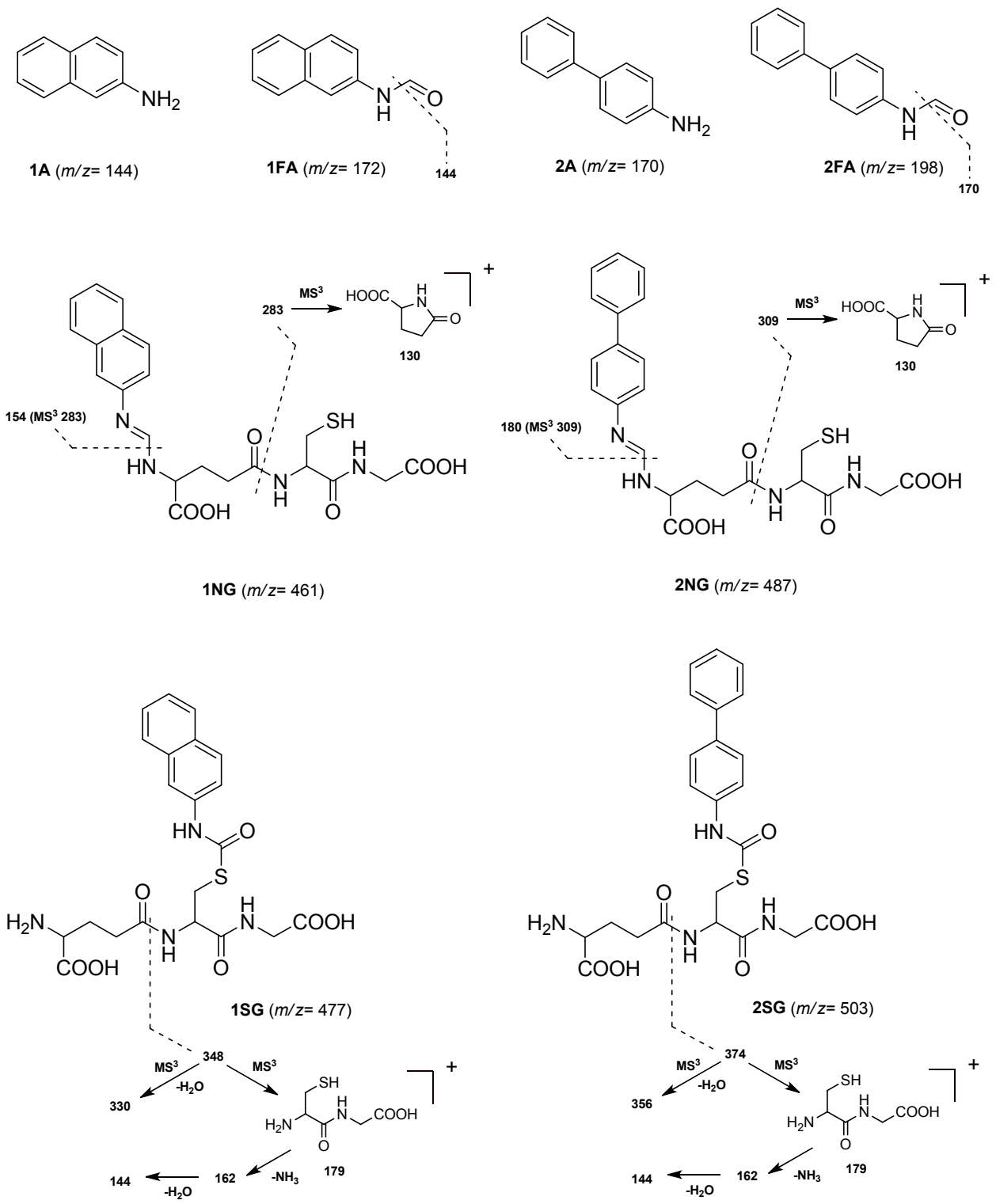


Figure 4. Structures and MS^n fragmentation pathways of metabolites from the isocyanides **1** and **2**.

For the additional metabolites **1SG** (*m/z* 477) and **2SG** (*m/z* 503), which were formed under microsomal oxidative conditions, the mass shift of 16+307 Da suggested the formation of a thiocarbamate analogue deriving from the reaction between the thiol group of GSH and the

isocyanate intermediate formed by oxidation of isocyanide (Figure 4). The MS^2 spectra of these ions showed the main fragments at *m/z* 348 and 374 respectively (neutral loss of pyroglutamic acid, 129 Da) which further fragmentation (MS^3) generated four ions at *m/z* 330, 179, 162, and 144 for **1SG**, and 356, 179, 162, and 144 for **2SG** (Figure 4 and Figures S2-S5). It is worth of mention that similar adducts, associated with the same fragmentation pathways, have been previously reported for phenyl isocyanate (-N=C=O) adducts with GSH.[22] For confirmatory purpose, the molecular masses of **1NG**, **1SG**, **2NG**, **2SG** adducts were also confirmed by high resolution mass spectrometry (See Figures S6-S7 for experimental data).

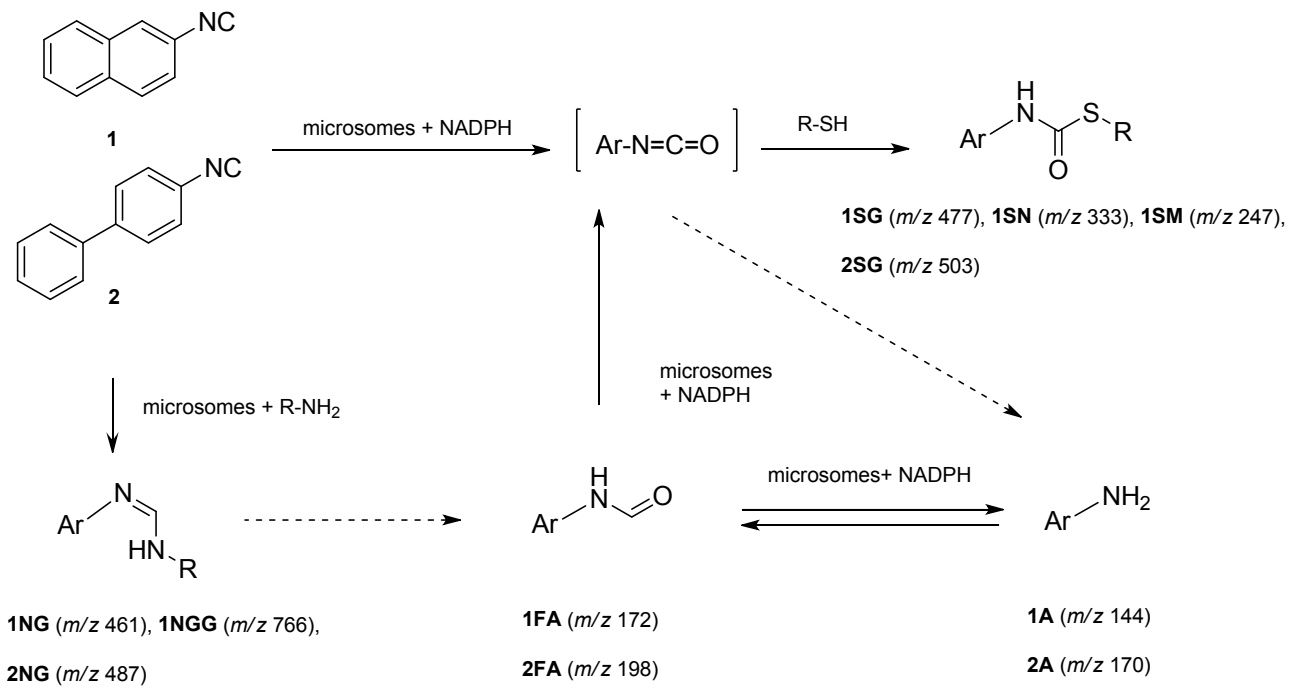
Furthermore, the reactivity of isocyanide **1** was studied in RLM activated by NADPH and in the presence of other trapping agents such as: GSSG, NAC, and MEA. Incubation supplied by GSSG gave rise to the metabolite **1NGG** (*m/z* 766) and, similarly to incubation with GSH, both **1FA** and **1NA** were also formed. Reactions with NAC or MEA, only generated **1SN** (*m/z* 333) and **1SM** (*m/z* 247) via cysteinyl thiol nucleophilic attack, whilst both **1FA** and **1NA** were absent (Scheme 1) (See Figures S8-S9 for structures and spectral data). The structures of these three adducts were confirmed by the presence of several diagnostic ions detected in their positive product ion spectra. **1NGG** gave two abundant ions: *m/z* 637 (neutral loss of pyroglutamic acid, 129 Da) and *m/z* 623 (formamidine cleavage) which were submitted to further fragmentation stage (MS^3). The diagnostic MS^3 fragment ions, *m/z* 548 (loss of glycine), 494 (formamidine cleavage and loss of pyroglutamic acid), and 355 (amide cleavage) confirmed the proposed structures for these metabolites. Finally, product ion scan spectrum of isocyanide **1** adduct with NAC, **1SN** (*m/z* 333) gave an abundant ion at *m/z* 164 (acetylcysteine loss) which gave, when submitted to MS^3 stage, two abundant ions at *m/z* 146 and 122 corresponding to neutral losses of water and the acetyl group. Similarly, isocyanide **1** reacted with MEA to form the metabolite **1SM** (*m/z* 247) which fragmentation pathways gave rise to the diagnostic ions at *m/z* 230 (loss of ammonia), 170 (mercaptoethylamine cleavage), and 144 (N-C cleavage) (See Figure S9 for spectral data and structures).

In order to demonstrate the metabolic pathway leading to the formation of amine **1A**, an additional incubation of formamide **1FA** was carried out in the presence of RLM activated by NADPH and supplied with GSH. The same peaks attributable to **1A** and **1SG**, revealed in isocyanide **1** incubations, were detected (Figure S10). This means that *N*-aryl formamide **1FA** could be oxidized to isocyanate which can either react with GSH to give **1SG** or with water to give **1A** after spontaneous decarboxylation of carbamic acid. Alternatively, it could be deacylated in liver microsomes giving the corresponding amine **1A** (Scheme 1).

Finally, adduct **1NG** was isolated by semipreparative liquid chromatography: on standing, it spontaneously hydrolysed to **1FA**. (Figure S11).

Differently from incubations in rat and human liver microsomes, incubation of **1** in the presence of recombinant CYP3A4 isoform supplied by NADPH and GSH only gave small traces of **1NG** and **1SG** adducts, whereas formamide **1FA** and amine **1A** were absent (data not shown): however, these data were obtained from a single CYP isoform, therefore, if on the one hand, it is not possible to exclude the role of other CYPs in the formation of isocyanide adducts; on the other hand, further metalloenzymes or enzymes present in the microsomal fraction could actively participate in the biotransformation of isocyanides.

Overall, the metabolic pathways summarized in Scheme 1 demonstrate that aryl isocyanides **1** and **2** are not stable in *in vitro* metabolism; indeed, in liver they are prone to react with nucleophilic agents: mainly via direct nucleophilic attack of the glutamic acid α-amino group of GSH and, in a lesser extent, via the cysteinyl thiol of GSH after oxidation to isocyanate intermediate.



Scheme 1. Proposed i*n vitro* metabolic pathways of aryl isocyanides **1** and **2**. (Dashed lines indicate non enzymatic transformations; structure in parenthesis indicates undetected intermediate; R indicates: reduced and oxidised glutathione, *N*-acetylcysteine, and mercaptoethylamine scaffolds).

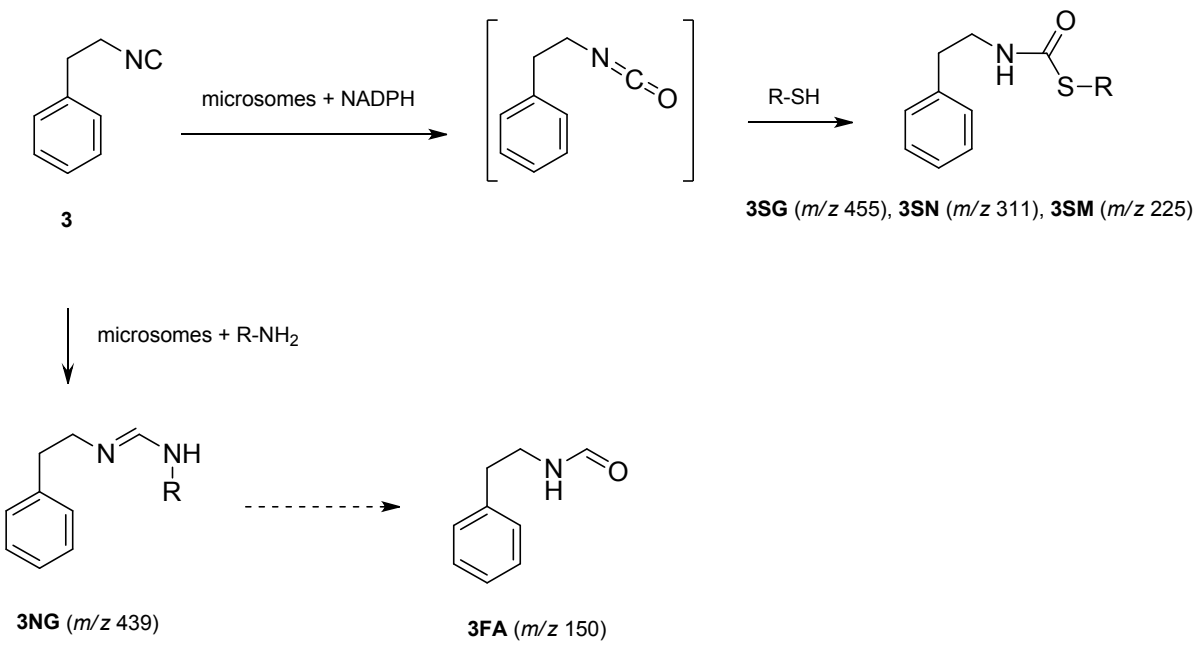
Microsomal metabolism of (2-isocyanoethyl)benzene (3).

Incubations of **3** was performed in RLM activated by NADPH and supplied with nucleophilic trapping agents. When incubated with GSH, **3** formed in traces the adduct **3NG** (*m/z* 439) and formamide **3FA** (*m/z* 150), thus suggesting a limited substrate depletion; furthermore, the adduct **3SG** (*m/z* 455) was also revealed (Scheme 2 and Figure S12). Even, when **3** was incubated in the absence of NADPH, only traces of **3NG** were revealed (data not shown). Hence, for isocyanide **3**, reaction with GSH occurred either via nucleophilic attack by the cysteinyl thiol or the α-amino group of glutamic acid in a similar manner but to a minor extent with respect to aryl isocyanides **1** and **2**. The structures of these two adducts were confirmed by the presence of several diagnostic ions revealed in their MS^n spectra (Figure S12). Indeed, MS^2 spectrum of **3NG** showed an abundant fragment ion at *m/z* 261 (neutral loss of Cys-Gly dipeptide, 178 Da) which further fragmentation (MS^3) gave two prominent fragment ions at *m/z* 130 (loss of pyroglutamic acid), and 105 (N-C cleavage). **3SG** metabolite also showed an abundant fragment ion at *m/z* 326 (loss of pyroglutamic

acid) which, when submitted to MS^3 stage, gave four fragment ions being the most abundant at *m/z* 179 due to the loss of the Cys-Gly dipeptide.

Microsomal incubation of **3** activated by NADPH in the presence of NAC or MEA generated both the adducts **3SN** (*m/z* 311) and **3SM** (*m/z* 225) exhibiting only traces of formamide **3FA** (*m/z* 150), (Scheme 2 and Figure S13). Product ion scan spectrum of **3SN** gave the same intense ion at *m/z* 164 (acetylcysteine loss) already observed for **1SN**: MS^3 stage gave the same two abundant ions at *m/z* 146 and 122 corresponding to neutral loss of water and the acetyl group respectively. **3SM** gave rise to three abundant and diagnostic ions at *m/z* 208 (loss of ammonia), 148 (loss of mercaptoethylamine) and 78 (protonated mercaptoethylamine). No adducts were detected when **3** was incubated in the presence of GSSG trapping agent.

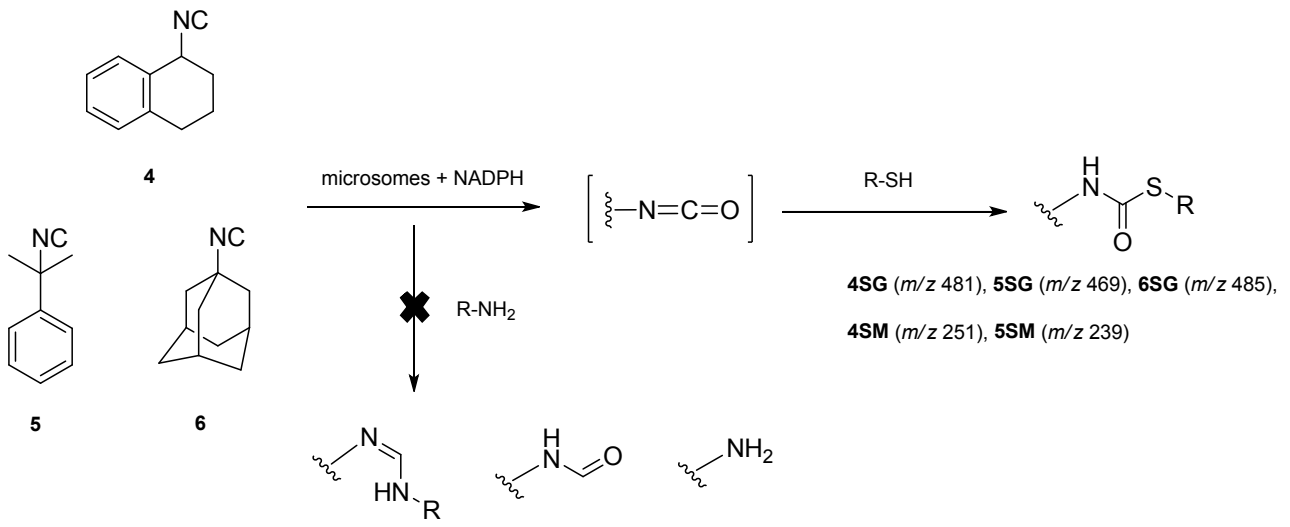
These data (summarized in Scheme 2) indicate that isocyanide **3** shares the same metabolic pathways of aryl isocyanides **1** and **2**.



Scheme 2. Proposed *in vitro* metabolic pathways of isocyanide **3**. (Dashed line indicates non enzymatic transformation; structure in parenthesis indicates undetected intermediate; R indicates: reduced glutathione, *N*-acetylcysteine, and mercaptoethylamine scaffolds).

Microsomal metabolism of 1-isocyano-1,2,3,4-tetrahydronaphthalene (4), (2-isocyanopropan-2-yl)benzene (5), and adamantyl isocyanide (6).

When compounds **4** and **5** were incubated in RLM activated by NADPH and supplied with GSH or MEA, the adducts **4SG** (*m/z* 481) and **5SG** (*m/z* 469) or **4SM** (*m/z* 251) and **5SM** (*m/z* 239) were formed respectively, (Scheme 3), (see Figure S14 for structures and spectral data). This demonstrates that secondary and tertiary aliphatic isocyanides only react with the nucleophilic thiols via isocyanate intermediate, being absent the adducts with the amino groups of GSSG and NAC and also the formamides and amines revealed for the other isocyanides. This strongly suggests their high hepatic metabolic stability. Adamantyl isocyanide **6** revealed to be even more metabolically stable, only giving traces of the adduct **6SG** (*m/z* 485), (Scheme 3 and Figure S15).



Scheme 3. Proposed *in vitro* metabolic fate of isocyanides **4**, **5**, **6**. (Structure in parenthesis indicates undetected intermediate; R indicates: reduced glutathione and mercaptoethylamine scaffolds).

Isocyanides incubations with rat plasma and human hemoglobin.

After identifying the metabolic pathways involved in the isocyanide hepatic degradation, we investigated their blood stability, a mandatory condition to consider isocyanides as potential drug candidates. This was done through an indirect way, evaluating the stability of all the isocyanides both in plasma and in human hemoglobin.

They were incubated at 1 mM concentration for 1 hour in plasma or human hemoglobin and the residual substrate evaluated by LC-UV analysis. Overall, all the isocyanides resulted stable in these conditions except **1** and **2**; when incubated with human hemoglobin supplied with GSH, they gave the same adducts detected in rat and human liver microsomes: **1-2SG** and **1-2NG** (the latter present in traces), (Scheme 1), (data not shown). These results suggested that hemoglobin can spontaneously catalyse the same reactions catalyzed in liver microsomes: the direct nucleophilic attack of the glutamic acid α-amino group, and the cysteinyl thiol of GSH via isocyanate intermediate.

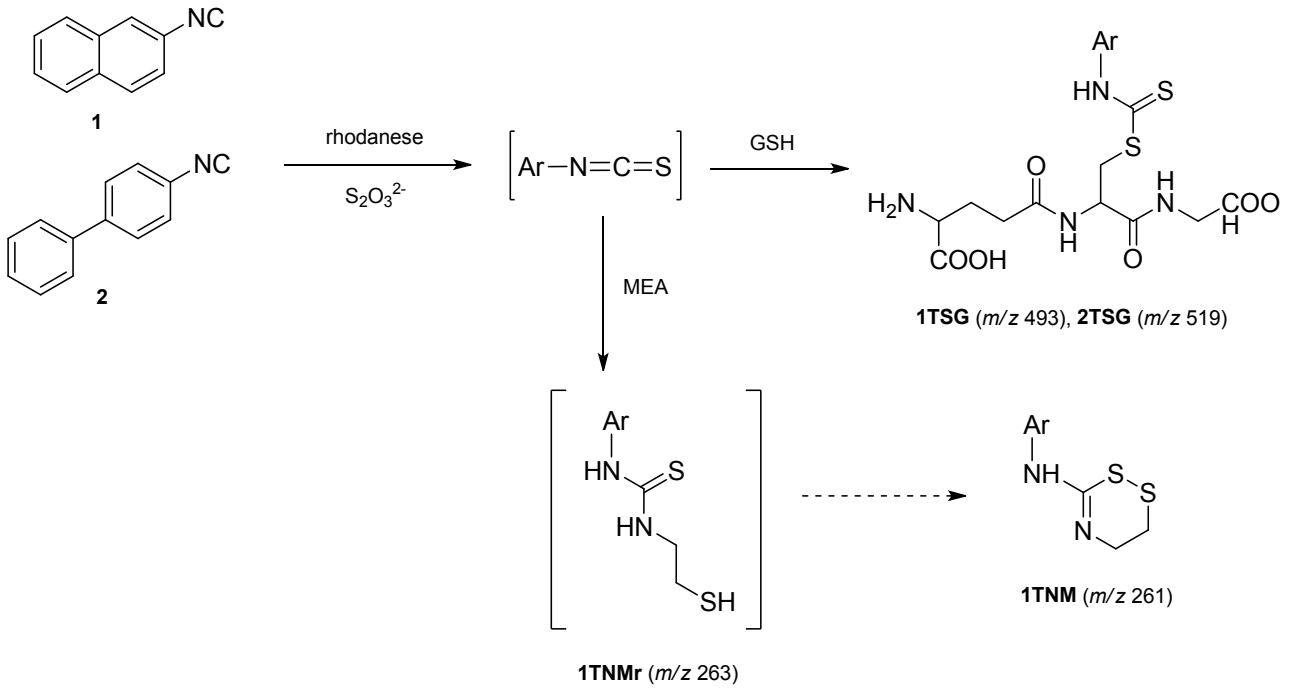
Isocyanides incubations with bovine liver rhodanese.

As rhodanese (thiosulfate sulfurtransferase, EC 2.8.1.1) is a hepatic enzyme involved in the direct detoxification of cyanide ions forming thiocyanates, we were intrigued to whether isocyanides might react with this enzyme.

*Experiments with 2-isocyanonaphthalene (**1**) and 4-isocyano-1,1'-biphenyl (**2**).*

Incubation of isocyanides **1** or **2** with rhodanese in the presence of sodium thiosulfate and supplied with GSH gave rise to the formation of the thiourea adducts **1TSG** (*m/z* 493) or **2TSG** (*m/z* 519) respectively, suggesting the formation of a GSH adduct via isothiocyanate intermediate (scheme 4). The MS^2 spectrum of **1TSG** showed a main fragment at *m/z* 364 (neutral loss of pyroglutamic acid, 129 Da) and other diagnostic ions at *m/z* 418, 330, 289, 22 and 144 which structures were proposed in figure S16. Similar fragmentations occurred for **2TSG** (Figure S18). Alternatively, incubation of **1** in the presence of MEA gave the adduct **1TNM** (*m/z* 261) as a result of the nucleophilic attack via isothiocyanate intermediate by the amino group of MEA. **1TNM**, which is featured by the fragmentation pathways reported in Figure S17 was supposed to be generated from thiourea intermediate oxidation to give the corresponding disulfide derivative. To support this hypothesis, incubation of **1** was treated with dithiothreitol (DTT); as the results **1TNM** peak intensity decreased

and a new peak **1TNMr** (*m/z* 263) was observed. Product ion scan spectrum of *m/z* 263 gave the fragmentation pathways depicted in Figure S17 supporting the structure of the metabolite which was definitively confirmed by comparison of its retention time and spectral data with those of a synthetic reference compound.

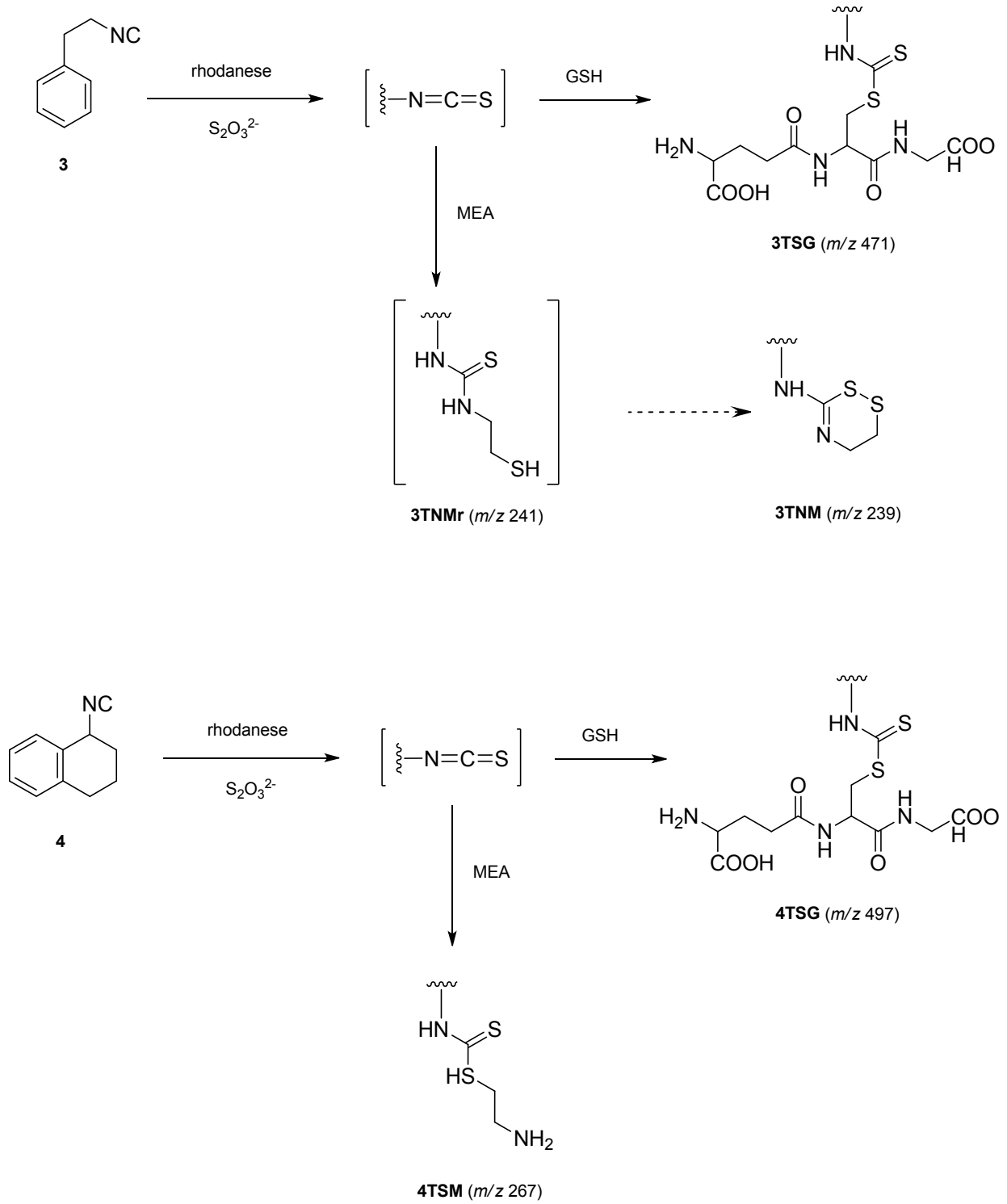


Scheme 4. *In vitro* metabolic pathways of isocyanides **1** and **2** in the presence of rhodanese. (Dashed lines indicate non enzymatic transformation; structures in parenthesis indicate undetected intermediate).

*Experiment with (2-isocyanoethyl)benzene **(3)** and 1-isocyano-1,2,3,4-tetrahydronaphthalene **(4).***

Incubation of **3** with rhodanese in the presence of GSH gave rise to metabolic transformations similar to those of compound **1** albeit to a lesser extent. (Scheme 5). Rhodanese catalysed the formation of isothiocyanate intermediate of **3**, which reacted with GSH or MEA via thiol or amino nucleophilic attack respectively to form the adducts **3TSG** (*m/z* 471) and **3TNM** (*m/z* 239) (Scheme 5); these structures and fragmentation pathways are depicted in Figure S19. Also, for **4**, rhodanese catalysed the formation of **4TSG**, (*m/z* 497) and **4TSM**, (*m/z* 267) (Scheme 5). The presence of the diagnostic fragment ion at *m/z* 368 (neutral loss of pyroglutamic acid, 129 Da) in **4TSG** MS^2

spectrum confirmed that the nucleophilic attack of GSH via SH group occurred. Differently, nucleophilic attack of MEA on **4** isothiocyanate occurred via SH group instead of NH_2 to form **4TSM**; its structure was unequivocally confirmed by the diagnostic ion fragments and identical retention time with respect to the synthetic reference compound (Figure S20).



Scheme 5. *In vitro* metabolic pathways of isocyanides **3**, **4** with rhodanese. (Dashed lines indicate non enzymatic transformation; structure in parenthesis indicates undetected intermediate).

Cytochrome P450 inhibition: aminopyrine N-demethylase assay

To evaluate the CYPs inhibition by means of isocyanides **1-6,** the aminopyrine *N*-demethylase assay was performed.[23] The results indicated that both aromatic and the aliphatic isocyanides can inhibit CYPs with an IC_{50} comprised in a micromolar range (0.5 – 32.1 μM), (Table 1). However, except for **3** and **6**, the isocyanides inhibited CYPs more efficiently with respect to the inhibitors used as a control (*i.e.* ketoconazole for CYP3A, and quinidine for CYP2D6) which in our assay gave IC_{50} = 14.0 and 12.1 μM respectively.

Isocyanide 1 reactivity toward BSA sulphydryl groups

Taking into account the above described reactivity of the isocyanide moiety toward nucleophilic functions during the metabolism, we evaluated its ability to react with the free cysteine SH residues of bovine serum albumin (BSA) using the previously reported[24] spectrophotometric assay based on Ellman's reagent. The results expressed as residual [SH]/[BSA] molar ratio demonstrated the inability of isocyanides **1** and **2** to bind the free SH group of BSA in comparison to the positive control *N*-ethylmaleimide (NEM) which reduced the ratio from 0.215 to 0.021 (Table 1).

compound	CYP inhibition IC_{50} μM[a,b]	Ellman's assay [SH]/[BSA][c]
1	3.2	0.172
2	2.1	0.230
3	32.1	-
4	3.6	-
5	0.5	-
6	19.3	-

Table 1.

[a] IC_{50} for aminopyrine *N*-demethylase inhibition was determined from the formaldehyde formation velocity (nmol/min/protein mg) versus log isocyanides concentration (0.01-100 μM) using GraphPad PRISM 6.0 software.

[b] Control incubations with known CYP inhibitors, gave IC_{50} values of 14.0 μM for ketoconazole (CYP3A) and 12.1 μM for quinidine (CYP2D6).

[c] Control incubation without isocyanides [SH]/[BSA]= 0.215; positive control with NEM [SH]/[BSA]= 0.021

Discussion

In this work, we investigated the metabolic stability of the isocyanide functional group by studying the metabolic profile of some aryl (**1**-**2**) and aliphatic (**3**-**6**) isocyanides models.

Depending on their structural features, the metabolic stability of drugs could be strictly related to their chemical stability; hence, the integration of both aspects could be conveniently performed defining a unique chemical and metabolic stability space. For this reason, chemical stability of isocyanides in aqueous media at physiological conditions was first investigated confirming substantially no degradation. Similar results were obtained in preliminary plasma and hemoglobin solution stability studies. Next, exploiting the sensitivity and the power of electrospray ionization tandem mass spectrometry (ESI-MS^n) for structural characterization, we elucidated the structures of isocyanides metabolites generated in liver microsomes supplied with nucleophilic trapping agents (*e.g.* GSH). Indeed, we first supposed that the interaction with metal containing enzymes (*e.g.* CYPs), may increases the reactivity of the isocyanide group toward nucleophiles triggering a plethora of biotransformations.

Our results proved that both aryl and aliphatic isocyanides undergo hepatic metabolism. However, if the aryl isocyanides **1** and **2** and the primary aliphatic isocyanide **3**, reacting with nucleophilic trapping agents, undergo an extensive biotransformation in liver microsomes, (Scheme 1-2), secondary and tertiary isocyanides are stable, undergoing only a mild NADPH dependent microsomal oxidation. Both aryl isocyanides **1-2** and the primary aliphatic isocyanide **3** react via the direct nucleophilic attack of the -NH_2 of GSH to give the formamidine adducts, which can be partially hydrolysed into formamide.

A possible mechanism to account for the formation of **1NG** has to consider the involvement of a metal catalyst. Indeed, it is well-know that, even at temperature higher than 100 °C neither amines nor thiols can give α-addition with the isocyano moiety.[1b] This reaction usually requires the presence of a transition metal of group IB or IIB. In particular, isocyanides coordinate the metal to

form the complex. The isocyanide carbon is then inserted into a -NH_2 or -SH bond to form the corresponding adduct as it was demonstrated by Segusa in the sixties and seventies[1a] (Figure 5). The prevalence for the NH insertion over SH insertion cannot be explained at this stage of research, but it is reasonable that the metalloenzyme involved in this transformation dictates the chemoselectivity. Interestingly, microsomal incubations of isocyanide **1**, activated by NADPH and in the presence of lysine as trapping agent instead of GSH, failed to give the corresponding formamidine derivative. This result corroborates the hypothesis that the nucleophilic attach on the isocyanide carbon group is driven by a catalytic enzymatic mechanism.

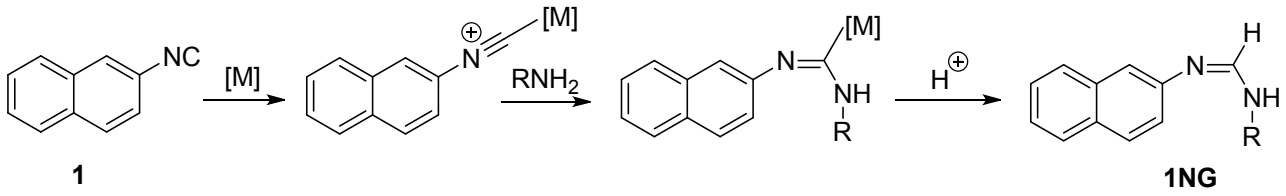


Figure 5. Proposed mechanism for the formation of **1NG**.

The observation of these biotransformations, which are driven by the presence of a nucleophilic agent (*e.g.* GSH), led us to speculate why aromatic isocyanide (**E**) was reported to be stable toward liver microsomal assay performed in the absence of a nucleophilic trapping agent.[16c]

Finally, at least for aryl isocyanide **1**, we demonstrated that formamidine **1NG** spontaneously degrades to formamide **1FA**. It was previously established that in mammalian liver, *N*-arylamines can undergo *N*-acetylation/formylation; on the contrary, the corresponding *N*-aryl formamides are de-formylated in liver cytosol by formamidases, and, to a lesser extent, in microsomes, where carboxylesterases are supposed to play a role.[25]

Isocyanides **1** and **2** reactivity toward BSA sulphydryl groups demonstrated their inability to bind the free SH group of BSA. This further corroborates the hypothesis that nucleophilic attack of the glutamic acid α-amino group of GSH to isocyanides is not spontaneous, but enzymatically driven. Further phenotyping studies will be worthy of investigation to evaluate the role of different hepatic

metabolizing enzymes to catalyse the reaction of isocyanide moiety with GSH. In addition, all the aryl and aliphatic isocyanides considered in this study react with the nucleophilic thiols via isocyanate intermediate generated by NADPH dependent oxidation. Interestingly, Cross and Hyland reported that in liver homogenate fractions, *N*-alkylformamides undergo formyl oxidation throughout carbamoyl intermediate formation followed by nucleophilic thiol attack by GSH or NAC.[26]

Surprisingly, when isocyanide **1** was incubated with CYP3A4 (one of the most representative CYP isoforms in liver) activated by NADPH in the presence of GSH, it only gave small traces of adducts generated by nucleophilic attack, whereas formamide **1FA** and amine **1A** were totally absent. This suggests that, albeit we investigated only one of the numerous CYP isoforms involved in hepatic drug metabolism, other microsomal enzymes are probably involved in the formation of the formamidine adduct **1NG**, the most abundant metabolite of **1**. However, interaction of isocyanides toward CYPs seem to occur; indeed, the isocyanides considered in this study inhibited CYPs, sometimes even more efficiently compared to the well known CYP inhibitors ketoconazole and quinidine.

As the enzyme rhodanese (a thiosulfate sulfurtransferase) is an ubiquitous mitochondrial enzyme[27] present in all living organisms from bacteria to mammals, catalysing the transfer of a sulfur atom from thiosulfate (sulfur donor) to cyanide (sulfur acceptor), we evaluated the metabolism of isocyanides through incubation with bovine liver rhodanese activated by sodium thiosulfate in the presence of GSH or MEA as trapping agents. Incubation of both aliphatic and aromatic isocyanides with rhodanese supplied with thiosulfate and GSH gave rise to the formation of the thiourea adducts via the corresponding isothiocyanate intermediate.

Conclusions

In conclusion, the aim of this work was to evaluate for the first time the metabolic stability of the isocyanide functional group by means of six model isocyanides with different steric and electronic

features. The results obtained show how aromatic and primary isocyanides suffer from a severe *in vitro* hepatic metabolic degradation probably due to a lack of a steric shield able to prevent the addition of a nucleophilic group (*e.g.* the amino group of GSH). Favorable π-π interactions between the phenyl ring of aromatic isocyanides and aromatic amino acids in the active site can also play a role in the stabilization of the drug-enzyme interaction.[4b]

A good balance between coordinating properties and metabolic stability due to steric effects is present both in secondary and above all in tertiary isocyanides. To this point is useful to emphasize that in nature no aromatic and primary isocyanides have ever been isolated, while only *tert*-alkyl and, to a lesser extent, *sec*-alkyl isocyanides are produced. Although no experimental data was available, very recently the Vanderwal's group reported a simplified analogue of kalihinol B (**D**) which was characterized by significant metabolic stability when incubated with human microsomes.[28]

Due their well-recognized metal coordinating ability with the most important transition metals (*e.g.* Fe, Cu, Zn) we hypothesize that the tertiary isocyanide group might be considered as an alternative war-head for the inhibition of metalloenzymes or as strategic group for the design of covalent inhibitors directed to the active site. Rationally designed synthetic isocyanides which escape from the chemical complexity of natural isocyanoterpenes might therefore be a novel promising class of drugs of the future.

Supporting information

Schemes for the synthesis of formamides and isocyanides from amines; schemes for synthesis of metabolites **1TNMr**, **4TSM**; purity data; LC-MS data of isocyanides incubations are provided free of charge in the supporting information.

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Abbreviations

BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, DL-dithiothreitol; GSH, reduced glutathione; GSSG, oxidised glutathione; HLM, human liver microsomes; LC-MS^n, liquid chromatography coupled to tandem mass spectrometry detector; LC-UV, liquid chromatography coupled to ultraviolet-visible detector; MEA, mercaptoethylamine; NAC, *N*-acetylcysteine; NEM, *N*-ethylmaleimide; RLM, rat liver microsomes; TCA, trichloroacetic acid; THF, tetrahydrofuran; TLC, thin layer chromatography; TRIS, tris[hydroxymethyl]aminomethane.

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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