SUPPLEMENTARY INFORMATION

Toxicity of 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NKK) in early development: a wide-scope metabolomics assay in zebrafish embryos

Carla Merino[1,2,3], Marta Casado[4], Benjamí Piña[4], Maria Vinaixa*[1,2,3], Noelia Ramírez*[1,2,3]

1. Universitat Rovira i Virgili, Departament d'Enginyeria Electrònica, Elèctrica i Automàtica, Tarragona, Spain.
2. Institut d'Investigació Sanitària Pere Virgili, Tarragona, Spain.
3. CIBER de Diabetes y Enfermedades Metabólicas Asociadas (CIBERDEM), Instituto de Salud Carlos III, Madrid, Spain.
4. Department of Environmental Chemistry, Institute of Environmental Assessment and Water Research, Spanish Council for Scientific Research (IDAEA-CSIC), Barcelona, Spain.

***Corresponding authors:**
Noelia Ramírez, PhD
IISPV-URV-CIBERDEM
Av. Països Catalans, 26
43007-Tarragona, Spain

Maria Vinaixa, PhD
URV, Department of Electronic Engineering
Av. Països Catalans, 26
43007-Tarragona, Spain

Table S1. Parameters assessed to classify embryo phenotypes

Phenotype	Parameters*
Death embryos	Non-viable embryos; coagulated embryos; lack of heartbeat; and death embryos/larvae.
Severe phenotype	Lack of movement; malformation of eyes, head, mouth, and pectoral fins; modified chorda structure; no-hatched embryos; pericardial and yolk sac oedema; scoliosis; and yolk deformation.
Normal phenotype	Hatched embryos without any of the above commented phenotypic parameters.

* The observation of a single parameter assigned the phenotype

Table S2. List of previously reported NNK metabolites (Dator et al., 2018; Hecht, 1998), their abbreviation, theoretical and empirical protonated mass [M+H], empirical retention time (t_R), identification level (ID level), and source of LC-MS/MS data.

Chemical name	Abbreviation	Chemical formula	Theoretical [M+H]	Empirical [M+H]	Empirical t_R (min)	ID level*	MS/MS Refs.
4-Hydroxy-1-(3-pyridyl)-1-butanone	**HPB**	$C_9H_{11}NO_2$	166.0863	166.0867	4.83	*Not identified*	HMDB
3-Hydroxy-1-(3-pyridyl)-1-butanol	**1,3-Diol**	$C_9H_{13}NO_2$	168.1019	168.1016	1.74	*Not identified*	(Dator et al., 2018)
4-Hydroxy-1-(3-pyridyl)-1-butanol	**1,4-Diol**	$C_9H_{13}NO_2$	168.1019	168.1016	1.74	*Not identified*	(Dator et al., 2018)
1,1-Diol	**Unknown 1 (Diol 3)**	$C_9H_{13}NO_2$	168.1019	168.1016	1.74	*Not identified*	(Dator et al., 2018)
4-Oxo-4-(3-pyridyl)butyric acid	**OPBA**	$C_9H_9NO_3$	180.0655	*Not found*	*Not found*	-	-
4-Hydroxy-4-(3-pyridyl)-butyric acid	**HPBA**	$C_9H_{11}NO_3$	182.0812	182.0809	2	**1**	STDR
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone	**NNK**	$C_{10}H_{13}N_3O_2$	208.1081	208.1078	5.87	**1**	STDR
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol	**NNAL**	$C_{10}H_{15}N_3O_2$	210.1237	210.1228	4.7	*Not identified*	STDR
4-(Methylnitrosamino)-1-[3-(6-hydroxypyridyl)-1-butanone	**6-OH NNK**	$C_{10}H_{13}N_3O_3$	224.103	*Not found*	*Not found*	-	-
4-[Methyl(nitroso)amino]-1-(1-oxido-3-pyridinyl)-1-butanone	**NNK-*N*-oxide**	$C_{10}H_{13}N_3O_3$	224.103	*Not found*	*Not found*	-	-
N-(3,4-Dihydroxy-4-(pyridin-3-yl)butyl)-*N*-methylnitrous amide	**γ-OH NNAL (OH-NNAL 2)**	$C_{10}H_{15}N_3O_3$	226.1186	226.1187	5.18	*Not identified*	(Dator et al., 2018)
4-(Methylnitrosamino)-1-(3-pyridyl-N-oxide)-1-butanol	**NNAL-*N*-oxide**	$C_{10}H_{15}N_3O_3$	226.1186	226.1187	5.18	**2**	(Dator et al., 2018)
-	**Unknown 2 (OH-NNAL 1)**	$C_{10}H_{15}N_3O_3$	226.1186	226.1187	5.18	*Not identified*	(Dator et al., 2018)
3-(4-(Methyl(nitro)amino)butanoyl)pyridine 1-oxide	**nitro-NK-*N*-oxide**	$C_{10}H_{13}N_3O_4$	240.0979	240.0981	5.18	*Not identified*	(Dator et al., 2018)
3-(1-Hydroxy-4-(methyl(nitro)amino)butyl)pyridine 1-oxide	**nitro-NAL-*N*-oxide**	$C_{10}H_{15}N_3O_4$	242.1135	*Not found*	*Not found*	-	-
-	**Unknown 3**	-	254.1135	*Not found*	*Not found*	-	-
N-acetylcysteine-PHB 1	**NAC-PHB 1**	$C_{14}H_{20}N_2O_4S$	313.1216	313.1212	5.22	*Not identified*	(Dator et al., 2018)
N-acetylcysteine-PHB 2	**NAC-PHB 2**	$C_{14}H_{20}N_2O_4S$	313.1216	313.1212	5.22	*Not identified*	(Dator et al., 2018)
N-acetylcysteine-PHB 3	**NAC-PHB 3**	$C_{14}H_{20}N_2O_4S$	313.1216	313.1212	5.22	*Not identified*	(Dator et al., 2018)

4-Hydroxy-1-(3-pyridyl)-1-butanone glucuronide	**HPB-Gluc**	$C_{15}H_{19}NO_8$	342.1183	*Not found*	*Not found*	-	-
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol-O-glucuronide	**NNAL-O-Gluc**	$C_{16}H_{23}N_3O_8$	386.1558	*Not found*	*Not found*	-	-
3,4,5-trihydroxy-6-((nitroso(4-oxo-4-(pyridin-3-yl)butyl)amino)methoxy)tetrahydro-2H-pyran-2-carboxylic acid	**α-OH-methyl-NNK-Gluc**	$C_{16}H_{21}N_3O_9$	400.1351	*Not found*	*Not found*	-	-
3,4,5-Trihydroxy-6-(((4-hydroxy-4-(pyridin-3-yl)butyl)(nitroso)amino)methoxy)tetrahydro-2H-pyran-2-carboxylic acid	**α-OH-methyl-NNAL-Gluc**	$C_{16}H_{23}N_3O_9$	402.1507	402.1492	5.03	*Not identified*	(Dator et al., 2018)

***** Identification level according to Schymanski et al. (Schymanski et al., 2014). Identification was performed by matching the empirical MS/MS spectra with the MS/MS of NNK metabolites pure chemical standards (STDR) (Level 1). When pure chemical standards were not available, annotation was performed by comparing the empirical MS/MS spectra with the MS/MS spectra available in the HMDB (Wishart et al., 2018) or in the literature (Dator et al., 2018) (level 2). *Not found*: NNK metabolites are those whose empirical protonated mass was not within 5 ppm mass error. *Not identified*: NNK metabolites are those whose empirical MS/MS spectra did not match with the MS/MS spectra of NNK metabolites pure chemical standards, databases, or literature. *Unknow*: compounds are novel NNK metabolites described by Dator et.al. (Dator et al., 2018).

REFERENCES

Dator, R., Von Weymarn, L.B., Villalta, P.W., Hooyman, C.J., Maertens, L.A., Upadhyaya, P., Murphy, S.E., Balbo, S., 2018. In Vivo Stable-Isotope Labeling and Mass-Spectrometry-Based Metabolic Profiling of a Potent Tobacco-Specific Carcinogen in Rats. Anal. Chem. 90, 11863–11872. https://doi.org/10.1021/acs.analchem.8b01881

Hecht, S.S., 1998. Biochemistry, Biology, and Carcinogenicity of Tobacco-Specific N-Nitrosamines. Chem. Res. Toxicol. 11, 559–603. https://doi.org/10.1021/TX980005Y

Schymanski, E.L., Jeon, J., Gulde, R., Fenner, K., Ruff, M., Singer, H.P., Hollender, J., 2014. Identifying small molecules via high resolution mass spectrometry: Communicating confidence. Environ. Sci. Technol. https://doi.org/10.1021/es5002105

Wishart, D.S., Feunang, Y.D., Marcu, A., Guo, A.C., Liang, K., Vázquez-Fresno, R., Sajed, T., Johnson, D., Li, C., Karu, N., Sayeeda, Z., Lo, E., Assempour, N., Berjanskii, M., Singhal, S., Arndt, D., Liang, Y., Badran, H., Grant, J., Serra-Cayuela, A., Liu, Y., Mandal, R., Neveu, V., Pon, A., Knox, C., Wilson, M., Manach, C., Scalbert, A., 2018. HMDB 4.0: The human metabolome database for 2018. Nucleic Acids Res. 46, D608–D617. https://doi.org/10.1093/nar/gkx1089