Nicotine, Cotinine or a cotinine metabolite inhibits NNK-induced DNA-strand break in metabolically competent hepatic cells

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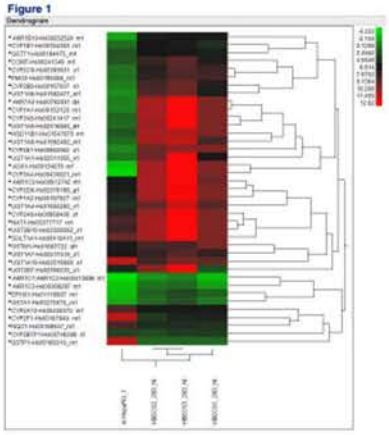
Background: Nicotine is not considered to be genotoxic; however, nicotine has been reported to enhance tumor multiplicity in A/J mice treated with the tobacco nitrosamine NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) (Davis *et al.*, 2009). In contrast, other *in vivo* studies with A/J mice concluded that nicotine had no influence on NNK-induced tumor multiplicity and progression (Murphy *et al.*, 2011). Furthermore, a previous A/J mice study has also suggested a protective effect of nicotine against metabolic activation of NNK (Brown *et al.*, 1999). Recent *in vitro* work using purified enzymes demonstrated that nicotine and/or a nicotine metabolite could inhibit CYPs (CYP2A6, 2A13) involved in NNK bioactivation by a mechanism-based inhibition. Therefore, we hypothesized that nicotine or a nicotine metabolite such as cotinine might contribute to the inhibition of NNK-induced DNA strand breaks by inhibiting CYPs enzymes. The effect of nicotine and cotinine on DNA strand break were evaluated using the COMET assay in CYP competent HepaRG cells incubated with bioactive CYP-dependent NNK and CYP-independent NNKOAc (4-(acetoxymethylnitrosoamino)-1-(3-pyridyl)-1-butanone).

Methods: HepaRG, HBECs, and BEAS-2B culture conditions, mRNA extraction, QRT-PCR and enzymatic probe assays have been descibed in Garcia-Canton *et al.*, 2013 and Newland *et al.*, 2011. The Alkaline COMET assay was based on the methods described by Tice et al. (Tice *et al.*, 2000), Thorne et al. (Thorne *et al.*, 2009) and the Comet assay interested group (http://www.cometassay.com) with slight modifications. Data were analysed by using the parametric statistical approach published by Bright et al. (Bright *et al.*, 2011). The median by plate of the logarithmic tail intensity were analysed in a mixed model in Minitab 16 software with treatment as fixed effect and run as random effect, differences between the treatments variances was specified. Post-hoc multiple comparisons were adjusted by Tukey's.

Results:

Hierarchical cluster representing the gene expression profiles of 39 selected metabolic genes tested in HBECs (3 subjects) and HepaRG is shown in **Figure 1**. Columns represent individual samples and rows represent genes. Green, black, and red indicate high signal intensity, moderate to low signal intensity or no signal in normalized gene expression data (ΔCt), respectively.

Figure 1



A comparison of coumarin 7-hydroxylation (Coum) activity in primary human bronchial epithelial cells (HEBCs) from 3 donors, BEAS-2B cells, and HepaRG cells is shown in **Figure 2**. 8-methoxypsoralen (8-mop) was used as inhibitor of CYP2A6/CYP2A13. Results are presented as mean of three measurements ± standard deviation.

Figure 2

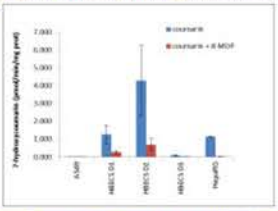


Table 1A shows a summary of the inhibition kinetics reported in the literature for nicotine and the nicotine iminium ion metabolite. **Table 1B** presents the IC50 values we obtained following incubation of [^{3}H]-NNK with CYP2A13 bactosomes in the presence of nicotine, cotinine, and PPITC.

Table 1A

		Inhibitor	Ki (μM)	Kinact (min^{-1})	$t_{1/2}$ (min)	References
CYP2A13		nicotine	17	0.1	7	He *et al.*, 2004
	nic	iminium	30	0.04	15.5	Von Weyman *et al.*, 2013
CYP2A6		nicotine	21	0.021	33	He *et al.*, 2005
	nic	iminium	300	0.03	27	Von Weyman *et al.*, 2013

Table 1B

	IC50 (μM)		
	Nicotine	Cotinine	PPITC
HPB	70.15 ± 15.18	16.32 ± 7.5	0.016 ± 0.0035
Total CYP2A13 NNK metabolism	30.56 ± 10.11	3.61 ± 0.99	0.01 ± 0.002

Figure 3 shows the COMET assay results following exposure of HepaRG to NNK and increasing doses of nicotine (**A**), cotinine (**B**), and β-nicotyrine (**C**). The Individual value plots include the values for each nuclei acquired during the COMET assay expressed as **%** tail intensity. The COMET assay was performed with NNK (100 μM) and increasing doses of nicotine, cotinine, and β-nicotyrine. NT indicates non treated control samples and PPITC was used as a control inhibitor for CYP1A2, CYP2A13, and CYP2A6. NNKOAc was used as a positive control for DNA damage. The mean values for each condition tested are shown in blue (•) and the median values are shown in red (•). Pairwise comparison results (Tukey's test) for significant differences between tested conditions at 95% confidence are shown in **Table 2A, B, C**. N indicates the total number of slides counted from 3 independent experiments with the overall total nuclei counted. The mean (Mean Med Log) is the average of the median of the log transformed tail intensity calculated for each independent experiment. Means that do not share a letter are significantly different.

Figure 3

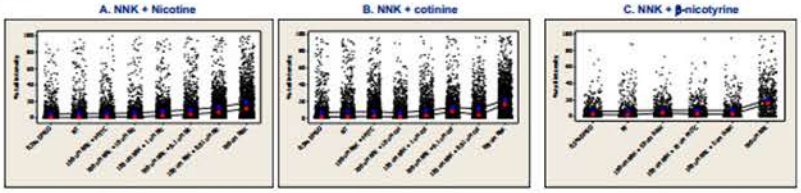
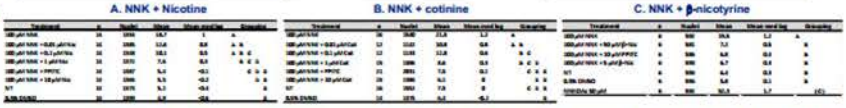


Table 2



An individual value plot showing the COMET % tail intensity for each acquired nuclei following the incubation of HepaRG with NNKOAc (25 μM) for 3 hrs and HepaRG pre-incubated with cotinine, nicotine, and PPITC (10 μM) for 12 hours and NNKOAc (25 μM) added for the last 3 hours is shown in **Figure 4**. Mean value for each condition tested are shown in blue (•) and median values are shown in red (•). Pairwise comparison (Tukey test) for significant differences between tested conditions at 95% confidence is shown in **Table 3**. N indicates the total number of slides counted from a minimum of 3 independent experiments with the overall total nuclei counted (approximately 100 per slide). The mean is the average of the median of the log transformed tail intensity calculated for each independent experiment. Means that do not share a letter are significantly different.

Figure 4

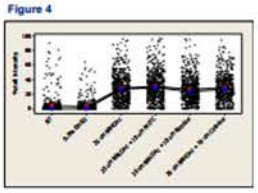


Table 3

Treatment	n	Nuclei	Mean	Mean med log	Grouping
25 μM NNKOAc	6	595	28.8	1.4	a
25 μM NNKOAc + 10 μM Nic	6	600	26.7	1.4	a
25 μM NNKOAc + 10 μM Cot	6	564	28.8	1.4	a
25 μM NNKOAc + 10 μM PPITC	6	592	30.9	1.5	a
NT	6	593	4.2	-0.8	b
0.5% DMSO	6	590	3.7	-0.6	b

Conclusion: We have developed and applied a robust protocol to assess DNA damage by COMET in HepaRG cells, a cell line that shares greater metabolic and morphological similarities with normal human hepatocytes. Using the COMET assay, we showed that nicotine, cotinine, or a metabolite of cotinine can protect against NNK-induced DNA damage in HepaRG by inhibiting metabolic enzymes, possibly of the CYP2A or CYP1A family. Importantly, this and other researches highlight the current limitations of the classical paradigm of single toxicants assessment when a complex mixture such as tobacco smoke is considered. Single toxicant assessments do not take into account complex interactions between chemicals and biological systems.

Conflicts of interest

The authors report no conflict of interest. E. Minet, A. Banerjee, and O.M. Camacho and D. Waters were employees of British American Tobacco or contracted by British American Tobacco during the conduct of this study. Part of the work was contracted to Vivotecnia Research S.L., Santiago Grisolia 2, Tres Cantos, Madrid, Spain and Charles River, Tranent, Edinburgh, EH33 2NE, UK

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