Monoamine oxidase inhibitory activity in tobacco particulate matter: Are harman and norharman the only physiologically relevant inhibitors?

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\section*{A B S T R A C T}

Monoamine oxidase inhibition is significant in smokers, but it is still unclear how the inhibition that is seen in the brains and bodies of smokers is brought about. Our aim was to test the contribution of the harman and norharman in tobacco smoke to MAO-A inhibition from tobacco smoke preparations, as part of a re-examination of harman and norharman as the cause of the inhibition of MAO-A inhibition in the brain.

Tobacco smoke particulate matter and cigarette smoke particulate matter were prepared and the amounts of harman and norharman measured. The results were compared with the total monoamine oxidase-A inhibitory activity.

At a nicotine concentration of 0.6 \textmu M (a "physiological" concentration in blood) the total monoamine oxidase-A inhibitory activity measured in these samples was sufficient to inhibit the enzyme by approximately 10\%. Of this inhibitory activity, only a small proportion of the total was found to be due to harman and norharman.

These results show that harman and norharman provide only a moderate contribution to the total monoamine oxidase-A inhibitory activity of tobacco smoke, perhaps under 10\%. This suggests that other inhibitors (either known or unknown) may be more significant contributors to total inhibitory activity than has yet been established, and deserve closer examination.

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\section*{1. Introduction}

It is well established that monoamine oxidase (MAO) activity is reduced in the brains and bodies of long-term smokers. The average reduction in brain MAO-A is 30\% (MAO-B, 40\%) in established smokers (Fowler et al., 1996, 2003). MAO activity recovers slowly over the course of a few weeks when smokers stop smoking (Fowler et al., 2003; Rose et al., 2001) and this time course is believed to imply that the activity is irreversibly inhibited by smoke components (Rose et al., 2001; Fowler et al., 2003; Lewis et al., 2007; Yu and Boulton, 1987). MAO enzymes metabolise dopamine (Lewis et al., 2007; van Amsterdam et al., 2006) the reinforcement stimulus instigated by nicotine and most closely associated with addiction (Le Foll and Goldberg, 2009; Pontieri et al., 1996). Impairment of MAO activity is thus predicted to have an impact on addiction to tobacco (reviewed in Hogg, 2016). Indeed, MAO inhibitors have enhanced responses to nicotine in rat self-administration tests (Kapelewski et al., 2011; Villegier et al., 2011; Lotfpour et al., 2011; Smith et al., 2016). MAO inhibitors have been trialled for smoking cessation (George and Weinberger, 2008).

Although the fact of MAO activity reduction in smokers and its potential to affect the brain's responses to nicotine is clear, it is less clear how this reduction in activity is brought about. A variety of MAO inhibitors have been found in tobacco smoke. The most notable are harman and norharman, \beta-carbolines which are reversible inhibitors of MAO-A and -B. Smoking is the major source of human exposure to harman and norharman (Pfau and Skog, 2016).
2004; Rommelspacher et al., 2002), which have been proposed as major contributors towards reducing MAO activity in smokers (van Amsterdam et al., 2006; Herrera and Chaparro, 2005; Rommelspacher et al., 2002).

Nevertheless, there are challenges to this view. Harman and norharman are both reversible inhibitors (Lewis et al., 2007). Their half-life in blood is reported to be close to one hour (Herrera and Chaparro, 2005) or less (Fekkes and Bode, 1993). In contrast, nicotine has a half-life of closer to two hours (Benowitz et al., 2009) and should be cleared from the body more slowly than these MAO inhibitors. The concentration of nicotine in arterial blood can reach 100 ng/mL (0.6 μM) in smokers but it is more usually around half of this. The concentrations of harman and norharman measured in tobacco smoke, compared to the concentration of nicotine, suggest that blood concentrations after smoking should be in the low nanomolar range (Brennan et al., 2013, 2015; Lewis et al., 2012), and this is indeed what is observed (van Amsterdam et al., 2006; Rommelspacher et al., 2002). Yet the MAO-B activity in blood platelets will be inhibited by 50% only if norharman reaches low micromolar concentrations (Ki, 1.12 μM). Similarly the most potent inhibitor of MAO-A, harman, has a Ki of 55 nM (Herrera and Chaparro, 2005). Can these reversible inhibitors, harman and norharman, reach the concentrations required for 30–40% inhibition of MAO-A and –B activity?

Attempting to explain this discrepancy, Rommelspacher et al. have found evidence for a longer half-life for norharman in platelets (Rommelspacher et al., 2002) suggesting that it may be possible for its concentration (and inhibitory effects) to build up over time in specific compartments in the body. Partitioning effects may be occurring in the brain as well (Rommelspacher et al., 2002; Fekkes and Bode, 1993). However, there is as yet no direct evidence that harman and/or norharman in tobacco smoke cause the MAO inhibition seen in smokers, and no evidence for accumulation of either harman or norharman in the brain to the mid nanomolar or low micromolar concentrations required to cause the observed inhibition of enzyme activity.

In this paper we report the results of experiments aimed at determining the proportion of the total MAO-A inhibitory activity in a preparation of tobacco smoke which is attributable to harman and norharman. This is a first step towards re-examining the significance of harman and norharman in causing the reduction of MAO-A activity experienced by smokers.

2. Materials and methods

2.1. Preparation of monoamine oxidase inhibitors

Tobacco smoke preparations (tobacco particulate matter; TPM) were prepared as previously described (Lewis et al., 2012) using particulate matter collected from the smoke under ISO 3308 conditions (LabStat international, Kitchener, Canada). The tobacco and cigarettes samples used were each purchased commercially within New Zealand and were chosen from commonly used brands. The tobacco was Drum, rolled into cigarettes containing 1 g of tobacco, and the cigarettes were Holiday Red. Filters with TPM were extracted with ethanol, and the ethanol extracts used directly in enzyme inhibition assays (Lewis et al., 2012). TPM prepared in this way will lack the more volatile components of tobacco smoke (e.g. carbon monoxide and ammonia). Furthermore small particles insoluble in ethanol (e.g. partially burnt plant material) largely stay on the filter.

Harman and norharman were obtained from Sigma Aldrich (St Louis, MO) and made up in ethanol to a stock concentration of 100 mM. For concentration: response curves inhibitors were diluted in ethanol and 5 μL of inhibitor in ethanol (or ethanol alone) added to the assay mix.

2.2. Monoamine oxidase inhibition assays

Human recombinant monoamine oxidase A (MAO-A) was obtained from Sigma Aldrich, aliquoted and stored at −80°C until use. Enzyme inhibition assays were performed essentially as previously described (Lewis et al., 2012; Lewis, 2010) but with minor modifications. The kynuramine substrate was used at 100 μM and the final ethanol concentration was 5% (v/v) in each well. Finally, incubations were for 15 min only, before the reaction was stopped by addition of sodium hydroxide.

Blank wells containing buffer and inhibitor only, at the same concentration as in the assay, were included to allow removal of fluorescence due to the sample itself, prior to calculation of the inhibition of MAO-A activity caused by the sample. Controls with no ethanol or inhibitor, and for the intrinsic fluorescence of the substrate were also included. Harman, at close to its IC50 concentration, was used as positive control when TPM samples were being analysed, to ensure assay consistency.

All assays were performed in triplicate and repeated at least once. Prism 5 (GraphPad Software Inc) was used for fitting a sigmoidal concentration: response curve of log [inhibitor concentration] against relative fluorescence units. % inhibition at specific nicotine or inhibitor concentrations (see Tables 1 and 2) was obtained by interpolation from the fitted curve.

2.3. Nicotine, harman, and norharman analyses

Nicotine concentrations were determined by gas chromatography-mass spectrometry as previously described (Ambrose et al., 2007).

Harman and norharman concentrations were assessed by liquid chromatography- mass spectrometry using an Agilent 1200 liquid chromatograph coupled to an Agilent 6410 triple quadrupole mass spectrometer fitted with an Agilent Multimode ion source and run in ESI mode. Samples in ethanol were diluted 50- to 300-fold in acetonitrile before separation on a Zorbax SB-C18 column run at 35°C under isocratic buffer conditions (50% water/acetonitrile 0.02% in formic acid). A small amount of deuterated harman (D4 harman, BDG Synthesis) was added to each sample and standard and was used to correct for ionisation suppression. Five standards of each analyte were run separately (0.1–10 ppb in acetonitrile) and the concentrations of harman and norharman in the samples were assessed by comparison with the standard curves. Mass spectrometric detection was in positive ion mode (harman m/z 183, norharman m/z 169, with subsequent determination of daughter ions at m/z 154 (harman) and 145 (norharman) as well as m/z 115 for both analytes).

3. Results

3.1. Inhibition of MAO-A by ethanol

Addition of ethanol to 5% reduced the enzyme activity by 19.1–25.6% in these experiments, with the extent of reduction depending on the batch of the monoamine oxidase enzyme (unpublished data), but being consistent within each enzyme batch.

3.2. Inhibition of MAO-A by tobacco smoke

Both of the tobacco smoke samples inhibited MAO-A, as expected (Fig. 1A). Half maximal inhibition of MAO-A was achieved by addition of 0.053 μL of the tobacco smoke sample (5 μL of a 1/94 dilution; 95% confidence limits 0.036–0.079 μL) and 0.13 μL of the cigarette smoke sample (5 μL of a 1/39 dilution; 95% confidence limits 0.11–0.15 μL).