Identification of Pyridine Compounds in Cigarette Smoke Solution That Inhibit Growth of the Chick Chorioallantoic Membrane

Lin Ji, Goar Melkonian, Karen Riveles, and Prue Talbot

Department of Cell Biology and Neuroscience, 135 Spieth Hall, University of California, Riverside, California 92521-0146

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Based on prior work, we hypothesized that cigarette smoke contains chemicals that can inhibit growth of the chick chorioallantoic membrane (CAM). In this study, gas chromatography and mass spectrometry were used to identify 12 pyridine derivatives in the inhibitory fractions of smoke eluted from solid phase extraction cartridges. These pyridine derivatives were further studied individually in dose response experiments to determine their effects on CAM growth. A correlation was observed between the functional group substitutions on pyridine and the relative toxicity of each pyridine derivative. In the CAM growth assay, pyridine derivatives with single methyl or single ethyl substitutions had lowest observed adverse effect levels (LOAELs) of $5 \times 10^{-8}$ and $5 \times 10^{-12}$ M, respectively. Other pyridine derivatives and pyridine itself had LOAELs in the micromolar range. One of the most inhibitory derivatives, 3-ethylpyridine, was studied further and inhibited cell proliferation, as measured by BrdU incorporation. Since 3-ethylpyridine inhibited growth at picomolar doses and is added to consumer products including cosmetics, food, drinks, and tobacco, it will be important to perform further toxicological testing to determine its effect on human health.

Key Words: pyridine; pyridine derivative; 3-ethylpyridine; growth; chick chorioallantoic membrane; cigarette smoke; passive smoking; active smoking.

Sidestream smoke, which burns off the end of cigarettes, is the main component of environmental tobacco smoke and is inhaled by passive smokers (U.S. EPA, 1992). Mainstream smoke is the bolus of smoke inhaled by active smokers. Mainstream and sidestream smoke contain over 4000 chemicals, some of which, such as nicotine, have been studied extensively (U.S. EPA, 1992). However, the effects of most of the chemicals in cigarette smoke have not been well characterized, and many of the chemicals in smoke may be unrecognized toxicants.

Several lines of in vivo evidence indicate that components in cigarette smoke can inhibit growth. We recently showed that mainstream whole, sidestream whole, and sidestream gas phase smoke solutions significantly inhibited growth of the chick chorioallantoic membrane (CAM) in a dose-dependent manner and that sidestream whole and sidestream gas solutions were more inhibitory than the mainstream whole solutions (Melkonian et al., 2000, 2002a). It was deduced from these data that growth inhibitory chemicals in sidestream whole smoke partition mainly with the gas phase, while in mainstream smoke inhibitory chemicals partition mainly with the particulate phase. Inhibition of growth was shown by BrdU labeling to be due to inhibition of cell division (Melkonian et al., 2002a). Several other processes in smokers are linked to inhibition of cell proliferation. In the rat, cigarette smoke inhibited gastric ulcer healing by inhibiting cell proliferation (Ma et al., 2000). Slower healing of wounds has also been observed clinically in smokers (Silverstein, 1992), and could be due to an inhibition of cell proliferation by chemicals in smoke. In addition, incomplete development in the placentas of human smokers has been correlated with a decrease in the number of cytotrophoblasts in the S phase of the cell cycle (Genbacev et al., 2000).

Based on prior work, we hypothesized that cigarette smoke contains chemicals that can inhibit growth of the CAM. Our current research goal was to identify the chemicals in cigarette smoke solutions that inhibit tissue growth. To accomplish this goal, we first screened a number of solid phase extraction cartridges to identify one that retained almost 100% of the CAM growth inhibitory activity. The eluate from this cartridge was subsequently analyzed by gas chromatography-mass spectrometry (GC-MS) and 12 pyridine derivatives were identified as the main class of compounds in the cartridge eluate. The pyridine identities were then confirmed using purified standards matched by mass spectrum and retention time. In the present study, the lowest observed adverse effect level (LOAEL) and no observed adverse affect level (NOAEL) were determined for each purified pyridine derivative using the day 5 CAM growth assay. The majority of the pyridine derivatives and pyridine itself had LOAELs and NOAELs in the micromolar range. However, several chemicals were identified that had LOAELs 10,000 to 10 million times lower than pyridine. A correlation was observed between the functional group substitutions and the relative toxicity of each pyridine derivative. At least one of the chemicals that inhibited growth in the picomolar range is an approved additive in consumer products.
MATERIALS AND METHODS

Media and reagents. All buffers, including phosphate buffered saline (PBS), Earle’s Balanced Salt Solution (EBSS), and acrylamide were made using Barnstead/Thermolyne nanopure water (Fisher Scientific, Tusin, CA). EBSS was made fresh daily from a 10X stock solution. To a 1X salt solution, sodium bicarbonate and HEPES were added to make EBSS-H (Talbot et al., 1998). The pH of EBSS-H was adjusted to 7.4 with NaOH and was used as the control solution to dilute test chemicals in all experiments. Glutaraldehyde (50%) was purchased from Electron Microscopy Supplies (Fort Washington, PA) and diluted to 3% in 0.1 M cacodylate buffer (pH 7.4) for use in fixation. Pyridine was purchased from VWR Scientific (San Francisco, CA), 2-methylpyridine, 3-methylpyridine, 2,3-dimethylpyridine, 2,4-dimethylpyridine, 2,4,6-trimethylpyridine, 2-ethylpyridine, 3-ethylpyridine, and 4-ethylpyridine were purchased from Sigma-Aldrich Incorporated (Milwaukee, WI). 2,4,6-trimethylpyridine, 2-ethylpyridine, 3-ethylpyridine, and 4-ethylpyridine were purchased from Sigma-Aldrich Incorporated (Madison, WI). 2-ethylpyridine and 3-(1-pyrroldin-2-yl)pyridine (nornicotine) was purchased from Toronto Research Chemicals (North York, Ontario, Canada).

Smoke solutions. Smoke solutions were made fresh in EBSS-H using 2R1 research grade cigarettes (University of Kentucky, Louisville, KY) as described in detail previously (Knoll and Talbot, 1998). Sidestream (SS) whole smoke solutions were made by collecting the smoke that was produced at the burning end of a cigarette during 30 puffs of MS smoke and pushing it through 10 ml of EBSS-H. The gas phase of SS (SSG) smoke was made by passing whole SS smoke through a Cambridge filter prior to bubbling it into EBSS-H (Knoll and Talbot, 1998). Smoke solutions and control medium (EBSS-H) applied to CAMs were passed through a 0.22 μm Acrodisc filter (Fisher Scientific, Tusin, CA) and handled using sterile technique. The pH of each solution was adjusted to 7.4, and solutions were added to CAMs immediately after preparation.

Solid phase extraction of smoke solutions. Solid phase extraction (SPE) cartridges were used to precollect smoke solutions and concentrate chemicals that inhibit growth. All SPE cartridges were Bond Elut cartridges 3 cc with 500 g capacity purchased from Phenomenex, Torrance, CA. Cartridges that were screened for their ability to bind inhibitory chemicals included NH2, 2OH, CN, CBA, SCX, SAX, C18, C8, C2, CH, SL, and PH. This range of cartridges was chosen with the expectation that one or several of them would be effective at retaining the growth inhibitory chemicals in cigarette smoke.

The solid phase extraction cartridges were activated with 50 ml of HPLC grade methanol (Fisher Scientific, Tusin, CA), and then rinsed with 200 ml of deionized water. A column control was collected to assure that the methanol had been rinsed from the cartridge and that any toxic effect observed was solely due to the smoke solution. The smoke solution was run through the cartridge and the filtrate was collected and tested in the CAM assay. The cartridge was then either eluted immediately or frozen at −20°C for later elution. Solid phase extraction cartridges were eluted with 1 ml of HPLC grade methanol. The eluate was collected and used in the GC-MS analysis and the biological assays. For the biological assays, the methanol was evaporated under nitrogen, and the residue was resuspended in EBSS-H. A methanol control was used to assure that any observed activity was not due to residual methanol in the sample, and in no case did the methanol control affect growth.

GC-MS. A preliminary screen of solid phase extraction cartridges had shown that the C2 cartridge retained most of the inhibitory activity in the CAM growth assay. To identify the components in aqueous smoke solutions that inhibit growth, mainstream and sidestream smoke solutions were analyzed with GC-MS after solid phase extraction on a C2 column. The equipment used was a Hewlett Packard 5890 GC interfaced to an HP-5971A MSD (quadrupole mass selective detector) with a Zebron ZB1701 cyanopropyl phenyl column 30 m × 0.32 mm and with a 1 μm phase thickness (Phenomenex, Torrance, CA.). Helium was used as the carrier gas. The mass range examined was 40 to 350 with a threshold of 1500 with a sampling of 2.3 scans per second. The temperature program was an initial temperature of 45°C for 1 min with an increase of 10°C per min to a final temperature of 280°C after 10 min. The total run time was 34.5 min. Two μl of the eluate sample were injected directly into the GC using a Hamilton gas-tight syringe. Identifications of compounds were made using the mass spectrometry data matched to library entries. Compound identities were confirmed using purified standards matched by mass spectrum and retention time.

CAM assay. CAM growth was evaluated by measuring CAM area using the CAM assay as modified by Melkonian et al. (2002b). Because the CAM undergoes very rapid growth, increasing in size 20-fold between days 5 and 6 of development (Melkonian et al., 2002b), it can be used as a screening assay to quickly identify chemicals that inhibit tissue growth (Melkonian et al., 2002a). The CAM assay is both economical and rapid. Since it is an in vitro assay, it may provide more meaningful data than in vitro screens using cultured cells. Fertilized chicken eggs were purchased from Hy-Line International (Lakeview, CA), and then incubated at 37°C at 85–90% relative humidity throughout the experiment. Albumin (2.5–3.0 cc) was removed on the fourth day after fertilization to drop the embryo away from the shell. Windows were then made in shells, and sealed with transparent tape to allow subsequent access to the CAM for experimental treatment. Two hundred μl of either EBSS-H or different concentrations of pure chemicals were added to the surface of each CAM at 1000 h on the fifth day after fertilization. A sham control group in which the window was opened and resealed was also included in each experiment. In all cases, the following 5 concentrations of test reagents were used, 5 × 10−3 M, 5 × 10−4 M, 5 × 10−5 M, 5 × 10−6 M, and 5 × 10−7 M. For 2-ethylpyridine and 3-ethylpyridine additional concentrations of 5 × 10−5 M, 5 × 10−6 M, 5 × 10−7 M, and 5 × 10−8 M were used. For nicotine and nornicotine, concentrations of 5 × 10−5 M, 5 × 10−6 M, 5 × 10−7 M, and 5 × 10−8 M were also used. Development was terminated at 1000 h on the sixth day after fertilization by fixing the embryo and the CAM with 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 2 h at room temperature. A small volume of fixative was also injected under the CAM. The CAMs were then dissected from eggs, postfixed in the same fixative for 24 h at room temperature, then thoroughly rinsed in PBS.

Quantification and imaging of CAMs. At least 6 CAMs were used in each control and experimental group. The area of each CAM was measured by placing the CAMs in PBS in a Petri dish under a Wild-M5A dissecting microscope (Max ERB Instrument Co., Burbank, CA), then measuring the longest and shortest length of each CAM with a ruler to the precision of 0.5 mm. The data were recorded into an Excel spreadsheet. CAM area was calculated using the formula:

\[
\text{Area} = (1/2 \times A) \times (1/2 \times B) \times (\pi) \text{ where } A = \text{longest length}, \ B = \text{longest width}, \ \pi = 3.14.
\]

Means and SD were then calculated for each control and treatment group. The treated group means were used to compare with control means to determine the effect of each chemical on CAM growth (the LOAEL, the NOAEL, and the maximum percentage of inhibition for each chemical were determined).

BrdU labeling. EBSS-H or 3-ethylpyridine were placed on CAMs between 1100–1200 h on day 5 after fertilization. At 8 h after the start of an experiment, control and treated CAMs were exposed to 100 μl of BrdU (10 mM) for 55 min; they were then transferred to a −20°C freezer for 2–3 min and fixed at −20°C in 70% ethanol containing 50 mM glycine buffer, pH 2.0 for 1.5 h. CAMs were cut out of eggs and washed in PBS and dehydrated in an ethanol series, and further processed in a Hypercenter II Tissue Processing System (Shandon Inc., Pittsburgh, PA), then embedded in paraffin using a Sakura Tissue-TE (Shandon Inc., Pittsburgh, PA). Sections (5 μm) were cut on an S20 Spencer microtome (Labequip, American Optical Corp., Ontario, Canada), and placed on Superfrost/plus glass slides (Fisher Scientific, Tusin, CA). Sections were deparaffinized with 3 changes of Hemo-De for 15 min each, washed in 100% ethanol 2 times for 5 min each, then rehydrated to PBS. Sections were incubated in 1N HCl for 1 h at 40°C, washed in PBS for 10–15 min, then incubated in 1% sodium borohydride (Sigma, St. Louis, MO) for 10 min, and washed again in PBS. Sections were incubated in anti-BrdU IgG

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Twelve pyridine derivatives were identified as the major group of chemicals in the eluate and were individually tested at various concentrations for their effects on CAM growth. In no experiments did treatment of CAMs with only EBSS-H, the vehicle used to dissolve the test compounds, affect CAM growth (Figs. 2–7; compare CN to EBSS-H).

**Pyridine and Bipyridine Inhibited CAM Growth at Micromolar Doses**

Pyridine is the parent compound of the various derivatives identified in the C2 eluate. Pyridine was not found in the C2 eluate but was tested in the CAM assay so that it could serve as a reference to investigate the effects of various substituted functional groups on CAM growth (Fig. 2A). Concentrations of pyridine between $5 \times 10^{-6}$ M to $5 \times 10^{-4}$ M did not significantly affect CAM growth when compared to the control and EBSS-H treated groups. The LOAEL for pyridine was determined to be $5 \times 10^{-5}$ M ($3.96 \times 10^{-3}$ mg/ml), which significantly inhibited CAM growth when compared to the controls.

Using the LOAEL of the pyridine ring as a comparative reference, the 12 pyridine derivatives identified in sidestream smoke solution were then tested for their effects in the CAM assay. 4,4-Bipyridine, which has two pyridine rings dimerized at the 4,4 position, increased the LOAEL to $5 \times 10^{-4}$ M. The 12 pyridine derivatives identified in C2 eluates of sidestream smoke had pyrrole substitutions (Fig. 3). 3-(1-Methylpyrrol-2-yl)pyridine ($\beta$-nicotyrine), 3-(1-pyrrolidin-2-yl)pyridine (nornicotine), and 1-methyl-2-(3-pyridyl)pyrrolidine (nicotine) were tested on day 5 CAMs and produced a bimodal effect in which CAM growth decreased at high concentrations and increased at low concentrations. The LOAELs for inhibiting growth for $\beta$-nicotyrine, nornicotine, and nicotine were $5 \times 10^{-5}$ M ($7.90 \times 10^{-3}$ mg/ml), $5 \times 10^{-4}$ M ($7.40 \times 10^{-2}$ mg/ml), and $5 \times 10^{-4}$ M ($7.40 \times 10^{-2}$ mg/ml), respectively.
In contrast to pyridines with single methyl substitutions, the LOAELs for 2,3-dimethylpyridine and 2,4-dimethylpyridine were $5 \times 10^{-5}$ M ($5.36 \times 10^{-7}$ mg/ml) and the LOAEL for $10^{-1}$ mg/ml), and $5 \times 10^{-4}$ M ($8.10 \times 10^{-2}$ mg/ml), respectively. Unlike all other pyridine derivatives tested, a significant stimulatory effect was observed on growth for nornicotine and nicotine at $5 \times 10^{-11}$ M. There was also a slight stimulation for $\beta$-nicotyrine, but this was not significantly different from the controls.

**Single Linear Substitutions to Pyridine Inhibited CAM Growth at Nano and Picomolar Concentrations**

Two of the pyridine derivatives identified in the C2 eluate had single methyl substitutions. Treatment of CAMs with either 2-methylpyridine or 3-methylpyridine significantly decreased CAM growth in a dose-dependent manner (Figs. 4A and 4B). Both 2-methylpyridine and 3-methylpyridine were effective at doses as low as $5 \times 10^{-9}$ M ($4.66 \times 10^{-7}$ mg/ml), which is 10,000 times lower than the LOAEL of pyridine.
2,4,6-trimethylpyridine was $5 \times 10^{-3}$ M ($6.10 \times 10^{-3}$ mg/ml; Fig. 5). Thus LOAELs for the double and triple methyl substituted derivatives were the same as for pyridine.

Two of the compounds in the C2 eluate had single ethyl substitutions. In the CAM growth assay, substitution of a single ethyl group at either position 2 or 3 of the pyridine ring decreased the LOAEL to at least $5 \times 10^{-11}$ M, a concentration that was ineffective for all other chemicals tested (Figs. 6A and 6B). Since all doses were highly inhibitory in this set of experiments, a second set of experiments was performed over the concentration range of $5 \times 10^{-9}$ M to $5 \times 10^{-14}$ M to define the LOAEL (Figs. 6C and 6D). For both 2-ethylpyridine and 3-ethylpyridine, the LOAEL was $5 \times 10^{-12}$ M ($5.36 \times 10^{-10}$ mg/ml), which is 10 million times lower than the LOAEL of pyridine.

One compound identified in the C2 eluate, 3-ethenylpyridine, is neither commercially available nor readily synthesized, therefore, its isomer, 4-ethenylpyridine, which is commonly used as its substitute, was tested in the CAM growth assay (Fig. 7). The LOAEL for 4-ethenylpyridine was $5 \times 10^{-6}$ M (5.26 $\times 10^{-10}$ mg/ml).
10^{-3} mg/ml), which was equivalent to that of the pyridine.
Addition of a double bond to the ethyl functional group created an ethenyl substitution and completely reversed the highly inhibitory effect of 3-ethylpyridine.

3-Ethylpyridine Inhibited CAM Growth by Inhibiting Cell Proliferation

To determine if inhibition of CAM growth correlated with inhibition of cell proliferation, BrdU labeling was done on CAMs treated with 10^{-8} M 3-ethylpyridine for 8 h (Fig. 8). 3-Ethylpyridine was chosen for this assay as it was one of the chemicals that inhibited growth at picomolar doses. Treated CAMs had significantly (p < 0.001) fewer BrdU labeled nuclei than the controls (control = 16.6 ± 3.3 labeled nuclei/7.8 mm^2; 10^{-8} M 3-ethylpyridine = 7.25 ± 2.5 labeled nuclei/7.8 mm^2), indicating that DNA synthesis had been inhibited by 3-ethylpyridine.

FIG. 6. (A) 2-Ethylpyridine and (B) 3-ethylpyridine inhibited CAM growth at picomolar doses. EBSS alone did not affect CAM growth compared to the untreated controls (CN). However, 5 × 10^{-13} M to 5 × 10^{-3} M 2-ethylpyridine and 3-ethylpyridine produced a significant dose-dependent decrease in CAM growth. (C, D) Additional experiments over the lower range of 5 × 10^{-13} M to 5 × 10^{-11} M showed the lowest effective dose was at 5 × 10^{-12} M for both chemicals. Each group had 6 CAMs. Values are plotted as means ± SD. *p < 0.05, **p < 0.01.

FIG. 7. 4-Ethenylpyridine inhibited CAM growth at micromolar doses. EBSS alone did not affect CAM growth compared to the untreated controls (CN). However, 5 × 10^{-9} M to 5 × 10^{-7} M doses of 4-ethenylpyridine produced a significant decrease in CAM growth. Each group had 6 CAMs. Values are plotted as means ± SD. **p < 0.01.
One of the goals of this study was to determine which chemicals in sidestream smoke inhibit CAM growth. Twelve pyridine derivatives were identified by MS in the eluate of C2 cartridges and were individually tested in dose response experiments to determine their relative effectiveness in inhibiting CAM growth. Each of the 12 pyridine derivatives inhibited CAM growth in a dose-dependent manner. The NOAELs, LOAELs, and the maximum percentage of inhibition for each chemical tested are summarized in Table 1. Their hierarchy of potency in the CAM growth assay based on their LOAELs was as follows: [2-ethylpyridine = 3-ethylpyridine (5 × 10⁻⁶ M)] < β-nicotyrine (5 × 10⁻⁷ M) < [4-ethenylpyridine = 2,3-dimethylpyridine = 2,4-dimethylpyridine = 2,4,6-trimethylpyridine = nornicotine = pyridine (5 × 10⁻⁵ M)] < [4,4-bipyridine = nicotine (5 × 10⁻⁴ M)]. One of the most remarkable findings in this hierarchy is the range of the LOAELs. 2-Ethylpyridine and 3-ethylpyridine were 10 million times more potent in the CAM growth assay than pyridine itself and demonstrate that a simple ethyl substitution to pyridine dra-

<table>
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<tr>
<th>Chemical Tested</th>
<th>NOAEL</th>
<th>LOAEL</th>
<th>MAXIMUM % INHIBITION</th>
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<tr>
<td>Pyridine</td>
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<td>5e-5M</td>
<td>ND</td>
</tr>
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<td>4,4-bipyridine</td>
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Note. In some cases, values for the maximum percentage inhibition may be underestimated as the maximum response may not have been reached for all chemicals.
matically alters its effect on tissue growth. Although the concentration of these derivatives in smokers is likely less than nicotine, the fact that several of them can exert an inhibitory effect on tissue growth at picomolar doses raises the possibility that they are active in smokers. Table 1 also shows the maximum percentage of inhibition of growth for each chemical. These values should be interpreted as estimates as a maximum achievable response may not have been obtained for all chemicals tested. The percentage of maximum inhibition was over 70% for all chemicals tested except for di- and trimethyl substituted pyridines. The two chemicals that were most potent, 2- and 3-ethylpyridine, also had the greatest efficacy (maximum percentage inhibition of at least 87%).

A second goal of these experiments was to compare the inhibitory activity of each pyridine derivative identified in the C2 eluate to pyridine to determine how different functional group substitutions affect CAM growth. An interesting consistency was found in the type of substitution and the effect observed on CAM growth. Pyridine was not particularly potent in the CAM growth assay having a LOAEL of 5 × 10⁻⁵ M. Single linear substitutions to pyridine substantially altered its inhibitory activity. Single linear ethyl substitutions (-CH₂CH₃) increased pyridine’s inhibitory activity 10 million fold, while single linear methyl substitutions (-CH₃) increased its inhibitory activity 10,000 fold. The enhanced effect of ethyl substitution was negated by incorporation of a single double bond into the ethyl group to create an ethenyl substitution. In the case of the methyl substitutions, their effect was negated by double and triple methyl substitutions to the pyridine ring.

Most other substitutions had very minor effects on the LOAEL of pyridine and in the case of bipyridine and nicotine the LOAEL actually increased relative to pyridine. The data with ethyl and methyl pyridine derivatives show that minor modifications to the pyridine ring can greatly affect its behavior in the CAM growth assay.

BrdU labeling indicated that 3-ethylpyridine blocked CAM growth by inhibiting cell division. Both the number of labeled nuclei and the intensity of the fluorescence in labeled nuclei was decreased by treatment with 3-ethylpyridine, which is not generally regarded as a toxicant and has not previously been reported to inhibit cell growth at picomolar doses. It is not yet known how 3-ethylpyridine affected cell division but inhibition by such low doses suggests that it acts through a receptor. Interestingly, our data also show a slight but significant stimulation of CAM growth at very low concentrations (5 × 10⁻¹¹ M) of nornicotine and nicotine. This is consistent with the previous finding that 10⁻¹¹ to 10⁻⁴ M nicotine stimulates DNA synthesis and proliferation in vascular endothelial cells (Heesch et al., 2001; Villablanca, 1998). Nicotine induced stimulation of endothelial cell division can be inhibited by hexamethonium indicating it acts through a nicotinic receptor (Villablanca, 1998). However, the effects of nicotine on cultured cells appear to be complex as others have reported that nicotine inhibited proliferation of cultured human umbilical vein endothelial cells at 10⁻⁸ M (Albaugh et al., 2001).

The pyridine derivatives we tested were identified in sidestream cigarette smoke, the major component of environmental tobacco smoke, and most, including 2-ethylpyridine and 3-ethylpyridine, are also present in mainstream smoke (Brunneman et al., 1978), indicating that both active and passive smokers receive exposure to these chemicals. Moreover, the methyl and ethyl substituted pyridines that were most effective in the CAM growth assay are 7-13 times more abundant in sidestream smoke than in mainstream smoke (Brunneman et al., 1978).

The aggregate concentration of the four most potent pyridine derivatives (2- and 3-methylpyridine and 2- and 3-ethylpyridine) in mainstream and sidestream smoke from one unfiltered cigarette are 46.8 μg/cigarette and 529 μg/cigarette, respectively (Brunneman et al., 1978). Although the concentrations of these chemicals in smokers have not yet been determined, the effective doses of single methyl and ethyl pyridines are so low that they are likely to be important in human smokers.

The identification of pyridines in sidestream and mainstream cigarette smoke that inhibit growth in nanomolar and picomolar doses could be very important in explaining why human smokers often have difficulty with processes requiring growth of new tissue. It is generally well accepted that smoking impairs wound healing (Frick and Seals, 1994). As an example, in the rat model, gastric ulcers heal much more slowly in animals exposed to cigarette smoke than in unexposed controls (Ma et al., 1999, 2000). In addition, reproductive processes that require growth appear to be affected by cigarette smoke (Stillman et al., 1986). Establishing and maintaining pregnancies require extensive growth in the embryo/fetus, corpus luteum, and the placenta (Findlay, 1986). Our previous in vivo study showed that inhalation of cigarette smoke by hamsters before and during pregnancy reduced vascular area in their corpora lutea (Magers et al., 1995). It is also well established that both active (Stillman et al., 1986) and passive (Martin and Bracken, 1986) smokers produce fetuses with lower than normal birth weights. This reduction in birth weight is thought to be due to fetal hypoxia caused by nicotine-induced vasostriction and decreased placental blood flow and by increased carboxyhemoglobin leading to decreased fetal oxygenation (Tourmaa, 1995). Our current data indicate that 2- and 3-ethylpyridine, which retard growth at picomolar doses, could impair wound healing and contribute to the low birth weights in fetuses delivered by active and passive smokers.

3-Ethylpyridine, which inhibited CAM growth at picomolar doses, is on the FEMA GRAS list of chemicals (“generally regarded as safe”; R. J. Reynolds Tobacco Company) and on the FDA EAFUS list (“everything added to food in the United States”; U.S. FDA). 3-Ethylpyridine is also one of the 599 chemicals reported by tobacco companies to be added to cigarettes to enhance flavor (R. J. Reynolds Tobacco Company). Extracts of the unburned 2R1 research grade cigarettes used to make our smoke solutions did not contain 3-ethylpyridine, thus...
manufacture to have 3-ethylpyridine, which was presumably added during combustion. However, several brands of unburned commercial cigarettes did have 3-ethylpyridine, which was presumably added during manufacture to flavor the smoke.

Because it is generally assumed safe and has not been subjected to toxicological testing, 3-ethylpyridine is added to food, cosmetics, and tobacco. Although the amounts added to consumer products are generally not made available to the public, tobacco companies add up to 100 µg/cigarette (R. J. Reynolds Tobacco Company). The doses that are present in humans have not been determined and might be difficult to measure as they are probably low. However, our study shows that very low doses of 3-ethylpyridine have significant inhibitory effects on tissue growth and indicate that further toxicological testing on pyridine derivatives is warranted.

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