VARIABLE CYP2A6-MEDIATED NICOTINE METABOLISM ALTERS SMOKING BEHAVIOR AND RISK

RACHEL F. TYNDALE AND EDWARD M. SELLERS

Centre for Addictions and Mental Health, Toronto, Ontario, Canada (R.F.T., E.M.S.); Departments of Pharmacology, (R.F.T., E.M.S.), Psychiatry, and Medicine (E.M.S.), University of Toronto, Ontario, Canada; and Centre for Research in Women’s Health, University of Toronto, Ontario, Canada (R.F.T., E.M.S.)

ABSTRACT:

Nicotine is the psychoactive substance responsible for tobacco dependence; smokers adjust their cigarette consumption to maintain brain nicotine levels. In humans, 70 to 80% of nicotine is metabolized to the inactive metabolite cotinine by the enzyme CYP2A6. CYP2A6 can also activate tobacco smoke procarcinogens [e.g., NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone]. In initial studies we found that there was an under-representation of individuals carrying defective CYP2A6 alleles in a tobacco-dependent population, and that among smokers, those with deficient nicotine metabolism smoked fewer cigarettes. We have since reproduced this data in a prospective smoking study (400 male and female, heavy and light smokers) examining the role of the CYP2A6 genotype on carbon monoxide levels, plasma and urine nicotine and cotinine levels, and cigarette counts. We have also recently identified deletion and duplication variants in the CYP2A6 gene locus and have examined their impact on smoking. These data provide the impetus to examine how inhibition of CYP2A6 activity might be useful in a therapeutic context. Both kinetic and behavioral experiments in human smokers demonstrated that inhibiting CYP2A6 in vivo decreased nicotine metabolism and smoking behavior. This article summarizes the preliminary results from our studies.

Approximately one-third of the global population over 15 years old smokes; the frequency of dependent smokers varies by gender and ethnicity. Smoking is associated with a higher incidence of various types of cancers, respiratory and cardiovascular diseases, gastrointestinal disorders, as well as many other medical complications (Lee and D’Alonzo, 1993). It has been estimated that approximately 50% of the initiation of smoking dependence is genetically influenced, while maintenance of dependent smoking behavior, and amount smoked, have approximately a 70% genetic contribution (True et al., 1997). Nicotine, and not the other tobacco constituents, is responsible for establishing and maintaining cigarette dependence (Henningfield et al., 1985). It has been demonstrated that among dependent smokers, smoking behavior is adjusted to maintain peripheral and central nicotine levels (McMorrow and Foxx, 1983; Russel, 1987).

CYP2A6 and Hepatic Nicotine Metabolism

Determining the variation in nicotine inactivation is important because of nicotine’s role in producing tobacco dependence and regulating smoking behavior. In humans, approximately 70 to 80% of nicotine is metabolized by inactivation to cotinine (Benowitz et al., 1994). We identified the genetically polymorphic CYP2A6 enzyme as responsible for the majority of the metabolic conversion/inactivation of nicotine to cotinine (Messina et al., 1997). These studies included correlating nicotine metabolism to immunodetectable hepatic and CYP2A6 (n = 31 human livers), chemical- and immuno-inhibition studies as well as cDNA P450 expression studies. The involvement of CYP2A6 in the metabolism of nicotine to cotinine and further to trans-3-hydroxycotinine, 5′-hydroxycotinine and possibly norcotinine has also been demonstrated (Nakajima et al., 1996a,b; Murphy et al., 1999).

CYP2A6 Polymorphism

Both in vitro and in vivo studies have demonstrated considerable interindividual variation in CYP2A6 activity (Yamano et al., 1990; Cholerton et al., 1992; Rautio et al., 1992; Iscan et al., 1994), which is due primarily to genetic variation in the CYP2A6 gene locus. Initially three CYP2A6 alleles were identified: wild-type (CYP2A6*1) and two defective alleles (CYP2A6*2 and CYP2A6*3; Yamano et al., 1990; Fernandez-Salgueiro et al., 1995). In vitro and in vivo studies have demonstrated that the CYP2A6*2 allele is a null allele having no activity toward probe substrates (Yamano et al., 1990; Fernandez-Salgueiro et al., 1995). There are multiple mutations in the CYP2A6*3 allele that resemble the alterations found in the neighboring CYP2A7 pseudogene (Fernandez-Salgueiro et al., 1995). Individuals with the CYP2A6*2/*3 and CYP2A6*3/*3 genotype have no CYP2A6-mediated metabolism (Rautio et al., 1996). Furthermore, using nicotine as a substrate in vitro with Caucasian human livers, we have shown that heterozygous livers (CYP2A6*1/*2 and CYP2A6*1/*3) have 50% of the CYP2A6-mediated nicotine metabolism. They also have 50% of the nicotine to cotinine Vmax values, when compared with homozygous wild-type (CYP2A6*1/*1) livers (R. F. Tyndale and E. M. Sellers, unpublished observations). In other words, each individual

1 Abbreviations used are: P450, cytochrome P450; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol.

Send reprint requests to: R. F. Tyndale, Ph.D., Rm. 4336, Department of Pharmacology, University of Toronto, 1 King’s College Circle, Toronto, Ontario M5S 1A8, Canada. E-mail: r.tyndale@utoronto.ca
Polymorphic CYP2A6 and Risk of Tobacco-Dependence: An Epidemiology Study

We hypothesized that individuals with impaired nicotine metabolism [carriers of a defective CYP2A6 allele(s)] would be protected from becoming tobacco-dependent. When learning to smoke, individuals often find the nicotine unpleasant (e.g., causing dizziness or nausea). We anticipated that if nicotine metabolism was decreased in some individuals, due to defects in the CYP2A6 gene, the aversive nicotine effects might last longer or the nicotine levels might be higher than in unimpaired individuals.

We tested whether impaired metabolism protected individuals with defective alleles (CYP2A6*2, CYP2A6*3) from becoming dependent on nicotine by examining the genotype frequencies in populations of smokers and nonsmokers (Pianezza et al., 1998). Specifically, tobacco-dependent only, alcohol- and tobacco-dependent, and never-tobacco-dependent [dependence defined using the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) APA for 1994] Caucasians were genotyped for CYP2A6*2 and CYP2A6*3 alleles (Fernandez-Salguero et al., 1995). The never-tobacco-dependent group represented an exposure control group who had each tried smoking at least once, but had never become tobacco-dependent. In contrast to the nonsmokers, in the dependent-smokers with or without alcohol dependence, fewer of the individuals had CYP2A6 defective alleles ($p < 0.01$, $\chi^2$-square; odds ratio = 1.9). A similar decrease in the frequency of individuals carrying CYP2A6 null alleles was seen in tobacco-dependent individuals without (odds ratio = 1.8) or with (odds ratio = 2.04) alcohol dependence. Furthermore, if only males were studied the protection due to defective CYP2A6 alleles was observed (odds ratio = 1.7) as well as when only females were examined (odds ratio = 2.2). These data provide the initial evidence that impaired nicotine metabolism due to defective CYP2A6 alleles is protective against becoming tobacco-dependent; however, while individuals with the CYP2A6*3 allele possess decreased nicotine metabolism, we are aware that there are discrepancies in the genotyping methods for this allele; we are addressing this by repeating the study with larger numbers of individuals and improved genotyping assays (Oscarson et al., 1998, 1999).

Polymorphic CYP2A6 and Amount Smoked by Dependent Smokers

The second half of the original study (Pianezza et al., 1998) examined the impact of defective metabolism on the number of cigarettes smoked. Dependent smokers adjust their smoking to maintain constant blood and brain nicotine concentrations levels (McMorrow and Fox, 1983; Russel, 1987). This suggests that dependent smokers who have defective CYP2A6 alleles resulting in impaired nicotine metabolism will need to smoke fewer cigarettes to maintain their nicotine levels. Within the tobacco-dependent only group (DSM-IV) those who had one defective (CYP2A6*2 or CYP2A6*3) allele and one active (CYP2A6*1) allele (e.g., heterozygotes) smoked significantly fewer cigarettes per day and per week than smokers without impaired nicotine metabolism (homozygotes for the wild type CYP2A6*1 allele, 129 versus 159 cigarettes/week, $t$ test, $p < 0.02$). These data again provided evidence that CYP2A6-mediated nicotine metabolism is a significant determinant of smoking behavior; heterozygosity in a single gene, the CYP2A6 gene, significantly decreases both initiation of tobacco dependence and the amount of drug consumed.

CYP2A6 and Smoking Indices

The initial study (Pianezza et al., 1998) was limited both by the alleles studied (CYP2A6*2 and CYP2A6*3) and by assessing only self-reported cigarette smoking. We have subsequently performed a prospective epidemiological study in female ($n = 100$) and male ($n = 100$) light (1–15 cigarettes/day) smokers and female ($n = 100$) and male ($n = 100$) heavy (>16 cigarettes/day) smokers examining the role of the CYP2A6 genotype on cigarette number as well as plasma and urine nicotine and cotinine levels. We also did carbon monoxide measurements because these are an independent measure of smoke exposure and can be used to verify cigarette estimations. In addition to the CYP2A6*2-defective allele, we and others have identified a CYP2A6 gene deletion, which has been named CYP2A6*4 (Nunoya et al., 1999; Oscarson et al., 1999). In this prospective epidemiological study we have found complete concordance between our one-step unpublished CYP2A6*4 assay and the two-step assay of Oscarson et al. (1999). Our preliminary results demonstrate that people with defective (CYP2A6*2 and CYP2A6*4) alleles consumed fewer cigarettes per day (12.5 versus 18.5, $p < 0.02$) and had lower carbon monoxide levels (13 versus 19 ppm, $p < 0.005$), a measure of smoke exposure (Rao et al., 2000). Furthermore, we have initial evidence for a CYP2A6 gene duplication. In a preliminary assessment of the data we observed a descending rank order for plasma cotinine levels between individuals with our newly identified CYP2A6 gene duplication, wild-type (CYP2A6*1) alleles and those with defective (CYP2A6*2 and CYP2A6*4) alleles (365, 257, and 203 ng/ml, respectively). The plasma nicotine/cotinine ratios also followed an expected ascending rank order for these three genotype groups (0.085, 0.119, and 0.179, respectively). These preliminary results (Rao et al., 2000) confirm and extend our previous findings and suggest a major influence by CYP2A6 genotype on nicotine kinetics and smoking behavior.

CYP2A6 and Tobacco-Related Cancer

Tobacco smoke contains a number of tobacco-specific procarcinogens; for example, N-nitrosodimethylamine, 4-(methylisotrosoamino)-1-(3-pyridyl)-1-butanone (NNK), and N'-nitrosornonicotine (NNN). These compounds are termed pro- or precarcinogens because they are activated by the body to carcinogens. CYP2A6 has been specifically demonstrated to activate NNN and NNN tobacco smoke procarcinogens via $\alpha$-hydroxylation (Crespi et al., 1990; Yamazaki et al., 1992; Patt et al., 1997); therefore, individuals who have CYP2A6 null alleles may also be less efficient at bioactivating tobacco smoke procarcinogens to carcinogens, while those with duplications would be more efficient. This is of particular interest as ethnic variation in frequencies for CYP2A6 variant alleles exist (Fernandez-Salguero et al., 1995; Nowak et al., 1998; Yokoi and Kamataki, 1998) and may be related to the ethnic differences in lung cancer incidence and histology (Groeger et al., 1997). Other genetically polymorphic P450 enzymes (e.g., CYP1A1, CYP2E1) may also contribute to increased risk for cancer, suggesting that variation in the drug- and toxin-metabolizing P450 enzymes may be very important as risk or protection factors for cancer-causing agents. Thus, individuals carrying CYP2A6-defective alleles may have a decreased risk of developing tobacco-related cancers and other medical complications for three reasons. First, they have a decreased risk of becoming a
tobacco-dependent smoker. Second, if they do become tobacco-dependent, they smoke less than those without impaired nicotine metabolism, resulting in lower exposures to tobacco-related procarcinogens. The amount of exposure is exponentially related to cancer risk (Law et al., 1997). Finally, they may activate less of the tobacco-gens. The amount of exposure is exponentially related to cancer risk metabolism, resulting in lower exposures to tobacco-related procarcinogens. These three factors suggest a significant reduction in tobacco-related cancers for carriers of a CYP2A6-defective allele(s), while those with duplicated CYP2A6 genes may be at increased risk.

To study the impact of CYP2A6 gene on tobacco-related cancers, it is necessary to perform some of the studies on nonsmokers (to avoid the impact of CYP2A6 on risk for dependence and altered cigarette consumption and resulting procarcinogen exposure) as well as on smokers. The interim analysis of our epidemiological studies indicates that individuals with defective CYP2A6 allele(s) are less likely to get bladder or lung cancer, have lower Ki-ras oncogene mutations in biopsies from lung tumors, and have lower incidences of lymphomas. The recent study by Miyamoto et al. (1999) supports this conclusion, finding that the CYP2A6 gene deletion allele (only CYP2A6 allele examined) resulted in a significant reduction in risk for lung cancer. However, the gene’s effects on smoking, which were not controlled for, confound interpretation of these results. Their control group was healthy volunteers, so the decrease observed in the lung cancer population could be due to the decreased risk for becoming a smoker, as well as the gene’s impact on amount smoked and activation of carcinogens.

In addition, we have blocked CYP2A6 activity in vivo in smokers, and our preliminary analysis suggests a significant rerouting of the NNK nitrosamines away from the mutagenic hydroxylations and to the nonmutagenic NNAL glucuronide (Sellers et al., 2000b). Subjects (n = 11) were instructed to maintain their normal cigarette consumption during 3 days of treatment with a CYP2A6 inhibitor. Although attempting to maintain normal smoking behavior while on the inhibitor, subjects decreased breath carbon monoxide (a measure of smoke inhalation), demonstrating that they had decreased their smoking behavior on the inhibitor. The alteration in smoking and nicotine kinetics resulted in a 32% increase in plasma nicotine/carbon monoxide ratios (p < 0.03). Urinary total NNAL, expressed relative to carbon monoxide to eliminate decreases in NNK exposure due to decreased smoking, doubled after CYP2A6, indicating a rerouting of the NNK to the less toxic NNAL glucuronides.

**CYP2A6 Inhibition In Vitro and in Vivo**

These data together provide evidence for a protective effect of impaired nicotine metabolism (carriers of CYP2A6 null alleles) on the risk for becoming tobacco-dependent and in lowering the number of cigarettes smoked, as well as in reduced procarcinogen activation. This suggests that mimicking the defect may provide the same benefits imparted by the genetic defect. In other words, the data suggest that inhibiting the activity of CYP2A6 may provide novel therapeutic approaches to prevention and treatment of tobacco smoking. This has particular appeal because the target is a hepatic enzyme that is already known to be completely missing in some people and is not importantly involved in the metabolism of clinically used drugs other than nicotine.

The subsequent studies were done to 1) investigate whether we could replicate the genetic findings using inhibition of CYP2A6 to phenocopy, or imitate, the defective nicotine metabolism and decreased smoking behavior that we had observed (Pianezza et al., 1998); and 2) to determine whether inhibition of the CYP2A6 could be useful therapeutically.

Coumarin is a prototype CYP2A6 substrate. In human liver microsomes, coumarin is metabolized with a K\textsubscript{m} of 0.95 \mu M and V\textsubscript{max} of 52 nM/min/mg, respectively, while nicotine is metabolized with a K\textsubscript{m} of 64 \mu M and a V\textsubscript{max} of 0.48 nM/min/mg. Coumarin inhibited nicotine metabolism in human liver microsomes with a K\textsubscript{i} of 1.8 \mu M (Messina et al., 1997), whereas it inhibited cDNA-expressed CYP2A6-mediated nicotine metabolism with a K\textsubscript{i} of 6.4 \mu M. In addition, we found using in vitro inhibition of nicotine metabolism by expressed CYP2A6 or human liver microsomes that tranylcypromine and methoxsalen were potent CYP2A6 inhibitors (K\textsubscript{i} = 0.05–6 \mu M; Zhang et al., 2000).

We then investigated whether we could block systemic nicotine metabolism with a CYP2A6 inhibitor given orally. We used the subcutaneous route to approximate nicotine kinetics following nicotine inhalation. In 18 tobacco-dependent smokers given three doses of nicotine (31 \mu g/kg subcutaneously, hourly), even high and multiple doses of coumarin (50 mg × 6; 100 mg × 3; 225 mg × 6) failed to increase nicotine after 8 h (area under the curve) compared with placebo (Tyndale et al., 2000). However, methoxsalen, 30 to 50 mg orally 30 min before nicotine (31 \mu g/kg subcutaneously, three doses, hourly), increased the 8-h mean plasma nicotine by 49% (p < 0.01) compared with placebo (Tyndale et al., 1999). These data suggest that coumarin is subject to rapid metabolism, which makes it an ineffective CYP2A6 inhibitor in vivo, while the methoxsalen data strongly suggest that potent oral inhibitors of CYP2A6-mediated nicotine metabolism could be useful in decreasing smoking due to prolonging the half-life of nicotine in the body.

Nicotine bioavailability is low (20–35%) and while high doses of nicotine, given orally, might produce nicotine levels sufficient for nicotine replacement therapy, this is not possible due to nicotine-mediated gastrointestinal distress. This high first-pass metabolism in combination with high dose intestinal disturbances has prohibited an oral formulation of nicotine as a nicotine replacement therapy. Therefore, we tested whether inhibition of nicotine metabolism, specifically the first-pass metabolism of oral nicotine, could produce systemic nicotine levels comparable with other nicotine replacement therapy formulations at doses that do not cause gastrointestinal distress. Initially, we tested coumarin as an in vivo inhibitor with oral nicotine, but due to the rapid metabolism of coumarin by CYP2A6 its utility as an in vivo first-pass CYP2A6 inhibitor was limited (Tyndale et al., 2000); thus, we tested two alternative inhibitors. Nicotine-abstinent dependent smokers coingested 4 mg of nicotine orally with either 10 or 30 mg of methoxsalen, 2.5 or 10 mg of tranylcypromine, or placebo. Compared with placebo, methoxsalen and tranylcypromine (at indicated doses) increased mean plasma nicotine concentrations 72, 83, 43, and 65%, respectively (p < 0.01), as well as reducing subjects’ self-rated current desire to smoke (p < 0.05; Sellers et al., 2000a). No indications of gastrointestinal distress were reported using this low dose of nicotine (4 mg). Thus, at doses considerably below those used therapeutically (1/3 of those used therapeutically for methoxsalen and 1/8 of those used therapeutically for tranylcypromine) CYP2A6 inhibitors can inhibit nicotine metabolism in vivo, providing a new approach to treatment of tobacco dependence by making an oral nicotine replacement therapy feasible.

**CYP2A6 Inhibition Decreases Smoking**

Having demonstrated that an oral formulation of low dose nicotine (4 mg) was possible kinetically, we investigated whether this combination of CYP2A6 inhibition with or without oral nicotine could decrease smoking behavior. We hypothesized that CYP2A6 inhibition
would decrease nicotine metabolism, decrease smoking, and decrease smoke exposure as reflected in smoke measures such as breath carbon monoxide. In these studies we used methoxsalen rather than translycyprome to avoid confusion between the potential central activities of the antidepressant tranylcyprome with its kinetic effects on CYP2A6. We modeled this study on the experimental design which was used to demonstrate that nicotine gum was effective at decreasing nicotine craving and which predicted that nicotine gum would have utility as a nicotine replacement therapy (Nesmuth-Coslett et al., 1987). Overnight nicotine-abstinent dependent smokers (six male and six female CYP2A6 extensive metabolizers without the duplication) smoked one cigarette in the morning, and were then given one of four oral drug treatment combinations in a crossover counterbalanced order: 30 mg of methoxsalen (CYP2A6 inhibitor, $K_i = 0.2 \mu M$) or placebo with either 4.0 mg of nicotine or placebo. At the end of the 60 min, subjects could smoke ad libitum for the next 90 min. Subjects when receiving the methoxsalen and oral nicotine combination smoked significantly less than in the placebo/placebo condition (e.g., 50% less increase in breath carbon monoxide; 83% increase in latency to the second cigarette, 24% decrease in the number of cigarettes smoked; 24% decrease in grams of tobacco burned; and a 25% decrease in the total number of puffs taken (all $p < 0.05$; Sellers et al., 2000a). Measures of the smoke exposure cost of nicotine acquisition decreased; in other words, the amount of smoke exposure (cost) decreased compared with the amount of nicotine acquired. The ratio of carbon monoxide increase to number of puffs was 30% lower, in other words, the amount of smoke exposure (cost) decreased compared with the amount of nicotine acquired. The ratio of carbon monoxide increase to number of puffs was 30% lower, indicating that subjects were taking shallower or shorter puffs. On several measures (e.g., latency to second cigarette), the rank order of response was methoxsalen/nicotine > methoxsalen/placebo > placebo/nicotine > placebo/placebo. This suggests that methoxsalen with nicotine caused the greatest change followed by the effects of methoxsalen/placebo, in the presence of nicotine from the first cigarette. This suggests a methoxsalen effect on systemic clearance of nicotine; once nicotine was present (from the first cigarette), CYP2A6 inhibition alone (the methoxsalen/placebo condition) decreased smoking indices. Conclusions

In summary, our data suggest that CYP2A6 inhibition alone could be used in an exposure reduction paradigm to decrease smoking and activation of tobacco-related procarcinogens. It is striking that the inhibition of CYP2A6 activity, using orally ingested inhibitors, results in a lowering of cigarette consumption that mirrors the findings of our epidemiology studies, wherein those individuals with defective CYP2A6 alleles smoked fewer cigarettes than those with higher CYP2A6 activity and fully functional alleles. In addition, our data suggest that individuals with increased CYP2A6 activity resulting from carrying duplicated forms of the CYP2A6 gene may be at much higher risk for becoming a smoker, may smoke more, and may activate more of the procarcinogens in tobacco. This suggests that they may be one group where inhibition of the enzymatic activity is of even greater therapeutic import than those with normal activity. Finally, we have provided evidence for a new oral formulation of nicotine in combination with a CYP2A6 inhibitor providing a more acceptable pill formulation for nicotine replacement therapy that may also decrease the risk from procarcinogens found in tobacco products. These smoking and cancer epidemiological studies in combination with the pharmacokinetic and behavioral studies provide extensive evidence that variation in CYP2A6 is an important determinant of smoking behavior and its medical consequences.

References

Diagnostic and Statistical Manual of Mental Disorders, 4th ed. (1994)
Sellers EM, Zeman M, Kaplan HL and Tyndale RF (2000b) Inhibition of cytochrome P450 2A6 (CYP2A6) decreases smoking and activation of the procarcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butane (Abstract). American Society for Clinical Therapeutics Meeting.


