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A Real-Time Fluorescence Assay for Measuring *N*-Dealkylation

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Non-standard abbreviations: 9AA, 9-aminoacridine; AAMC, 3-[2-(*N,N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin; BAA, 9-*N*-(1-aminobutyl)acridine; BzAA, 9-*N*-(1-aminobenzyl)acridine hydrochloride; BzR, 7-benzyloxyresorufin; CEC, 3-cyano-7-ethoxycoumarin; Cl_{int} , intrinsic clearance; EAA, 9-*N*-(ethylamino)acridine; EtR, 7-ethoxyresorufin; ΔG_{bind} , experimental binding energy; MAA, 9-*N*-(methylamino)acridine; MAMC, 7-methoxy-4-(aminomethyl)coumarin;

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NDA, no detectable activity; PAA, 9-*N*-(1-aminopropyl)acridine; PtAA, 9-*N*-(1-aminopentyl)acridine; PtR, 7-pentoxyresorufin

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ABSTRACT

A real-time fluorescence assay system utilizing a series of 9-*N*-(alkylamino)acridine derivatives (methyl, ethyl, *n*-propyl, *n*-butyl, *n*-pentyl, and benzyl) that are *N*-dealkylated to 9-aminoacridine (9AA) is described. The product, 9AA, is ca. 27-fold more fluorescent than the substrates using excitation and emission wavelengths of 405 and 455 nm, respectively. Tests using expressed CYPs 1A1, 1A2, 3A4, 3A5, 1B1, 2C9, 2C19, and 2D6 indicated that *N*-dealkylase activity is specific for CYP1A1 and CYP2D6. CYP2D6 *N*-dealkylated methyl, ethyl, *n*-propyl, and *n*-butyl substrates while CYP1A1 *N*-dealkylated these plus the *n*-pentyl derivative. Activities using 5 μ M 9-*N*-(alkylamino)acridine substrates ranged from 0.1 – 0.9 pmole 9AA/min/pmole CYP. Kinetic constants for CYP1A1 *N*-dealkylation of the 9-*N*-(methylamino)acridine (MAA) and 9-*N*-(ethylamino)acridine (EAA) were K_m 1.09 ± 0.68 and 0.35 ± 0.21 μ M and the V_{max} 61.9 ± 48.5 and 113.8 ± 8.4 pmol 9AA/min/pmol CYP1A1, respectively. Kinetic constants for CYP2D6 *N*-dealkylation of MAA and EAA were K_m 7.9 ± 5.4 and 3.2 ± 1.6 μ M and the V_{max} 501 ± 35.4 and 702.7 ± 257 pmol 9AA/min/pmol CYP2D6, respectively. The experimental binding energies (ΔG_{bind}) were calculated for MAA with CYP1A1 and CYP2D6 to be -8.266 and -7.074 kcal/mole, respectively. The ΔG_{bind} values for EAA with CYP1A1 and CYP2D6 were -8.950 and -7.618 kcal/mole, respectively. The substrates were suitable for monitoring *N*-dealkylase activity in microsomal preparations (human, rat, and monkey hepatic preparations) and human hepatocellular carcinoma cell suspensions. Assays were conducted by monitoring reactions either in 96-well microtiter plates using a fluorescence plate reader or in cuvettes using a spectrofluorimeter.

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INTRODUCTION

Cytochromes P450 are enzymes of particular interest to pharmacologists and toxicologists because of their major involvement in the Phase I metabolism of drugs and chemicals. In particular, CYP2D6 is a major player in hepatic metabolism of *N*-alkylated drugs. While there are many assays for the cytochrome P450 mediated *N*-dealkylations there are few that can be measured in real-time that are true *N*-dealkylation reactions and are cytochrome P450 isoform specific. Most assays require extraction of the product(s), conversion of the products to readable derivatives, radiolabeling, gas-liquid chromatography, HPLC or other product isolation techniques coupled with either UV-Vis, fluorescence, flame ionization, or mass spectral detection. For example, Moody *et al.* (1999) described automated inhibition screens for the major human hepatic cytochromes P450. Radiometric analysis of erythromycin *N*-demethylation for CYP3A4, dextromethorphan *O*-demethylation for CYP2D6, naproxen *O*-demethylation for CYP2C9, and diazepam *N*-demethylation for CYP2C19 were employed. For the radiometric assays greater than 99.7% of ¹⁴C-labelled substrate was routinely extracted from incubations by solid-phase extraction.

The measurement of formaldehyde formation via the Nash reaction for *N*-dealkylation is also a common method (Nerland and Mannering, 1978; Pinto *et al.*, 2004). However, this is labor intensive and time consuming.

Stresser *et al.* (2004) tested azamulin, an anti-infective, toward 18 different cytochromes P450 using human liver microsomes or microsomes from insect cells expressing single isoforms. The products from these chemical reactions were determined using HPLC which is time consuming and laborious.

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Crespi *et al.* (1997) reported a high-throughput microtiter plate fluorescence assay for CYP1A2, CYP2C9, CYP2C19, and CYP2D6. Assays of all but CYP3A4 (assayed using BzR) were based on using the substrate 3-cyano-7-ethoxycoumarin which is *O*-deethylated to 3-cyano-7-hydroxycoumarin. One problem with the use of CEC is that to differentiate between the different CYPs one must use either purified preparations or expressed forms of particular CYPs. Another concern is that the assay does not measure *N*-dealkylation *per se*. Later, recognizing the problem of selectivity for the high-throughput fluorescence assay of cytochromes P450, Miller *et al.* (2000) substituted the use of 3-[2-*N,N*-diethyl-*N*-methylammonium)ethyl]-7-methoxy-4-methylcoumarin as a selective substrate for CYP2D6, use of 7-benzyloxyquinoline and 7-benzyloxy-4-trifluoromethylcoumarin as substrates for CYP3A4, and the use of dibenzylfluorescein for monitoring CYP2C8 activity. However, *N*-dealkylation activity is not measured.

Netter (1966) reported a selective *N*-dealkylase spectrophotometric assay based on the *N*-demethylation of *N*-monomethyl-*p*-nitroaniline (absorbance maximum at 380 nm) and conversion to *p*-nitroaniline (absorbance maximum at 407 nm). Overlap of the substrate and product absorbance bands with those of inhibitors and other chemicals limits the application of the assay. Kupfer and Bruggeman (1966) used a coupled reaction with *p*-dimethylaminobenzaldehyde with the products (aniline and *p*-chloroaniline) to measure *N*-dealkylation of *N*-methylaniline and *p*-chloro-*N*-methylaniline. The coupled reaction increased the absorbance wavelength maxima but added additional steps in the assay. Similarly, van der Hoeven (1977) used fluorecamine to react with *p*-chloroaniline to generate a fluorescent product to measure *N*-dealkylation of *p*-chloro-*N*-methylaniline.

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Here we report a facile, direct, and CYP2D6 and CYP1A1 selective *N*-dealkylation fluorimetric assay that can be followed in real-time. Several 9-*N*-(alkylamino)acridine derivatives (methyl-, ethyl-, *n*-propyl-, *n*-butyl-, *n*-pentyl-, and benzyl-) were prepared and tested in reactions with microsomes and expressed CYP's. *N*-Dealkylation of these substrates produces the highly fluorescent product 9-aminoacridine (Fig. 1).

MATERIALS AND METHODS

Chemicals. 9-Aminoacridine, quinidine hydrochloride, 7,8-benzoflavone, resveratrol, and imipramine hydrochloride were obtained from Sigma-Aldrich (St. Louis, MO). Amitriptyline hydrochloride was obtained from Merck, Sharp, Dohme (Whitehouse Station, NJ). Expressed cytochromes P450 (Supersomes, CYP1A1, CYP1A2, CYP2C9, CYP2C19, CYP3A4, CYP3A5, CYP1B1, CYP2D6) were purchased from BD Gentest Co. (San Jose, CA). Hepatic microsomal preparations (adult human male and female, *Cynomolgus* sp. male monkey, and male rat (Sprague-Dawley)) were purchased from Sigma-Aldrich. Ethoxyresorufin and resorufin were prepared as described in Burke and Mayer (1974). The 9-*N*-(alkylamino)acridines were prepared following the procedure described here for the 9-*N*-(methylamino)acridine. Anhydrous phenol (22.0 g, 234.1 mmol) was placed into a 250 mL 3-neck round bottom flask equipped with a magnetic stirring bar, reflux condenser, thermometer, dry ice/acetone condenser and positive pressure nitrogen source. Anhydrous chloroform (100 mL) was added and the mixture stirred and cooled in a sodium chloride ice bath until the internal temperature was -10° C. The dry ice condenser was filled with acetone and dry ice while using caution that the system remained under a positive atmosphere of nitrogen. Methylamine gas (Fluka, sold

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through Sigma-Aldrich) flow was slowly initiated resulting in condensation of the gas to a liquid and this process was continued until the phenol/chloroform solution was saturated. Saturation was determined by initiation of refluxing of methylamine upon removal of the cooling bath. To this solution at an internal temperature of -10°C was added dropwise using an addition funnel a solution of 9-chloroacridine (Sigma-Aldrich; 5.0 g, 23.4 mmol) in 25 mL of anhydrous chloroform. This was followed by another 25 mL anhydrous chloroform to complete the addition. The reaction mixture was stirred for 1 h at -10°C then warmed to room temperature and stirred for 2 h. During this time the dry ice acetone condenser was kept full to help prevent escape of the methylamine from the reaction vessel. At the end of this 2 h period the solution was slowly warmed to an internal temperature of $50\text{--}55^{\circ}\text{C}$ using an oil bath. The mixture was maintained at this temperature overnight. Sampling of the reaction mixture and analysis by gas chromatography/mass spectroscopy indicated the absence of starting 9-chloroacridine ($t_{\text{R}} = 6.4$ min) and the appearance of a new peak at $t_{\text{R}} = 7.5$ min corresponding to the desired product. The GC/EI-MS was obtained using an Agilent GC model 6890 and 5973 mass spectrometer and an Agilent Technologies HP-5MS 30 m x 0.25 mm 0.25 μm capillary column utilizing the following temperature run profile: Starting at 150°C for 1 min then ramping at 25°C per min to 300°C and holding for 4 min. Flow rate was 1 mL He per min.

The reaction mixture was cooled to room temperature and transferred to a 500 mL separatory funnel with the aid of 50 mL of chloroform. This solution was washed with 100 mL of 13% aqueous sodium hydroxide solution. This aqueous phase was extracted with 100 mL chloroform. The pooled organic phases were washed with one 100 mL

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portion of 10% aqueous sodium hydroxide and two 100 mL portions of saturated aqueous sodium chloride solution, then dried over solid anhydrous sodium sulfate and finally filtered through a bed of sodium sulfate. This solution was concentrated to dryness by rotary evaporation to afford a yellow/brown solid. The solid was dissolved in 200 mL of boiling hot anhydrous acetonitrile and left to cool under a nitrogen environment at ambient temperature resulting in crystallization. The solid was collected by suction filtration and washed with hexanes. The mother liquor was concentrated and a second crop of crystals was obtained similar to that described above. The solid material was dried under high vacuum overnight to afford 4.431 g (91% yield) of a yellow powder as the free base. Several of the 9-*N*-(alkylamino)acridines would not crystallize as the free base and were converted to hydrochloride salts by bubbling dry hydrogen chloride into the crystallization solution.

Compound Characterization Data: 9-*N*-(Methylamino)acridine—Yield % reported in previous section; MP 172-173° C (uncorrected); GC/EI-MS $t_R = 7.47$ min; m/z 208; ^1H NMR (CDCl_3) δ 8.13 (d, 2 H, $J = 8.8$ Hz, aromatic), 8.03 (d, 2 H, $J = 8.7$ Hz, aromatic), 7.62 (t, 2 H, $J = 7.1$ and 8.1 Hz, aromatic), 7.29 (t, 2 H, $J = 7.4$ and 8.0 Hz, aromatic), 5.72 (br s, 1 H, NH), 3.51 (s, 3 H, CH_3); ^{13}C NMR (CDCl_3) δ 151.93, 149.12, 129.77, 128.93, 122.85, 122.47, 115.82, 37.44; Anal. Calcd. for $\text{C}_{14}\text{H}_{12}\text{N}_2$ (208): C, 80.74; H, 5.81; N, 13.45. Found: C, 80.09; H, 5.84; N, 13.22. 9-*N*-(Ethylamino)acridine—Yield 87%; MP 126-127° C (uncorrected); GC/EI-MS $t_R = 7.55$ min; m/z 222; ^1H NMR (CDCl_3) δ 8.04-8.08 (m, 4 H, aromatic), 7.63 (t, 2 H, $J = 7.6$ Hz, aromatic), 7.31 (t, 2 H, $J = 7.5$ and 7.7 Hz, aromatic), 5.22 (br s, 1 H, NH), 3.79 (q, 2 H, $J = 14.2$ Hz, NCH_2CH_3),

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1.34 (t, 3 H, $J = 7.11$ Hz, NCH_2CH_3); ^{13}C NMR (CDCl_3) δ 151.07, 149.14, 129.74, 129.20, 122.81, 122.68, 116.46, 45.31, 16.75; Anal. Calcd. for $\text{C}_{15}\text{H}_{14}\text{N}_2$ (222): C, 81.05; H, 6.35; N, 12.60. Found: C, 81.07; H, 6.33; N, 12.47. **9-N-(1-Aminopropyl)acridine hydrochloride** –Yield 77%; MP 248-249° C (uncorrected); GC/EI-MS $t_R = 7.91$ min; m/z 236 (free amine); ^1H NMR (D_2O , referenced to HDO at 4.80 ppm) δ 7.35-7.40 (m, 4 H, aromatic), 6.98 (t, 2 H, $J = 7.6$ Hz, aromatic), 6.74 (d, 2 H, $J = 8.6$ Hz, aromatic), 3.19 (t, 2 H, $J = 7.2$ and 7.9 Hz, $\text{NCH}_2\text{CH}_2\text{CH}_3$), 1.44-1.52 (m, 2 H, $\text{NCH}_2\text{CH}_2\text{CH}_3$), 0.85 (t, 3 H, $J = 7.3$ Hz, $\text{NCH}_2\text{CH}_2\text{CH}_3$); ^{13}C NMR (D_2O , external referenced to 1,4-dioxane at 66.65 ppm) δ 154.86, 137.66, 134.86, 123.64, 117.44, 110.35, 49.85, 22.64, 10.32; Anal. Calcd. for $\text{C}_{16}\text{H}_{17}\text{N}_2\text{Cl}$ (272): C, 70.45; H, 6.28; N, 10.27. Found: C, 70.58; H, 6.37; N, 10.07. **9-N-(1-Aminobutyl)acridine hydrochloride** –Yield 66%; MP 188-190° C (uncorrected); GC/EI-MS $t_R = 8.30$ min; m/z 250 (free amine); ^1H NMR (D_2O , referenced to HDO at 4.80 ppm) δ 7.10-7.30 (m, 4 H, aromatic), 6.84 (t, 2 H, $J = 7.4$ Hz, aromatic), 6.58 (d, 2 H, $J = 8.2$ Hz, aromatic), 2.99 (t, 2 H, $J = 7.6$ and 7.8 Hz, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.26-1.34 (m, 2 H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.13-1.21 (m, 2 H, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 0.79 (t, 3 H, $J = 7.3$ Hz, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_3$); ^{13}C NMR (D_2O , external referenced to 1,4-dioxane at 66.65 ppm) δ 154.31, 137.44, 134.79, 123.61, 117.36, 110.04, 49.96, 31.06, 19.32, 12.88; Anal. Calcd. for $\text{C}_{17}\text{H}_{19}\text{N}_2\text{Cl}$ (286): C, 71.19; H, 6.67; N, 9.76. Found: C, 71.03; H, 6.81; N, 9.66. **9-N-(1-Aminopentyl)acridine** –Yield 85%; MP 107-108° C (uncorrected); GC/EI-MS $t_R = 8.78$ min; m/z 264 (free amine); ^1H NMR (CDCl_3) δ 8.07 (d, 4 H, $J = 9.0$ Hz, aromatic), 7.65 (t, 2 H, $J = 8.2$ and 6.9 Hz, aromatic), 7.34 (t, 2 H, $J = 7.5$ Hz, aromatic), 5.10 (br s, 1 H, NH), 3.78 (t, 2 H, $J = 7.2$ Hz, $\text{NCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_3$), 1.31-1.44 (m, 2 H,

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NCH₂CH₂CH₂CH₂CH₃), 1.13-1.21 (m, 4 H, NCH₂CH₂CH₂CH₂CH₃), 0.89 (t, 3 H, *J* = 7.0 Hz, NCH₂CH₂CH₂CH₂CH₃); ¹³C NMR (CDCl₃) δ 151.28, 149.25, 129.77, 129.42, 122.87, 122.65, 116.42, 50.82, 31.40, 28.95, 22.32, 13.91; Anal. Calcd. for C₁₈H₂₀N₂ (264): C, 81.77; H, 7.62; N, 10.59. Found: C, 81.78; H, 7.82; N, 10.60. **9-N-(1-Aminobenzyl)acridine hydrochloride**– Yield 81%; MP 246-248° C (uncorrected); GC/EI-MS *t*_R = 10.78 min; *m/z* 284 (free amine); ESI-MS *m/z* 285 (M+H)⁺; Poor solubility in deuterium oxide, d₆-DMSO and CDCl₃ alone – finally a mixture as described was used; ¹H NMR (1:1 CDCl₃:d₆-DMSO referenced to DMSO at 2.50 ppm) δ 14.12 (s, 1 H, NH₃⁺), 10.57 (br s, 1 H, NH₃⁺), 8.47 (br s, 2 H, NH₃⁺), 8.01 (d, 1 H, *J* = 8.5 Hz, aromatic), 7.85 (t, 1 H, *J* = 7.5 and 7.8 Hz, aromatic), 7.25-7.50 (m, 13 H, aromatic), 3.98 (q, 2 H, *J* = 11.7 Hz, NCH₂Ar); ¹³C NMR (1:1 CDCl₃:d₆-DMSO referenced to DMSO at 39.5 ppm) δ 157.80, 136.39, 134.47, 133.47, 128.72, 128.69, 128.27, 128.21, 127.50, 126.27, 118.59, 51.02, 42.37.

***N*-Dealkylase Assays.** Stock solutions were as follows: 100 mM potassium phosphate (KPi) buffer (pH 7.2); 50 mM NADPH in 100 mM KPi buffer; 1 mM substrate (i.e., 9-*N*-(alkylamino)acridine) in DMSO was serially diluted to 10 μM substrate in 100 mM KPi buffer. Reactions were conducted in real-time using either a spectrofluorimeter (LS-55, Perkin-Elmer, Shelton, CT) or a fluorescence microplate reader (Synergy HT, Bio-Tek, Highland Park, VT). Spectrofluorimeter reaction mixtures were contained in 10 mm pathlength quartz cuvettes and contained 4-10 pmoles of expressed cytochrome P450 enzyme, 0.5-10 μL of 1 mM 9-*N*-(alkylamino)acridine stock in DMSO, 10 μL 50 mM NADPH (final 0.25 mM), and enough 100 mM KPi buffer to bring the final volume to 2

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mL; reactions were initiated by addition of NADPH. With regard to reactions using microsomes, 40 μ L of microsomal preparation (0.8 mg protein) were used and the amount of buffer adjusted to a final volume of 2 mL in the cuvette. The reaction mixtures are allowed to equilibrate to 30°C for 1-2 min in the temperature controlled sample compartment of the spectrofluorimeter. Mixing of the reactions was accomplished by a magnetic stirrer in the sample well of the spectrofluorimeter. Reactions were initiated with 10 μ L 50 mM NADPH (final concentration 0.25 mM). 9-Aminoacridine (product) formation was monitored by following the increase in fluorescence at 455 nm using a 401 nm excitation wavelength. Excitation and emission slit widths were 7.5 nm, using a horizontal polarizer on the emission side and vertical polarizer on the excitation side to reduce scatter. Concentration values for 50% inhibition (I_{50}) for CYP1A1 and CYP2D6 metabolism of 5 μ M MAA by varying 7-ethoxyresorufin concentrations (0-15 μ M) were determined graphically. The effects of quinidine and 7,8-benzoflavone on 5 μ M MAA metabolism by human liver microsome preparations (188 pmoles cytochrome P450/mL) were determined using the spectrofluorimeter.

Microplate reader reactions were conducted using the kinetic mode of the plate reader at 30°C and only expressed CYP2D6 (Supersomes) was used to determine the pH optimum for the *N*-dealkylation. I_{50} values for quinidine, amitriptyline, imipramine, resveratrol, and 7,8-benzoflavone were determined for expressed CYP1A1 and CYP2D6. Wavelength settings for monitoring formation of 9AA were 401 nm excitation (10 nm slit) and 460 nm filter with a 40 nm bandpass. For the inhibition studies the microplate (96-well) wells typically contained 6 μ L CYP2D6 (6 pmoles), 10-100 μ L 10 μ M MAA (final 5 μ M) in 100 mM KPi (pH 7.2), 5 μ L 50 mM NADPH (final concentration 1.25

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mM), various volumes of inhibitor stock solutions (0.1-200 μ M) in 100 mM KPi buffer, and enough 100 mM KPi buffer (pH 7.2) to make a final volume of 0.2 mL. The microplates were allowed to temperature equilibrate and shaken in the plate reader for 2 min prior to initiating the reactions by addition of NADPH. There was no preincubation period with the CYP2D6, inhibitor, and coenzyme (NADPH). Reactions were monitored for 10 min. I_{50} values were determined graphically and are reported as the means \pm standard deviations of a minimum of three determinations. For the pH studies a series of 100 mM potassium phosphate buffers were prepared with pH ranging from 6-9. Substrates were prepared in the buffer being tested and NADPH was prepared in water.

The amount of 9AA formed was calculated using a 9AA standard curve generated for each reaction series. Kinetic constants (K_m and V_{max}) were determined graphically and are reported as the means \pm standard deviations of a minimum of three determinations. The experimental binding energies (ΔG_{bind}) were calculated from the K_m values for CYP1A1 and CYP2D6 using the formula $\Delta G = RT \ln K_m$, where R is the gas constant (1.9872 cal/deg/mole, T is the absolute temperature, and $\ln K_m$ is the natural logarithm of the K_m (Lewis, 2003). Intrinsic clearance (Cl_{int}) is defined as V_{max}/K_m (Lewis, 2003).

Ethoxyresorufin O-Dealkylase Assays. Assays followed the procedure given by Prough *et al.* (1978).

Cell Suspension Assays. Human hepatocellular carcinoma cells (ATCC#CRL-10741) were cultured for four days with 25 μ M Rifampicin present in minimal essential medium

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containing 10% fetal bovine serum and sodium pyruvate (110 mg/L). Culture medium containing Rifampicin was changed daily. On the fifth day the cells were harvested and washed twice by centrifuging at 1000 x g for 10 min and then resuspending in 2 mL of Dulbecco's phosphate buffered saline (PBS), pH 7.3. The final resuspension was in a volume of 1 mL. An aliquot (0.5 mL) of the stock suspension was added to a 10 mm pathlength fluorescence cuvette containing 1.5 mL of Dulbecco's PBS at pH 7.3, and a magnetic stirring bar. The cuvette was then placed in the spectrofluorimeter cuvette holder that was thermostatted at 30°C and the magnetic stirrer at low speed. The reaction mixture in the cuvette contained final concentrations of 1.125×10^6 hepatocytes per mL, 5 μ M MAA, and 1.25 mM NADPH. The spectrofluorimeter settings were the same as those given above. The reaction was initiated by addition of NADPH.

Confirmation on *N*-Dealkylation 9-Aminoacridine Metabolic Product. Spent reactions using MAA as the substrate were pooled and frozen (-20°C) until extracted. An aliquot (1 mL) of this reaction was treated with 1 mL NaOH (1 M) and extracted into 2 mL chloroform. The chloroform layer was separated, centrifuged, evaporated to dryness and the residue dissolved in 0.25 mL methanol. The formation of 9AA was confirmed by gas chromatography/mass spectrometry using an Agilent 6890 GC/ 5973 MSD system. The column was a HP-5MS (30 m x 0.25 mm x 0.25 μ m film thickness). Conditions were as follows: splitless injection, flow rate 1 mL He per min, injector temperature 250° C, temperature program from 150°C for 1 min with a ramp of 25°C per min to 300° C with a 4 min hold. 9-Aminoacridine eluted at 7.67 min and was detected via its molecular ion peak at m/z 194.

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RESULTS

Spectral Characteristics of the 9-*N*-(Alkylamino)acridines and 9-Aminoacridine.

Figure 2 shows the corrected excitation and emission spectra of 9-aminoacridine vs. that of 9-*N*-(methylamino)acridine. Excitation maxima for 9AA were observed at 310, 325, 362 (shoulder), 382, 401, and 421 nm with a λ_{em} 455 nm. Wavelength emission maxima for 9AA were 428, 455, 483 (shoulder), and 520 (shoulder) nm with an λ_{ex} 401 nm. The wavelength emission maxima (λ_{ex} 401 nm) for MAA were 415, 439, and 461 nm while the excitation maxima (λ_{em} 439 nm) were 253, 385, 405 nm. At a λ_{ex} 401 nm the 9AA fluorescence at 455 nm is about 27-fold more intense than that of MAA. This comparison is typical of all of the 9-*N*-(alkylamino)acridines tested.

Reactivity of 9-*N*-(Alkylamino)acridines with Expressed Cytochromes P450.

Initial tests confirmed that CYP2D6 Supersomes were capable of *N*-dealkylating MAA. Subsequently the pH optimum for the *N*-demethylation reaction was established to be 7.0-7.4 (data not shown) and a pH of 7.2 was selected for assays. Figure 3 indicates the real-time *N*-dealkylation reaction traces of expressed CYP2D6, CYP1A1, CYP3A5, CYP1A2, CYP2C19, CYP2C9, and CYP1B1 with MAA. Only CYP2D6 and CYP1A1 had any detectable activity. 9-Aminoacridine was confirmed as the *N*-dealkylation product of the reactions by GC-MS. Table 1 contains reaction data for all of the 9-*N*-(alkylamino)acridines compared with 7-alkoxyresorufins at 5 μ M with all of the expressed CYPs tested. Only CYP1A1 and CYP2D6 showed any activity for *N*-dealkylation of the acridine compounds. There were differences in the reactions for the

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two CYPs with the different substrates. CYP2D6 *N*-dealkylated four of the substrates (MAA; EAA; PAA; and PtAA) with the highest *N*-dealkylation rates shown for the methyl and ethyl compounds. CYP1A1 was capable of *N*-dealkylation of all of the substrates with the exception of BzAA at about the same rates. 7-Ethoxyresorufin *O*-dealkylation was observed with CYP1A1, CYP1A2, and CYP1B1 while the 7-pentoxo- and benzyloxyresorufins were metabolized by CYP1A1 and CYP1A2. Kinetic constants for the MAA and EAA with CYP1A1 were K_m 1.09 ± 0.68 , 0.35 ± 0.21 μM and the V_{\max} 61.9 ± 48.5 , 113.8 ± 8.4 pmole 9AA/min/pmole CYP1A1, respectively. The Cl_{int} values were 56.7 and 325.1 $\mu\text{L}/\text{min}/\text{pmole}$ CYP1A1 for MAA and EAA, respectively. Kinetic constants for the MAA and EAA with CYP2D6 were K_m 7.9 ± 5.4 , 3.2 ± 1.6 μM and the V_{\max} 501.2 ± 35.4 , 702.7 ± 257 pmole 9AA/min/pmole CYP2D6, respectively. The Cl_{int} values were 63.4 and 219.6 $\mu\text{L}/\text{min}/\text{pmole}$ CYP2D6 for MAA and EAA, respectively. The experimental binding energies (ΔG_{bind}) were calculated for MAA with CYP1A1 and CYP2D6 to be -8.266 and -7.074 kcal/mole, respectively. The ΔG_{bind} values for EAA with CYP1A1 and CYP2D6 were -8.950 and -7.618 kcal/mole, respectively.

Reactivity of 9-*N*-(Alkylamino)acridines with Microsomal Preparations.

Several hepatic microsomal preparations from human, rat, and monkey were tested with 5 μM concentrations of the 9-*N*-(alkylamino)acridines. The *N*-dealkylation kinetic constants for the microsomal preparations with the various substrates are shown in Table 2 and significant differences were observed. All of the microsomal preparations were capable of metabolizing the MAA, EAA, PAA, and BAA substrates. Only rat liver microsomes were capable of *N*-dealkylating the PtAA. Rat liver microsomes also had the

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lowest K_m values for the MAA and EAA substrates (0.27 and 1 μM , respectively). Monkey liver microsomes had consistent K_m values (2.3 to 5.6 μM) and consistently high V_{max} values (21.0 - 40.4 fmole 9AA/min/pmole P450) for MAA, EAA, PAA, and BAA. Intrinsic clearance values (Cl_{int}) are also indicated in Table 2. Rat liver microsomes had the highest Cl_{int} values for MAA being 6.7- and 5.7-fold greater than those of human and monkey liver microsomes, respectively. The Cl_{int} values for EAA were approximately the same for all microsomal sources. Monkey liver Cl_{int} values for PAA ca. 30- and 17-fold higher than those for human and rat liver microsomes, respectively. Likewise, monkey liver Cl_{int} values for BAA were 12- and 9-fold higher than for human and rat liver microsomes, respectively.

Real-Time Assay of Cell Suspensions.

Human hepatocellular carcinoma cells (ATCC#CRL-10741) were cultured for four days with 25 μM Rifampicin present in the culture medium to induce cytochrome P450 activity. Figure 4 is a real-time trace of *N*-demethylation of MAA by hepatocellular carcinoma cell suspensions. After a ca. 90-120 s lag time following the addition of NADPH there was a steady increase in fluorescence. At the conditions stated, the activity was calculated to 1.6 pmole 9AA/20 min.

Inhibition of CYP2D6, CYP1A1, Human Liver Microsomal *N*-Dealkylation of 9-*N*-(Methylamino)acridine.

The inhibition effects of quinidine, imipramine, 7,8-benzoflavone, resveratrol, and amitriptyline on CYP2D6 and CYP1A1 metabolism of MAA were determined. The I_{50}

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values for CYP2D6 were determined to be 9.41 ± 0.55 nM for quinidine, 1.25 ± 0.14 μ M for imipramine, 1.64 ± 0.41 μ M for amitriptyline, and 27 ± 0.71 μ M for resveratrol. No I_{50} could be determined for 7,8-benzoflavone with the CYP2D6; the value was greater than 200 μ M. I_{50} values with CYP1A1 were determined to be 21.5 ± 3.5 μ M for quinidine, 800 ± 70.7 nM for resveratrol, and 39 ± 8.5 nM for 7,8-benzoflavone; no I_{50} values could be determined for imipramine and amitriptyline for CYP1A1 metabolism of MAA. Similarly, the I_{50} values for EtR inhibition of *N*-dealkylation of 9-*N*-(methylamino)acridine by CYP1A1 and CYP2D6 were respectively determined to be $0.86 \mu\text{M} \pm 0.12$ and $3.96 \pm 0.6 \mu\text{M}$.

Because it is possible to use specific inhibitors to block specific CYPs, tests were conducted using human liver microsomal preparations. The I_{50} experiments with the expressed CYP1A1 and C2D6 preparations indicated that 50 nM quinidine would inhibit 100% of the CYP2D6 metabolism of MAA while having no effect on CYP1A1 metabolism of MAA. Likewise, 500 nM 7,8-benzoflavone inhibited 98-100% of CYP1A1 metabolism of MAA with no effect on CYP2D6 activity. In tests with human liver microsomes, MAA metabolism was inhibited by 20% with 50 nM quinidine, 65% with 500 nM 7,8-benzoflavone, and by 95% when both inhibitors were used simultaneously.

DISCUSSION

Fluorescence methods for assaying enzyme activity in real-time are generally preferred because of high sensitivities, are usually not labor intensive, and can be adapted to high-throughput formats for screening purposes. With regard to *N*-dealkylase assays, there are

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few *N*-alkyl containing fluorescent compounds that show significant changes in fluorescence properties (e.g., quantum yield, excitation and emission wavelength shifts) when *N*-dealkylation occurs. 9-Aminoacridine is an ideal fluorescent compound for measuring *N*-dealkylation of 9-*N*-(alkylamino)acridines because of the large differences in quantum yield between the free amine and 9-*N*-alkyl forms of the molecule (Fig. 2). Although differences exist in the excitation and emission wavelength maxima between the 9-*N*-alkyl derivatives and the 9AA, the largest apparent difference is in the quantum yields. Typically the fluorescence intensity of 9AA at 455 nm (excitation 401 nm) is 25-30-fold greater than that of the 9-*N*-alkyl derivatives. Product formation (i.e., 9AA) resulting from *N*-dealkylase activity is easily distinguished from background fluorescence of the substrates (i.e., 9-*N*-(alkylamino)acridines). The excitation and emission wavelengths of 9AA are higher than those of NADPH (λ_{ex} 340 nm, λ_{em} 460 nm) and most proteins (λ_{ex} 280 nm, λ_{em} 300-360 nm) and consequently obviate fluorescence problems that might result from the presence of these reaction mixture components.

9-Aminoacridine has been reported as an antibacterial agent for the treatment of wounds (Albert, 1979). It has also been extensively used to measure the surface charge densities of various types of membranes (Chow and Barber, 1980; Rugolo *et al.*, 1991; Jones *et al.*, 1995; Brauer *et al.*, 2000). The fluorescence of 9AA is quenched upon binding to membranes and it can be displaced by addition of cations, thus eliminating the quenching effect. Membrane surface charge density can be calculated using the Gouy-Chapman theory and the differential effects of cations on 9AA fluorescence. Chow and Barber (1980) showed that 100 mM KCl was effective in displacing 9AA from binding to thylakoid membranes and subsequently allowing for full fluorescence of 9AA. In setting

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the reaction conditions we used 100 mM potassium phosphate buffers to minimize 9AA from binding to membranes and maximize 9AA fluorescence. Any quenching due to membrane binding should be compensated for by calibrating the fluorescence of known amounts of 9AA added to reaction mixtures. 9AA may be metabolized by CYP1A since tacrine (1,2,3,4-tetrahydro-9-aminoacridine), used in the treatment of Alzheimer's disease, has been reported to be metabolized by human liver microsomes (Spaldin *et al.*, 1994) and can induce hepatic CYP1A content and increase ethoxyresorufin *O*-deethylase activity (Sinz and Woolf, 1996).

Some of the 9-*N*-(alkylamino)acridines, such as the antimalarial mepacrine (Albert, 1979), have been reported to have biological activity that may be the result of binding to DNA (Galy *et al.*, 1987). Several of the 9-*N*-(alkylamino)acridines reported here (i.e., EAA, PAA, PtAA) have been tested and shown to bind about 5-fold more tightly to DNA than 9AA (Galy *et al.*, 1987).

The fluorescence characteristics of the 9-*N*-(alkylamino)acridines and 9AA make it possible to directly assay *N*-dealkylase activity in real-time without having to either derivatize and/or purify the product for analysis as has been necessary for other assays (Kupfer and Bruggeman, 1966; van der Hoeven, 1977; Nerland and Mannering, 1978; Niwa *et al.*, 1999; Pinto *et al.*, 2004; Stresser *et al.*, 2004). Several assays have been reported to measure CYP2D6 enzyme activity by following either the *O*-demethylation of AMMC (Miller *et al.*, 2000) or the *O*-deethylation of CEC (Crespi *et al.*, 1997), or the *O*-demethylation of MAMC (Onderwater *et al.*, 1999). Of these only the AMMC is enzyme selective. CEC can also be metabolized by CYP1A2, CYP2C19 (Miller *et al.*, 2000) while the MAMC can be metabolized by CYP2D6 and CYP1A2 (Onderwater *et al.*,

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1999). Although these assays reflect CYP2D6 activity and the I_{50} results with known CYP2D6 inhibitors (e.g., quinidine) compare well with those reported elsewhere (Moody *et al.*, 1999) these assays do not measure *N*-dealkylation *per se* as does the *N*-dealkylation of 9-*N*-(alkylamino)acridines assay described here.

Eight different expressed CYPs (Table 1) were tested with various 9-*N*-(alkylamino)acridines and only CYP1A1 and CYP2D6 showed any *N*-dealkylation activity. CYP1A1 apparently has a broader reaction range as it can *N*-dealkylate all of the substrates with the exception of the BzAA. CYP2D6 can *N*-dealkylate all of the substrates except BzAA and BAA.

Tests with the inhibitors quinidine, imipramine, and amitriptyline on the *N*-demethylation of MAA indicated that the I_{50} values compared very well with those reported elsewhere for CYP2D6 metabolizing other substrates (Crespi *et al.*, 1997; Onderwater *et al.*, 1999; Moody *et al.*, 1999; Yamamoto *et al.*, 2003). Resveratrol inhibition of CYP1A1 metabolism of MAA yielded an I_{50} of 27 μM which is close to the I_{50} values reported for 7-methoxy- and 7-ethoxyresorufin metabolism (11 μM and 23 μM , respectively; Chun *et al.*, 1999). The I_{50} reported here for 7,8-benzoflavone (α -naphthoflavone) with CYP1A1 metabolism of MAA is ca. 10-fold lower than that reported by Chang *et al.* (1994) for inhibition of 7-ethoxycoumarin *O*-dealkylation (i.e., present study is 39 nM vs. 0.4 μM). The lower I_{50} value reported here may be the result of using a different substrate to calculate the value. From the results with human liver microsomes metabolism of MAA it appears possible to isolate the enzyme activities due to CYP1A1 and CYP2D6 in complex mixtures using inhibitors such as quinidine and 7,8-

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benzoflavone. The high selectivity of the 9-*N*-(alkylamino)acridines for CYP1A1 and CYP2D6 in combination with selective inhibitors is what makes this possible.

We also observed that EtR was an inhibitor for the *N*-demethylation of MAA by CYP1A1 and CYP2D6. The I_{50} values for EtR inhibition of *N*-dealkylation of MAA by CYP1A1 and CYP2D6 were in the same range, i.e., $0.86 \mu\text{M} \pm 0.12$ (CYP1A1) and $3.96 \pm 0.6 \mu\text{M}$ (CYP2D6). We expected that the CYP1A1 *N*-demethylation reaction would be inhibited by the presence of EtR since it can dealkylate either substrate. However, since CYP2D6 cannot *O*-dealkylate EtR, we did not know if EtR could inhibit the *N*-demethylation of MAA. Lewis *et al.* (1999) broadly classified CYP1A1 substrates as being “planar polyaromatic hydrocarbons and their derivatives” while substrates for CYP2D are “basic compounds with a nitrogen atom protonable at physiological pH.” Recently de Graaf *et al.* (2006) published an extensive list of candidate substrates for CYP2D6 based on automated molecular docking which substantiates the characteristic of a protonable nitrogen atom within CYP2D6 substrate molecules. Certainly the 9-*N*-(alkylamino)acridines fit this characteristic as the 9-amino group would be protonated at physiological pHs (Galy *et al.*, 1987). It may be that CYP2D6 can accommodate neutral molecules such as EtR along with more polar molecules. De Graaf *et al.* (2006) reported GBR 30111 as a compound that had an affinity (I_{50} of $60 \mu\text{M}$) for CYP2D6, but did not have a charged nitrogen at physiological pH's. The affinity was attributed to GBR 30111 being able to form hydrogen bonds with Glu²¹⁶.

The Michaelis-Menten kinetics analyses showed significant differences in the K_m 's and V_{max} 's obtained using MAA and EAA with expressed preparations of CYP1A1 and CYP2D6. The K_m 's and V_{max} 's were lower for both substrates with CYP1A1 (MAA

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K_m 1.09 μM , V_{max} 61.9 pmole 9AA/min/pmole P450; EAA K_m 0.35 μM , V_{max} 113.8

9AA/min/pmole P450) than with CYP2D6 (MAA K_m 7.9 μM , V_{max} 501.2 pmole

9AA/min/pmole P450; EAA K_m 3.2 μM , V_{max} 702.7 9AA/min/pmole P450).

Interestingly, if the Cl_{int} values are compared for MAA and EAA with CYP1A1 and CYP2D6 they are roughly equivalent (e.g., for MAA the Cl_{int} is 56.7 for CYP1A1 vs.

63.4 for CYP2D6) indicating that metabolism by the two CYPs is proportionately the same for the same substrates. However, the Cl_{int} values are significantly different

between substrates as the Cl_{int} for EAA is 5.7-fold higher than MAA with CYP1A1.

There is only a 3.5-fold higher Cl_{int} value for EAA than MAA with CYP2D6.

Kinetic analyses of the *N*-dealkylation of the 9-*N*-(alkylamino)acridines by various hepatic microsomal preparations indicated differences in metabolism (Table 2). Generally higher Cl_{int} values were observed for MAA and EAA than the other substrates with human and rat hepatic microsomes. The Cl_{int} value for MAA with rat liver was the highest value observed for any substrate and microsomal preparation being 40 nL/min/pmole P450. This was 6.7- and 5.7-fold higher than the values obtained with human and monkey preparations. For rat and human hepatic preparations, the Cl_{int} generally decreases with increased alkyl chain length of the substrate. Monkey hepatic microsomal Cl_{int} values were more consistent for all substrates.

The *N*-dealkylase assay reported here is highly cytochrome P450 isoform specific, allowing for assay of CYP1A1 and CYP2D6 in many different tissue preparations including microsomes, expressed enzymes, cell suspensions, and it can be used in high-throughput formats (i.e., 96-well microtiter plates). No attempt was made to reduce assay costs by optimizing reaction times and enzyme concentrations as has been done with

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other high-throughput methods (Crespi *et al.*, 1997; Miller *et al.*, 2000). Further, the assay requires no extraction or any other post-reaction processing and thus, has a reduced handling time compared to other available assays.

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Figure Captions

Figure 1. 9-*N*-(Alkylamino)acridines are *N*-dealkylated by CYP1A1 and CYP2D6 in the presence of O₂ and NADPH to form 9-aminoacridine.

Figure 2. Spectral characteristics of 0.25 μM 9-*N*-(methylamino)acridine (MAA) and 9-aminoacridine (9AA) in 100 mM potassium phosphate buffer, pH 7.2. Corrected excitation (Ex) spectra were recorded using an emission wavelength of 455 nm. Corrected emission (Em) spectra were recorded using an excitation wavelength of 401 nm. Excitation and emission slits were 2.5 nm. - - - - 9AA excitation spectrum; -•••• 9AA emission spectrum; - - - - MAA excitation spectrum; ••••• MAA emission spectrum; ——— excitation and ■■■■ emission spectrum of 100 mM potassium phosphate buffer, pH 7.2.

Figure 3. Relative activities of various expressed CYP's with 9-*N*-(methylamino)acridine. Reactions contained 8 pmoles of CYP, 5 μM 9-*N*-(methylamino)acridine, 0.25 mM NADPH, in a total volume of 2 mL of 100 mM potassium phosphate buffer, pH 7.2. A. CYP2D6; B. CYP1A1; C. CYP3A4; D. CYP3A5; E. CYP1A2; F. CYP2C19, G. CYP2C9, H. CYP1B1.

Figure 4. Metabolism of 9-*N*-(methylamino)acridine by human hepatocellular carcinoma (ATCC#CRL-10741) cells at 30°C. Cells were induced for four days using 25 μM Rifampicin. Reactions contained final concentration of 1.125 X 10⁶ hepatocytes per mL,

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1.25 mM NADPH, and 5 μ M 9-*N*-(methylamino)acridine in a final volume of 2 mL

Dulbecco's phosphate buffered saline, pH 7.3. Under these conditions the rate was 1.6

pmole 9-aminoacridine/20 min.

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Table 1. Reactivity of 9-*N*-(Alkylamino)acridines with Cytochromes P450.

CYP	MAA	EAA	PAA	BAA	PtAA	BzAA	EtR	PtR	BzR
	pmole 9AA or Resorufin/min/pmole P450 ± SD								
1A1	0.6 ± 0.02	0.5 ± 0.01	0.7 ± 0.01	0.6 ± 0.03	0.4 ± 0.03	NDA	7.6 ± 1.2	0.4	0.8 ± 0.2
1A2	NDA	NDA	NDA	NDA	NDA	NDA	0.91 ± 0.1	0.02	0.06
3A4	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
3A5	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
1B1	NDA	NDA	NDA	NDA	NDA	NDA	0.38 ± 0.01	NDA	0.97 ± 0.2
2C9	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
2C19	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA
2D6	0.9 ± 0.02	0.9 ± 0.01	0.1 ± 0.01	NDA	0.1 ± 0.01	NDA	NDA	NDA	NDA

NDA = No Detectable Activity; 9AA = 9-Aminoacridine; MAA = 9-*N*-(Methylamino)acridine; EAA = 9-*N*-(Ethylamino)acridine; PAA = 9-*N*-(1-Aminopropyl)acridine hydrochloride; BAA = 9-*N*-(1-Aminobutyl)acridine hydrochloride; PtAA = 9-*N*-(1-Aminopentyl)acridine; BzAA = 9-*N*-(1-Aminobenzyl)acridine hydrochloride; EtR = 7-Ethoxyresorufin; PtR = 7-Pentoxyresorufin; BzR = 7-Benzoyloxyresorufin.

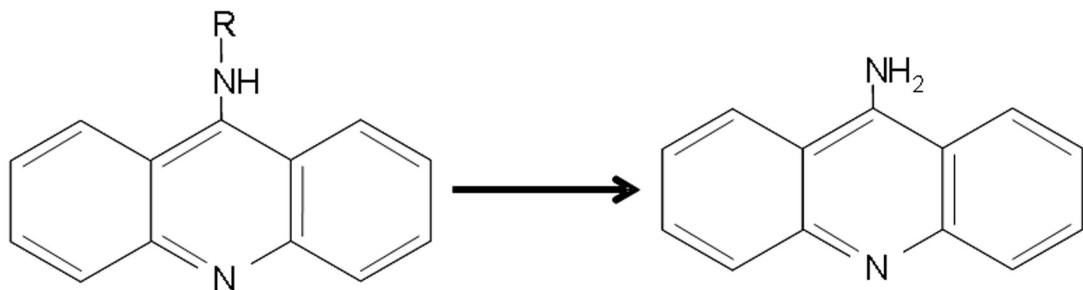
Table 2. Reactivity of 9-*N*-(Alkylamino)acridines with Microsomal Preparations.

Substrate	Microsome Source								
	Human Liver			Monkey Liver			Rat Liver		
	K_m (μ M) \pm SD	$V_{max} \pm SD$ (fmole 9AA/min/pmol P450)	Cl_{int} (nL/min/pmol P450)	K_m (μ M) \pm SD	$V_{max} \pm SD$ (fmole 9AA/min/pmol P450)	Cl_{int} (nL/min/pmol P450)	K_m (μ M) \pm SD	$V_{max} \pm SD$ (fmole 9AA/min/pmol P450)	Cl_{int} (nL/min/pmol P450)
MAA	3.7 \pm 1.9	23.6 \pm 6.6	6	5.6 \pm 0.4	39.5 \pm 2.7	7	0.27 \pm 0.1	10.7 \pm 0.9	40
EAA	2.6 \pm 0.1	27.3 \pm 8.1	11	3.1 \pm 0.3	24.5 \pm 1.6	8	1.0 \pm 0.4	8.7 \pm 8.0	9
PAA	16.3 \pm 12.2	9.3 \pm 3.1	0.6	2.4 \pm 0.1	40.4 \pm 3.6	17	12.1 \pm 4.8	16.0 \pm 5.3	1
BAA	12.3 \pm 8.1	9.3 \pm 3.1	0.7	2.3 \pm 0.2	21.0 \pm 4.3	9	21.8 \pm 17.2	26.3 \pm 9.8	1
PtAA	NDA	NDA	NDA	NDA	NDA	NDA	13.1 \pm 15.6	16.3 \pm 10.1	1
BzAA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA	NDA

NDA = No Detectable Activity; 9AA = 9-Aminoacridine; MAA = 9-*N*-(Methylamino)acridine; EAA = 9-*N*-(Ethylamino)acridine; PAA = 9-*N*-(1-Aminopropyl)acridine hydrochloride;

BAA = 9-*N*-(1-Aminobutyl)acridine hydrochloride; PtAA = 9-*N*-(1-Aminopentyl)acridine; BzAA = 9-*N*-(1-Aminobenzyl)acridine hydrochloride

N-Dealkylation of 9-*N*-Akylaminoacridines



R = H; CH₃; C₂H₅; C₃H₇; C₄H₉; C₅H₁₁; C₇H₈ (Benzyl)

Figure 1

Figure 2

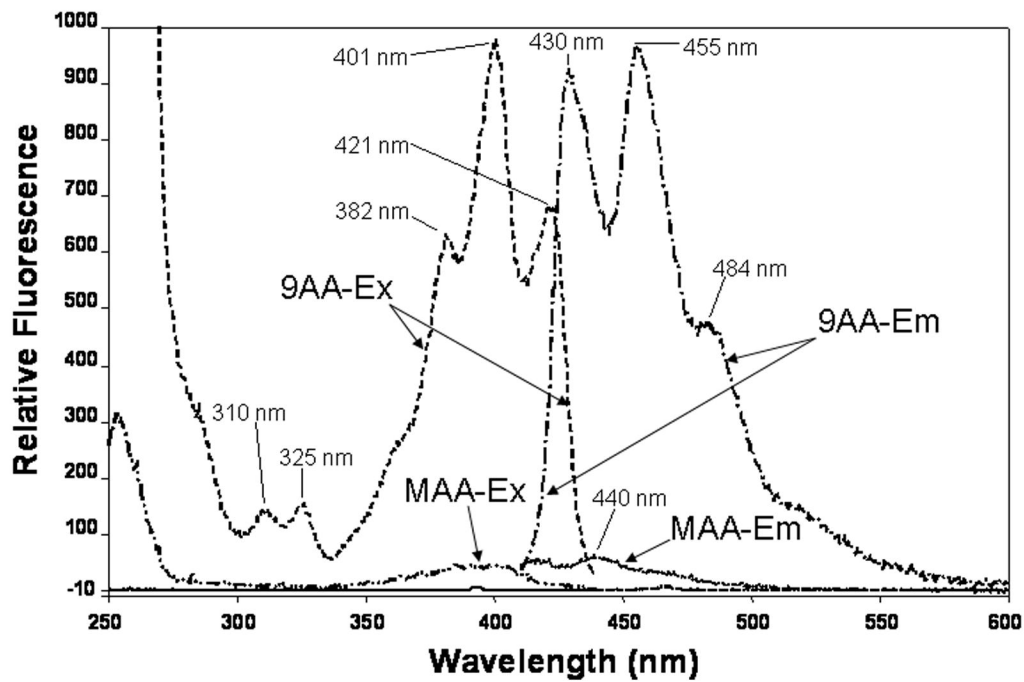


Figure 3

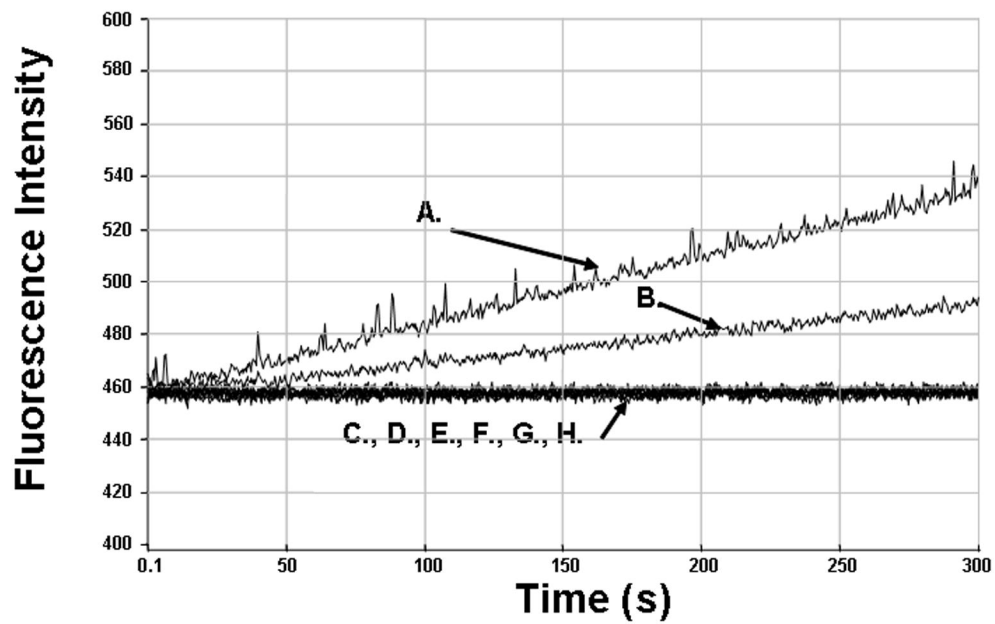


Figure 4

