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Note

High-performance liquid chromatographic assay and preclinical pharmacologic studies with the experimental antitumor agent batracylin

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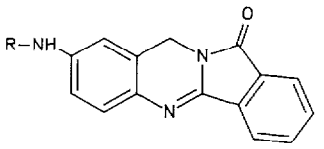
Batracylin (NSC-320846) (Fig. 1) is a quinazolineone synthesized by Bayer and currently under preclinical pharmacologic and toxicologic evaluation by the National Cancer Institute. This agent was selected for study based on its unusual in vivo activity in mice outbearing early-stage and advanced colon adenocarcinoma 38 [1]. While the drug was ineffective against L1210 leukemia, mouse B16 melanoma, CD8F1 mammary, and Lewis lung carcinoma, there was activity against P388 sublines with acquired resistance to adriamycin, cisplatinum, and methotrexate [1]. Due to very limited water solubility, all in



R–	Compound
H–	Batracylin
CH_3CH_2CO–	NSC–611002 (IS)

Fig. 1. Structure of batracylin and internal standard (IS).

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vivo activity studies were based on oral administration. The combined sex dose lethal to 10% of the treated animals (LD_{10}) following oral administration of batracylin to CD2F1 mice was 5655 mg/m^2 (1885 mg/kg) [2]. Rats were much more sensitive to batracylin, with renal, gastrointestinal, and testicular toxicity observed at 1/80th the mouse equivalent LD_{10} dose [2]. Data for beagle dogs are not yet available.

The mechanism of action of batracylin has not been described. However, the polyheteroaromatic ring structure is reminiscent of many molecules which intercalate into DNA. Specifically, there are structural features (fused planar cationic aromatic ring) in common with daunorubicin, ethidium bromide, and ellipticines, all of which intercalate into DNA.

In preparation for detailed preclinical and clinical pharmacologic studies, we have developed a high-performance liquid chromatographic (HPLC) assay for batracylin and used the assay to obtain preclinical pharmacologic data.

EXPERIMENTAL

Reagents

Batracylin (8-aminoisoindolo[1,2-*b*]quinazolin-12(10*H*)-one) and the 8-amino-N-propionyl analogue internal standard (see Fig. 1) were supplied by the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.). The ring-open product **2** (see Fig. 4) was prepared, characterized, and provided by Dr. V. Stella, University of Kansas (Lawrence, KS, U.S.A.). Solid-phase extraction columns (C_{18}, 1 ml) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Analytical HPLC columns (C_{18}, Nova-pak, 4 μm particle size, 15 cm $\times$ 3.9 mm I.D.) were purchased from Millipore Waters Chromatography Division (Milford, MA, U.S.A.) while guard columns (RP-18) were purchased from Brownlee Labs. (Santa Clara, CA, U.S.A.). All solvents were of HPLC grade.

Instrumentation

HPLC analyses were performed with a Hewlett-Packard Model 1090M system equipped with a diode-array detector, autosampler, and appropriate software.

HPLC analysis of batracylin

Batracylin was analyzed by reversed-phase HPLC on a Nova-pak C_{18} column with a mobile phase system of acetonitrile–5.0 m*M* ammonium acetate (pH 3.5) (25:75, v/v) at a flow-rate of 1 ml/min. A 15 mm $\times$ 3.2 mm I.D. guard column prepacked with C_{18} pellicular resin was used for all plasma analyses. Detection of batracylin and internal standard was by ultraviolet absorption at 413 nm.

Plasma isolation procedure

Murine plasma (0.05–0.3 ml) was diluted by addition of normal saline to a final volume of 1.0 ml. Internal standard (100–200 ng, 10 ng per μl of 1% dimethylsulfoxide in distilled water) was added to each plasma sample. Samples were applied to C_{18} solid-phase extraction columns prepared by washing with 2 ml methanol and 2 ml distilled water. Columns were washed with 2 ml water, and batracylin and internal standard eluted with 1 ml methanol. Eluates were evaporated to dryness under a gentle stream of nitrogen and the residue was dissolved in 100–200 μl of mobile phase prior to HPLC analysis. Standard curves were prepared by adding known amounts of batracylin (0.025–5.0 μg per plasma sample) and internal standard (1.5 μg per plasma sample) to drug-free plasma. Standard curves were fitted by linear regression and unknown concentrations of batracylin determined with the standard curve equations.

Buffer, plasma and whole blood stability studies

To determine the pH stability of batracylin in buffer, the drug was added to 0.1 *M* sodium phosphate buffer (pH 7.4), mobile phase, dimethylsulfoxide, 0.25 *M* sodium acetate buffer (pH 3.0), and 0.25 *M* glycine buffer (pH 11.0). Fresh human and mouse whole blood (citrated) and plasma isolated from fresh human and mouse whole blood were employed for plasma and whole blood stability studies. Aliquots were removed at appropriate times and frozen ($-20°C$) until HPLC analysis.

Mouse pharmacology studies

CD2F1 mice (20–25 g) were employed for murine pharmacology studies. Batracylin was prepared as a suspension in saline–TWEEN 80 (75:25, v/v) at a concentration of 25 mg/ml. The suspension was prepared by homogenization with a cooled PTFE-glass homogenizer. Drug (1050 mg/m^2, 350 mg/kg) was administered orally to unanesthetized mice with a straight 20-gauge ball-tip lavage needle or by intraperitoneal injection. At appropriate times, animals were anesthesized with ether vapors, a midline incision was made, and the peritoneal cavity exposed. The heart was punctured and 0.5–1.0 ml whole blood obtained. Plasma was immediately isolated by centrifugation (25 °C, 1000 *g*, 15 min) and stored at $-20°C$. Batracylin was determined in plasma by the isolation and HPLC methods described.

RESULTS

Initial chromatographic conditions focused on reversed-phase C_8 and C_{18} columns with acetonitrile, pH 3.5 ammonium acetate and heptanesulfonic acid ion-pairing mobile phase systems. While chromatography was adequate with this system, superior peak shape was obtained when a Nova-pak C_{18} column

was substituted for standard C_8 or C_{18} columns. In fact, the ion-pairing reagent did not improve chromatography obtained with the Nova-pak column when compared to a mobile phase system consisting of only acetonitrile and pH 3.5 ammonium acetate. A chromatogram from an extract of plasma containing batracylin (75 ng) and internal standard (200 ng) is illustrated in Fig. 2. Two of the three small peaks are contaminants present in the internal standard (5 and 6.5 min) and the third is a constituent of plasma (8 min).

Initial efforts to isolate batracylin from plasma under basic conditions with organic solvents such as ethyl acetate, chloroform, and hexane were not satisfactory. Recoveries were particularly low with ethyl acetate (approximately 15%) and hexane (approximately 25%). Results with chloroform were better (70–85%), but not sufficiently reproducible for pharmacologic studies. It was then determined that batracylin and the internal standard could be recovered efficiently and reproducibly from C_{18} disposable extraction columns with methanol as the eluting solvent. Recoveries of batracylin and internal standard from plasma were 85–95% and 70–90%, respectively. Ratios of extracted parent drug and internal standard were consistent from experiment to experiment. The on-column limit of detection (signal-to-noise ratio = 3) following isolation of batracylin from plasma was 5.0–7.5 ng. Plasma volumes for murine pharmacology studies were 100–300 μl per sample. No loss in sensitivity was observed when larger plasma volumes (e.g. 1 ml) were used with the assay. The assay was linear over the working range (0.075–5.0 μg per plasma sample, $r^2 \geq 0.99$), and inter-assay coefficients of variation were 6.9 and 3.0% for concentrations of 0.5 and 10.0 μg/ml, respectively ($n = 10$).

The HPLC assay was first applied to determination of buffer, plasma, and

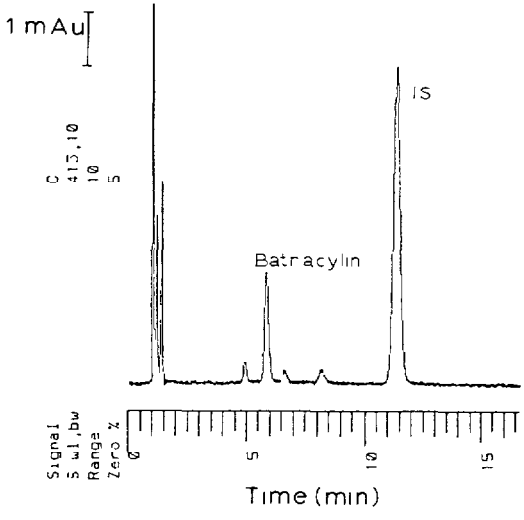


Fig. 2. Chromatogram of a plasma sample containing 75 ng batracylin per 300 μl and 200 ng internal standard (IS) following C_{18} column isolation procedure.

whole blood stability of batracylin. While the drug was stable in neutral (pH 7.4) phosphate buffer, mobile phase and dimethylsulfoxide, time-dependent disappearance of the parent drug was observed at pH 3.0 (acetate buffer), and more rapid disappearance was noted as pH 11.0 (glycine buffer) (Fig. 3).

Batracylin is known to hydrolyze reversibly to a ring-open product under acidic conditions [3]. Based on the chromatographic retention time of authentic material provided by Dr. Stella, we determined that disappearance of batracylin under acidic conditions was associated with concomitant appearance of the ring-open degradation product **2** shown in Fig. 4. Base-catalyzed decomposition was associated with formation of **2** and ultimate conversion to products not detected by the HPLC assay. There was no disappearance of batracylin in fresh mouse and human plasma for 48 h at temperatures from 6 to 37 °C. However, at a concentration of 10 μg/ml, disappearance of batracylin and concomittant appearance of a degradation product was detected following incubation in fresh mouse and human whole blood at 37 °C. The half-life of disappearance was 40 h. The breakdown product was not the acid degradation product **2**.

Because of very limited aqueous solubility, plasma elimination of batracylin was determined following oral and intraperitoneal administration (1050

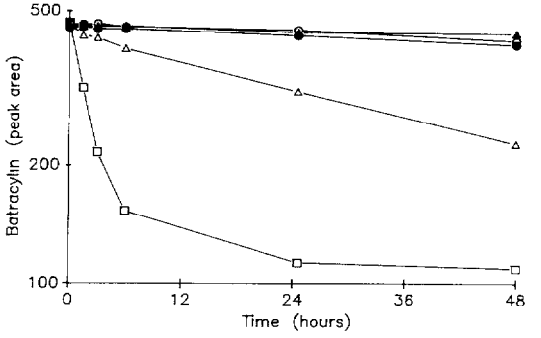
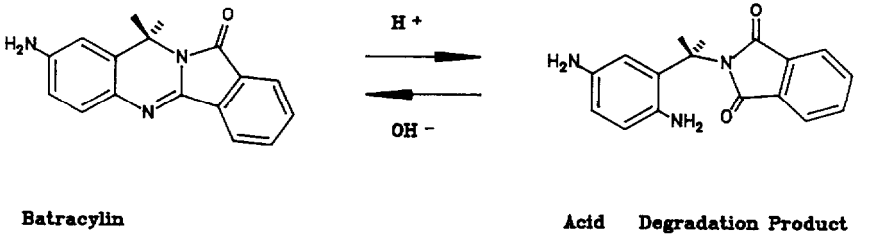


Fig. 3. Stability of batracylin as a function of time in HPLC mobile solvent (●), dimethylsulfoxide (○), sodium phosphate buffer, pH 7.4 (▲), sodium acetate buffer, pH 3.0 (△), and glycine buffer, pH 11.0 (□).



Batracylin **Acid Degradation Product**

Fig 4. Structure of batracylin and the acid-catalyzed ring-open product.

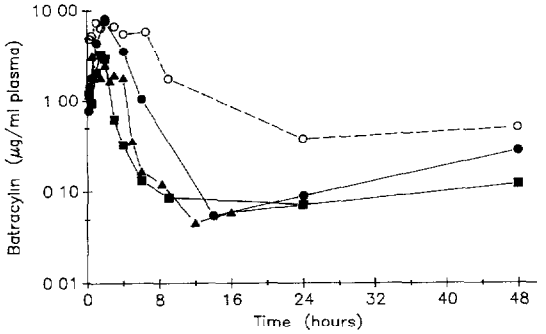


Fig. 5. Mouse plasma concentration-time data following oral administration of batracylin (1050 mg/m^2) (filled symbols) and intraperitoneal administration of the same dose (open symbols).

mg/m^2, 350 mg/kg) to mice. Representative plasma concentration–time profiles are illustrated in Fig. 5. Peak concentrations were observed from 1 to 2 h following administration of drug. Increased concentrations of parent drug were detected at 24 and 48 h following drug administration. This may have been due to enterohepatic cycling of drug and/or release from tissue storage sites. The relative bioavailability of batracylin following oral administration compared to intraperitoneal administration was approximately 20%. Several plasma metabolites of batracylin were detected in these experiments, but they did not include the acid degradation product.

DISCUSSION

The HPLC assay developed for batracylin provides adequate sensitivity and selectivity for both preclinical and anticipated clinical pharmacologic studies with this agent. We also determined that batracylin fluoresces with an emission maximum of 565 nm and an excitation maximum of 390 nm. Preliminary studies indicate that assay sensitivity could be significantly increased with fluorescence detection. Isolation of batracylin from plasma by extraction with organic solvents was not satisfactory. While there are lipophilic elements in this molecule (specifically the planar aromatic ring system), the presence of polar and charged nitrogen atoms, and the instability in base, complicates extraction. Isolation was readily accomplished with a simple procedure using C_{18} solid-phase extraction columns.

Pharmacologic studies with batracylin may be influenced by the irreversible degradation of the compound under alkaline pH conditions and the reversible hydrolysis of the molecule under acidic conditions. Batracylin was stable at physiologic pH in buffer and in plasma, but there was slow degradation in the presence of whole blood.

Batracylin was detected in plasma following oral and intraperitoneal admin-

istration to mice. An additional peak was detected following oral and intraperitoneal administration of batracylin to mice with a retention time identical to that of the whole blood breakdown product. Of note, batracylin was active in tumored mice when drug was administered by the oral route. Given the potential difficulties in formulating batracylin, oral administration may be relevant to the further evaluation of this compound. Additional preclinical pharmacologic studies are underway in our laboratory focusing on the metabolism of this agent and further characterization of the pharmacokinetics of this agent in mice and rats prior to Phase I clinical trials.

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