Site-specific folate conjugation to a cytotoxic protein

Bryan D. Smith, Joshua J. Higgin, and Ronald T. Raines*

Page **Contents**

Materials. K-562 cells, derived from a human chronic myelogenous leukemia line, and JAR cells, derived from a human choriocarcinoma line, were from the American Type Culture Collection (Manassas, VA). [*methyl*-^{3}H]Thymidine was from Perkin–Elmer Life Sciences (Boston, MA). The fluorogenic ribonuclease substrate, 6-FAM–dArUdAdA–6-TAMRA (where 6-FAM is a 5′ 6-carboxyfluorescein group and 6-TAMRA is a 3′ 6-carboxytetramethylrhodamine group), was from Integrated DNA Technologies (Coralville, IA). Folate-free RPMI medium and fetal bovine serum were from Life Technologies (Carlsbad, CA). To remove inhibitory contaminants, MES buffer was purified by anion-exchange chromatography prior to use.[1] All other chemicals and reagents were of commercial grade or better, and were used without further purification.

General Experimental Procedures. UV absorbance measurements were made with a Cary Model 3 spectrophotometer (Varian, Palo Alto, CA). Fluorescence measurements were made with a QuantaMaster 1 Photon Counting Fluorometer equipped with sample stirring (Photon Technology International, South Brunswick, NJ). Mass spectrometry was performed on a Voyager MALDI–TOF mass spectrometer (Perkin–Elmer, Boston, MA) in the Biophysics Instrumentation Facility at the University of Wisconsin–Madison.

The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water aspirator pressure (<20 torr) while maintaining the water-bath temperature below 50 °C. Residual solvent was removed from samples at high vacuum (<0.1 torr). The term "high vacuum" refers to vacuum achieved by a mechanical belt-drive oil pump.

Synthesis of Pteroyl Azide. Pteroyl azide was synthesized by methods described previously,[2] as summarized below. Folic acid (10 g, 20.9 mmol) was dissolved in THF (100 mL). The resulting solution was stirred for 10 min and then cooled to 0 °C. Trifloroaceticanhydride (16 mL, 113 mmol) was added slowly and with stirring over 30 min. The reaction mixture was allowed to warm to 25 °C and stirred overnight. The reaction mixture was then concentrated under reduced pressure to a thick dark viscous liquid that was dripped into benzene (150 mL). The precipitate was filtered, washed with ether, and dried under reduced pressure. THF (32 mL) was added to the solid along with ice (6.4 g), and this mixure was stirred for 4 h. The mixture was poured into ether, and the precipitate was collected by filtration, washed with ether, and dried under reduced pressure. The dried solid was then dissolved in DMSO (165 mL) and stirred at 25 °C in a water bath. Hydrazine (4.9 mL, 156 mmol) was added slowly, and the reaction mixture was stirred for 8 h. MeOH (300 mL) was added to precipitate the hydrazide, which was collected by filtration and washed with MeOH (3 × 50 mL) and ether (3 × 50 mL), and dried under reduced pressure. The hydrazide was dissolved in TFA at –10 °C containing KSCN (63 mg, 0.648 mmol). *t*BuONO (1.72 mL, 14.8 mmol) was added slowly, and the reaction mixture was stirred for 4 h at –10 °C. The reaction mixture was allowed to warm to 25 °C, NaN_3 (432 mg, 6.6 mmol) was added, and the resulting solution was stirred for 10 min before isopropanol (150 mL) was added slowly to precipitate the pteroyl azide, which was collected by centrifugation and washed with water (3 × 50 mL), acetonitrile (50 mL), and ether (2 × 50 mL). The product was dried under high vacuum to give pteroyl azide (2.84 g, 8.4 mmol) in a yield of 40% from folic acid.

Synthesis of N^{δ}-Bromoacetyl-N^{α}-pteroyl-L-ornithine (1). In a 25-mL solid phase peptide synthesis vessel, Wang resin (600 mg; 100–200 mesh, 1.2 mmol/g) was added to Fmoc-ornithine-N^{δ}-methyltrityl (880 mg, 1.3 mmol) and DMF (10 mL). 2,6-Dichlorobenzoylchloride (209 μL, 1.5 mmol) and pyridine (193 μL, 2.4 mmol) were added, and the reaction mixture was

shaken on an orbital shaker overnight. The resin was washed with DMF (3 × 20 mL) and CH_2Cl_2 (3 × 20 mL) and dried under reduced pressure. The resin was swelled in dichloroethane (8 mL), and benzoyl chloride (290 μL, 1.8 mmol) was added along with pyridine (290 μL, 3.6 mmol) to cap the resin. The mixture was allowed to shake overnight. The Fmoc group was removed with piperdine (20% v/v) in DMF (20 mL), and the resin was washed with DMF (3 × 20 mL) and CH_2Cl_2 (3 × 20 mL), and dried under reduced pressure. The resin was swelled with 24 mL of 1:1 DMSO/DMF, before the addition of pteroyl azide (790 mg; 2.3 mmol) and tetramethylguanidine (TMG; 400 μL, 3.2 mmol). The reaction mixture was shaken on an orbital shaker overnight. The resin was washed with 1:1 DMSO/DMF (20 mL) followed by CH_2Cl_2 (20 mL), and dried under vacuum. The methyl trityl group was removed by adding TFA (1% v/v) in CH_2Cl_2 until no more yellow color was apparent (~40 mL). The resin was washed with CH_2Cl_2 (40 mL) and dried under vacuum. The resin was then added to CH_2Cl_2 (5 mL) containing bromoacetylbromide (200 μL) and TMG (200 μL), and shaken on an orbital shaker overnight. The resin was washed with CH_2Cl_2 (40 mL), and dried under vacuum. The folate analogue was cleaved from the resin with TFA (10 mL) and dripped into ice-cold ether. The precipitate was collected by centrifugation and washed with ether (3 × 30 mL). The residue was dissolved in H_2O and lyophilized. The fluffy solid was purified by reverse-phase HPLC (20–35% v/v acetonitrile in water) on a Varian C18 column (250 × 21 mm), concentrated under reduced pressure to ~20 mL, and then lyophilized to give **1** (30 mg, 0.05 mmol) in an unoptimized yield of 2% from pteroyl azide.

ESI MS *m*/*z* (M^+) 547, 549 (characteristic Br doublet); ^{1}H NMR (TFA-*d*, 300 MHz) δ 9.0 (s, 1H, Ar), 8.0 (m, 2H, Ar), 7.7 (m, 2H, Ar), 6.6 (m, 2H,), 5.3 (s, 2H, NH), 5.1 (s, 1H, OH), 4.1 (m, 1H, CH), 3.8 (m, 2H, CH_2), 3.4 (m, 4H), 3.1 (m, 3H, NH_3), 2.4 (m, 1H), 2.1 (m, 4H, CH_2CH_2), 1.4 (m, 2H, CH_2).

Production of Ribonucleases. Plasmid pBXR directs the production of RNase A in *Escherichia coli*.[3] RNase A and its variants were produced and purified as described previously.[1] To prevent its inadvertant oxidation, the thiol group of the variants containing a cysteine residue at position 19 or 88 was protected with a 2-nitro-5-thiobenzoic acid (NTB) group as described previously.[4]

Random Conjugation of Folate to Ribonucleases. RNase A and its variants were conjugated randomly to folic acid as described previously.[5] Briefly, folic acid was reacted with a 5-fold molar excess of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in DMSO. A 4- to 10-fold molar excess of the EDC-modified folate was added to a solution containing a ribonuclease. The reaction was allowed to proceed for 2 h at room temperature. Random folate conjugates were purified twice by size-exclusion chromatography on a 5-mL Hi-Trap desalting column. The conjugate was then dialyzed overnight at 4 °C to remove any remaining folate. Protein concentration was determined using a bicinchroninic acid assay kit (Pierce, Rockford, IL). The average number of folates conjugated to a ribonuclease was determined by UV spectroscopy using an extinction coefficient of ε = 6197 $M^{-1}cm^{-1}$ at 363 nm. Mass spectrometry was used to assess the number of folates attached to the enzyme.

Site-specific Conjugation of Folate Analogue 1 to Ribonucleases. RNase A variants with free cysteines were stored as mixed disulfides with NTB. To remove the protecting group, the protein solution was adjusted to pH 8.0, and a 3-fold molar excess of DTT was added to the protein. A yellow color was observed, indicative of deprotection, which was complete after 5 min. The protein solution was purified twice by size-exclusion chromatography on a 5-mL HiTrap desalting column (Pharmacia, Piscataway, NJ). A 4-fold molar excess of analogue **1**

dissolved in DMF was then added to the deprotected protein, and the reaction mixture was stirred for 2 h at room temperature. To remove uncoupled analogue **1**, the protein was desalted again on a HiTrap desalting column. The ribonuclease–folate conjugate was dialyzed against buffer (3 L) overnight at 4 °C. The concentration of ribonuclease–folate conjugates was determined by UV spectroscopy using an extinction coefficient of ε = 35,745 $M^{-1}cm^{-1}$ at 280 nm, which is the sum of the extinction coefficients of RNase A and folic acid.

Assays of Catalytic Activity. Ribonucleolytic activity was assessed at 25 °C in 2.0 mL of 50 mM MES–NaOH buffer, pH 6.0, containing NaCl (0.10 M), and the fluorescent substrate 6-FAM–dArUdAdA–6-TAMRA (60 nM) as described previously.[6]

Assays of Ribonuclease Inhibitor Binding. An agarose gel-based assay was used to assess the affinity of RI for ribonuclease–folate conjugates as described previously.[7,8]

Assays of Cellular DNA Synthesis. Cellular DNA synthesis assays were performed as described previously,[9] with the following modifications. JAR cells grown in folate-free RPMI 1640 medium (FFRPMI) supplemented with fetal bovine serum (FBS; 10% v/v) were washed with FFRPMI and then resuspended in FFRPMI supplemented with dialyzed FBS (10% v/v) prior to the performance of the assays. The use of FFRPMI and dialyzed FBS removes from the medium free folic acid, which could compete with the ribonuclease–folate conjugates for folate receptors on the cell surface. After a 44-h incubation with a ribonuclease–folate conjugate, the JAR cells were treated with [methyl-^{3}H]thymidine for 4 h, and the incorporation of radioactive thymidine into cellular DNA was quantitated by liquid scintillation counting.

References and notes

1. Smith, B. D.; Soellner, M. B.; Raines, R. T. *J Biol Chem* **2003**, *278*, 20934.
2. Luo, J.; Smith, M. D.; Lantrip, D. A.; Want, S.; Fuchs, P. L. *J. Am. Chem. Soc.* **1997**, *119*, 10004.
3. delCardayré, S. B.; Ribó, M.; Yokel, E. M.; Quirk, D. J.; Rutter, W. J.; Raines, R. T. *Protein Eng.* **1995**, *8*, 261.
4. Abel, R. L.; Haigis, M. C.; Park, C.; Raines, R. T. *Anal. Biochem.* **2002**, *306*, 100.
5. Leamon, C. P.; Low, P. S. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 5572.
6. Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. *Nucleic Acids Res.* **1999**, *27*, 3696.
7. Wu, Y.; Mikulski, S. M.; Ardelt, W.; Rybak, S. M.; Youle, R. J. *J. Biol. Chem.* **1993**, *268*, 10686.
8. Leland, P. A.; Schultz, L. W.; Kim, B.-M.; Raines, R. T. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *98*, 10407.
9. Haigis, M. C.; Kurten, E. L.; Abel, R. L.; Raines, R. T. *J. Biol. Chem.* **2002**, *277*, 11576.


^{1}H NMR 300 MHz
TFA-*d*
1
PPM
0.94
1.85
1.91
1.88
1.10
0.41
0.77
1.75
4.18
2.47
1.04
3.21
0.24
1.77