Protein Arginine Deiminase 4: a target for an epigenetic cancer therapy[†]

Jessica L. Slack, Corey P. Causey, Paul R. Thompson[*]

Department of Chemistry & Biochemistry, University of South Carolina, 631 Sumter Street, Columbia, SC 29208

Running Title: PAD4 inhibition induces HL-60 differentiation

Key Words: Protein Arginine Deiminase, haloacetamidine, differentiation, HL-60, epigenetics

[†] This work was supported by NIH grant GM079357 to PRT.

Conflict of interest statement: The authors declare a conflict of interest. The University of South Carolina and P.R.T have a financial interest in F-amidine and Cl-amidine.

[*] To whom correspondence should be addressed: E-mail: thompson@mail.chem.sc.edu

This article contains supporting information online at

Supplemental Materials and Methods.

Cytotoxicity.

HL-60, MCF-7, NIH-3T3, and HT-29 cells were grown to a confluence of 1 x 10^6 cells/mL in the appropriate media. 50 µL of the MCF-7, NIH-3T3, and HT-29 cells were plated into each well of a 96 well plate with 40 µL of DMEM. Cells were allowed to adhere to the plate for 15 h at 37 °C and 5% CO_2. For the HL-60 cells, 90 µL of the cells were added to each well of a 96 well plate. Cl-amidine, F-amidine, doxorubicin, or ATRA (10 µL, 100 nM to 100 µM) were added to the plates and allowed to incubate with the cells for 24 h. Cell viability was determined using the CellTiter 96 Non-radioactive Cell Proliferation Assay (Promega). Cell viability was quantified as the percentage of control absorbance. Each condition was performed in triplicate. The use of 1% triton served as a 100% killing control. When possible, EC_{50} values were determined by fitting the dose response data to equation 1 using GraFit (version 5.0.11) (*1*), where [I] is the concentration of inhibitor (e.g., doxorubicin) and EC_{50} is the concentration of inhibitor that yields half-maximal cell survival.

$$\text{Fractional Activity} = 1/(1+([I]/EC_{50})) \quad (1)$$

Western blotting.

HL-60 cells (~5 x 10^5) were added to each well of a 12-well plate and treated for 0-48 h with either: ATRA (1 µM), Cl-amidine (100 nM, 1 µM, 100 µM), F-amidine (100 nm, 1 µM, 100 µM), or PBS as a negative control. Cells were collected after 6, 12, 24, and 48 h by centrifugation at 1000 rpm for 5 min at 4 °C. Cells were rinsed twice with cold PBS and resuspended in SDS lysis buffer (2% SDS, 62.5 mM Tris pH 6.8 and 10% glycerol) and boiled to afford cell lysis and protein denaturation. Proteins were then separated by 12% SDS-PAGE and transferred to PVDF for western blot analysis. The membranes were blocked with either 5% Nonfat dry milk or 5% BSA for 1 h at room temperature. The membranes were probed with either a polyclonal anti-PAD4 antibody (Abcam ab38772), a monoclonal anti-MYO antibody (Abcam, ab45977), a monoclonal anti-p21 antibody (Sigma, p1484), a monoclonal Alkaline phosphatase antibody (Abcam, ab54778), or a polyclonal anti-actin antibody (Abcam, ab1801).

Myeloperoxidase activity.

HL-60 cells (~5 x 10^5) were treated with either 1 µM ATRA, 1 µM Cl-amidine, 1 µM F-amidine, or PBS. Cells were harvested after 24 and 48 h by centrifugation and washed twice with cold PBS. The cells were resuspended in Tris lysis buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM PMSF, and 1% Triton X-100) and lysed by 3 freeze-thaw cycles. The lysates were clarified by centrifugation (5,000 rpm, 5 min) and the supernatant was used for the activity assays: 2 µg of total protein was added to 13.3 mM Guaiacol, 16.6 mM sodium phosphate buffer pH 7.3 (3.5 parts Na_2HPO_4, 1 part NaH_2PO_4), and 0.27 mM H_2O_2 in a 96 well plate. The

absorbance was obtained at 470 nm every 10 s for 15 min. The rate of change in absorbance was determined and the specific activity of the sample was determined as previously described (*2*).

Primers for Quantitative Real Time PCR.

Gene specific primers included: p21 Forward 5'-CTGGAGACTCTCAGGGTCGAA -3' and p21 reverse 5'-GGCGTTTGGAGTGGTAGAAATC -3'; PAD4 forward 5-CGAAGACCCCCAAGG -3' and PAD4 reverse 5'-AGGACAGTTTGCCCCGTG -3'; GAPDH forward 5'-CGAGATCCCTCAAAATCAA-3' and GAPDH reverse 5'-TGTGGTCATGAGTCCTTCCA -3'. The use of these primers has previously been described (*3*, *4*).

Supplemental References.

1. Leatherbarrow, R. J. (2004) Grafit Ver 5.0, Erathicus Software, Staines, UK.
2. Yamada, M., Mori, M., and Sugimura, T. (1981) Purification and characterization of small molecular weight myeloperoxidase from human promyelocytic leukemia HL-60 cells, Biochemistry 20: 766-771.
3. Dong, S., Zhang, Z., and Takahara, H. (2007) Estrogen-enhanced peptidylarginine deiminase type IV gene (PADI4) expression in MCF-7 cells is mediated by estrogen receptor-alpha-promoted transfactors activator protein-1, nuclear factor-Y, and Sp1, Mol Endocrinol 21: 1617-1629.
4. Li, P., Yao, H., Zhang, Z., Li, M., Luo, Y., Thompson, P. R., Gilmour, D. S., and Wang, Y. (2008) Regulation of p53 target gene expression by peptidylarginine deiminase 4, Mol Cell Biol 28: 4745-4758.

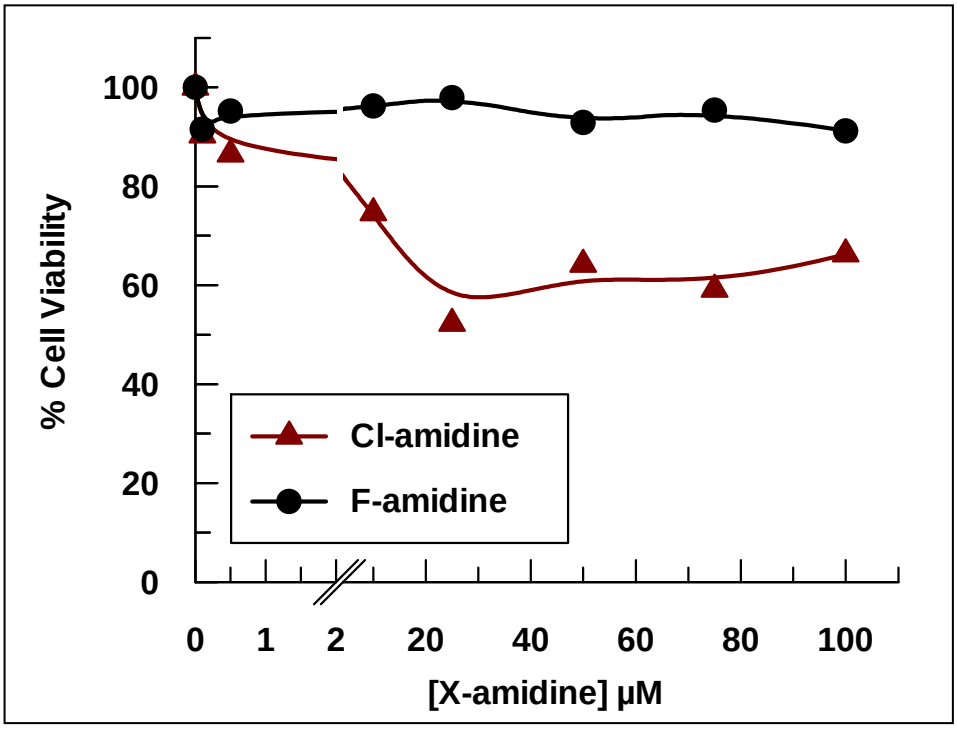

Figure S1. Cytotoxic effects of F- and Cl-amidine on HT-29 cells, a colon adenocarcinoma cell line. HT-29 cells were incubated with various concentrations of F- and Cl-amidine for 24 h, at which point cell viability was determined using a standard MTT assay. Each data point represents an average of three trials with a p value < 0.05.

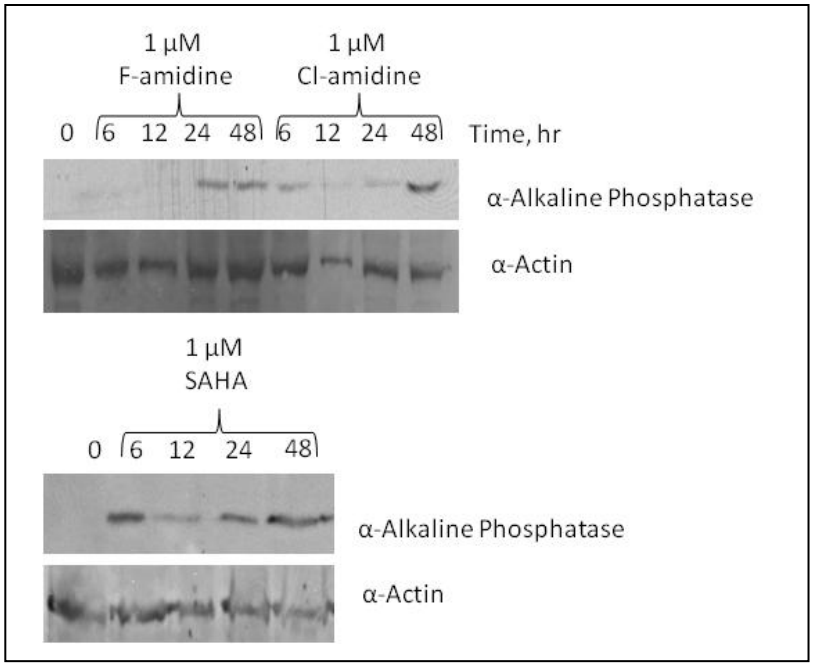

Figure S2. Protein levels of alkaline phosphatase in HT-29 cells after treatment with F-, Cl-amidine, or SAHA. SAHA served as a positive control for HT-29 differentiation.

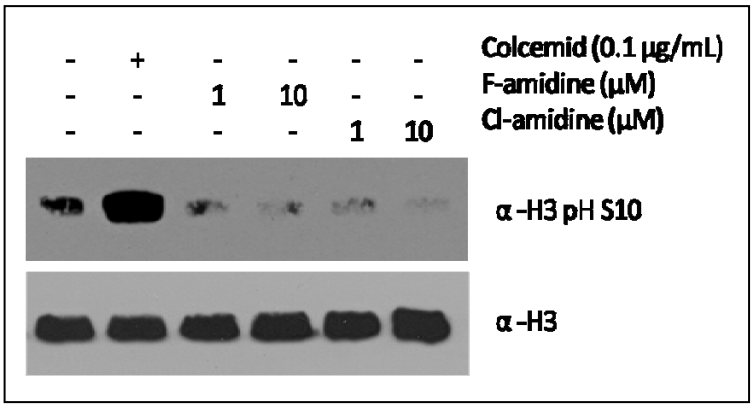

Figure S3. Protein levels of H3 phospho S10 and H3 in HL-60 cells after treatment with colcemid, F-, or Cl-amidine. Colcemid served as a positive control for mitotic cell arrest. The levels of histone H3 were measured as a loading control. The first lane corresponds to the PBS treated controls.