Carbonic anhydrase inhibitors (CAIs) are widely used in the clinic as diuretic and antiglaucoma (intratocular pressure-lowering) agents. However, new applications of isorm-selective CAIs have emerged, particularly in oncology. Identification of new chemotypes outside of the primary sulfonamide chemistry space (where the majority of known CAIs reside) can open new opportunities for designing such inhibitors. Herein, we report a serendipitous discovery of hitherto not reported class of CAIs via differential scanning fluorimetry (DSF; Thermal Shift Assay) screening of an unbiased, diverse set of compounds against bovine carbonic anhydrase IV. The compounds, all of which are a-aminonitriles attainable by the Strecker reaction, displayed modest (DTm = 1.0-1.8 °C) thermal shifts compared to the known and clinically used CAI acetazolamide (DTm = 5.0 °C). The compounds were further advanced to biochemical testing against a panel of human carbonic anhydrases (hCAI, hCAII, hCAIX, and hCAXIII) and showed submicromolar inhibition of certain isoforms with an apparent selectivity pattern. In this Communication, we provide preliminary insight into a possible inhibitory mechanism displayed by these compounds.

**Abstract**

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**Introduction**

CAIs are clinically used for decades as diuretics, antiglaucoma agents, antiepileptics, or more recently, antiseizure agents, whereas compounds targeting the tumor associated isoforms of CA IX and XII are in preclinical development as anticancer agents. Diagnostic tools for hypoxic tumors. Bovine CA was chosen as a model protein to validate our DSF high throughput screening platform due to the availability of the reference thermal shifts (acetazolamide) producing significant change in protein’s Tm.

**Methods**

**Screening set selection**

The compound screening set (~8000 compounds) was comprised of three distinct portions: 1) containing none of the known so-called privileged motifs (PMs); 2) containing only one PM per molecule and 3) smaller set with three PMs per molecule. While the results of this study will be disclosed elsewhere, herein we would like to report on the discovery of hitherto unknown class of CA inhibitors, namely, Strecker-type alpha-hommaments. Notably, these compounds were included in the ‘no-PM’ subset of the screening set and ultimately produced significant and dose-dependent thermal shift in bovine CA.

**Results**

CAIs are clinically used for decades as diuretics, antiglaucoma agents, antiepileptics, or more recently, antiseizure agents, whereas compounds targeting the tumor associated isoforms of CA IX and XII are in preclinical development as anticancer agents. Diagnostic tools for hypoxic tumors. Bovine CA was chosen as a model protein to validate our DSF high throughput screening platform due to the availability of the reference thermal shifts (acetazolamide) producing significant change in protein’s Tm.

**Thermal Shift Assay**

Primary screening on bovine CA was done in the presence of reporter dye Sypro Orange at 20 μM concentration of the tested compounds. Thermal scanning (40 to 85°C at 0.05°C/sec) with constant fluorescence read using 470/625nm filter set was performed in 96-well format using Applied Biosystems VICTOR™ 7 Real Time PCR System. All measurements were made in singletones. Compounds producing both positive and negative Tm shifts of >0.5°C upon derivative analysis of the melt curves were considered as hits and were reproped from dry powders at 3 concentrations (40, 20 and 10 μM) in quadruplicates.

**Applied Phytophytophys ot stopped-flow kinetics**

Functional activity of the discovered compounds was investigated by Dr. Claudia Supuran and his team at the University of Florence using carbonic dioxide hydration activity with phenol red as an indicator that works at the absorbance maximum of 557 nm. Phenol red has been used following the initial rates of the CA-catalyzed CO₂ hydration reaction for a period of 100-100 s. For each inhibitor at least six traces of the initial 5-10% of the reaction have been used for determining the initial velocity. The uncatalyzed rates were determined in the same manner and subtracted from the total observed rates. Four CA isoforms used were recombinant ones obtained in-house.

**Conclusion**

- A hitherto unknown class of carbonic anhydrase inhibitors has been identified using DSF method.
- The thermal shift was predictive of the actual biochemical inhibition of different isoforms of CA, as determined by the stopped-flow kinetics assay.
- The CA’s inhibition was not a result of adventitious cyanide that could be presented in minute quantities in Strecker-type products as demonstrated by the cyanide scavenging experiments with cobalmine.
- The compounds show micromolar inhibition of human CA I, II, IX and XII and some degree of isoform selectivity.