# Inhibition of $Ca^{2+}$/calmodulin mediated cAMP production via adenylyl cyclase type 1 for the treatment of chronic pain


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**Introduction:** Adenylyl cyclases (ACs) are effector enzymes downstream of various G protein-coupled receptors and ion channels that transduce signals via the catalysis of adenosine 5′-triphosphate (ATP) to 3′,5′-cyclic adenosine monophosphate (cAMP).[1] Modulation of ACs leads to a variety of physiological effects dependent on the AC isoform, interaction partners, and tissue localization. Group 1 ACs include AC1, AC3, and AC8 and are characterized by their positive modulation by $Ca^{2+}$/calmodulin (CaM).[2-4] AC1 and AC8 are primarily expressed in the central nervous system within regions such as, but not limited to, the hippocampus and the anterior cingulate cortex regions of the brain associated with learning, memory, and the development of chronic pain.[5-8] Evidence suggests that AC1 is responsible for propagation of inflammatory pain stimuli. (**Fig. 1**).[9] ACs also propagate signals downstream from the μ-opioid receptor (MOR), a well-characterized target for analgesic pharmacological therapy. MOR agonists act, in part, by negatively regulating AC1, thus reducing cAMP signal and dampening pain sensitization (**Fig. 1**).[10,11] AC1 knockout and AC1/8 double knockout mice exhibit nearly complete abrogation of behavioral pain response when treated with an inflammatory cocktail, complete Freund's adjuvant (CFA),[9] and display a lack of pain sensitization in a muscle pain model.[12] Thus, direct inhibition of AC1 could represent a novel therapeutic target to treat chronic pain.

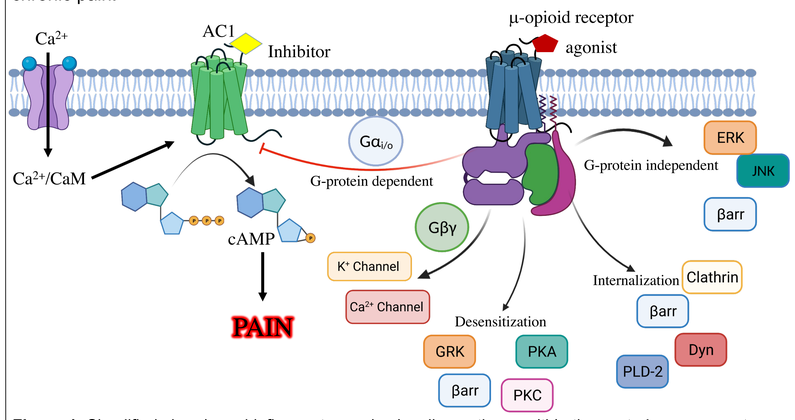



**Figure 1.** Simplified chronic and inflammatory pain signaling pathway within the central nervous system. Production of cAMP from AC1 leads to downstream signaling and the perception of pain with response to painful stimuli. Opioid agonist provide analgesic properties by binding to μ-opioid receptor, thereby releasing $G\alpha_{i/o}$ protein that negatively modulates AC1 and reduces pain sensitization.

**HTS Approach:** Our group has developed a cell-based platform to screen for, and evaluate, inhibitors of adenylyl cyclases. The endogenous AC isoforms 3 and 6 from HEK293 cells were knocked out using CRISPR/Cas9. These cells were then stably transfected with individual AC isoforms of interest, in this case AC1 of AC8[13] (**Fig. 2**). Using these cell lines we then quantified cAMP accumulation upon stimulation with calcium ionophore using homogenous time-resolved fluorescence (HTRF).

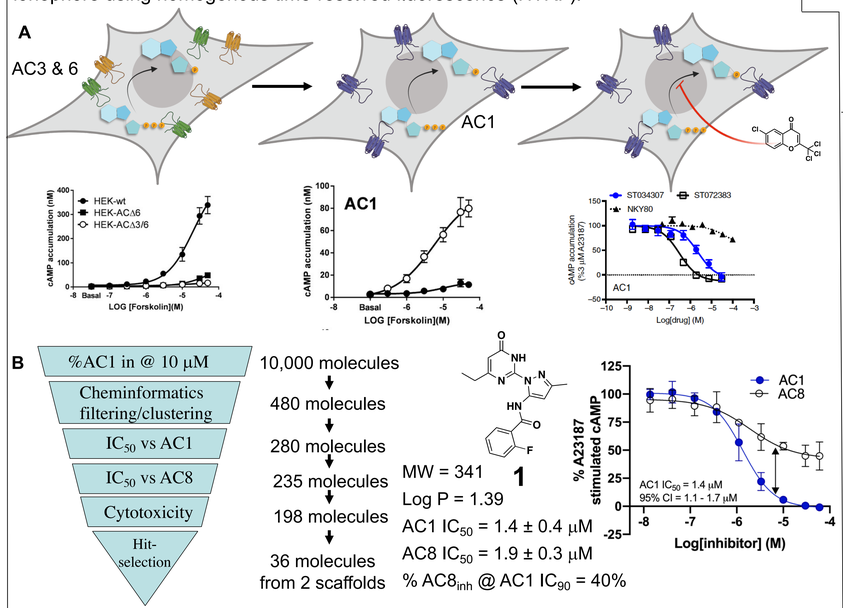



**Figure 2.** Cell-based model and high-throughput screening approach to identify inhibitors of AC1. (**A**) HEK293 cells have endogenous ACs knocked out followed by stable transfection of AC isoform of interest. These cells were then used to screen for inhibitors of AC1-mediated cAMP accumulation. (**B**) HTS funnel, hit molecule **1** and corresponding dose-response curves against AC1 and AC8

## Structure-Activity Relationship Studies

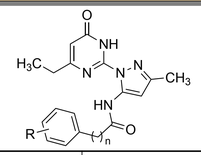


**Table 1. SAR for Pyrimidinone Series**

| Cpd | R | n | AC1 $IC_{50}$ (μM)[b] (95% C.I.) | AC8 $IC_{50}$ (μM)[b] (95% C.I.) | AC8 Inh[c] | Cpd | R | n | AC1 $IC_{50}$ (μM)[b] (95% C.I.) | AC8 $IC_{50}$ (μM)[b] (95% C.I.) | AC8 Inh[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 2-F | 0 | 1.4 (1.1-1.7) | 1.9 (ND) | 46% | 20 | 3-$C(CH_3)_3$ | 0 | 0.88 (0.63-1.25) | 0.38 (0.04-1.0) | 25% |
| 2 | H | 0 | 2.8 (1.9-4.6) | 35.9 (ND) | 0% | 21 | 4-$C(CH_3)_3$ | 0 | 0.97 (0.64-1.54) | 1.2 (0.44-4.2) | 35% |
| 3 | methyl[a] | 0 | > 30 | 10.6 (ND) | ND | **22** | **3-phenyl** | **0** | **0.25 (0.20-0.32)** | **0.57 (0.29-1.1)** | **37%** |
| 4 | 2-furan[a] | 0 | 6.7 (4.6-19.0) | 15.7 (ND) | 20% | 23 | 4-phenyl | 0 | 0.77 (0.53-1.12) | 0.70 (ND) | 32% |
| 5 | 2-pyridine[a] | 0 | 1.2 (1.0-1.5) | 2.8 (ND) | 46% | 24 | 3-$OCH_3$ | 0 | 1.42 (0.92-2.25) | 9.6 (ND) | 5% |
| 6 | 3-F | 0 | 1.4 (1.1-1.7) | 1.6 (0.88-3.7) | 55% | 25 | 4-$OCH_3$ | 0 | 0.90 (0.74-1.12) | > 60 | 0% |
| 7 | 4-F | 0 | 0.79 (0.63-0.99) | 0.78 (0.32-2.1) | 52% | 26 | 3-$SCH_3$ | 0 | 0.63 (0.40-0.94) | 1.3 (0.49-21.7) | 52% |
| 8 | 3-Cl | 0 | 0.47 (0.37-0.61) | 1.0 (ND) | 48% | 27 | 3-$OCH_2CH_3$ | 0 | 0.90 (0.73-1.1) | 4.4 (ND) | 11% |
| 9 | 4-Cl | 0 | 0.36 (ND) | 2.5 (1.1-12.4) | 53% | 28 | 4-$OCH_2OCH_3$ | 0 | 4.3 (3.0-6.6) | > 30 | ND |
| 10 | 3-Br | 0 | 0.61 (0.46-0.82) | 2.1 (ND) | 15% | 29 | 4-OH | 0 | > 60 | > 60 | ND |
| 11 | 2-$CH_3$ | 0 | 2.2 (1.6-2.9) | 15.7 (ND) | 11% | 30 | 4-$N(CH_3)_2$ | 0 | 2.3 (1.2-6.1) | 1.0 (ND) | 39% |
| 12 | **3-$CH_3$** | **0** | **0.41 (0.23-0.66)** | **2.7 (ND)** | **27%** | 31 | 2-F | 1 | 16.3 (ND) | 3.4 (ND) | ND |
| 13 | 4-$CH_3$ | 0 | 0.67 (0.44-0.92) | > 60 | 27% | 32 | 3-$CH_3$ | 1 | 9.2 (ND) | > 60 | 59% |
| 14 | 3-$CF_3$ | 0 | 0.68 (0.46-0.97) | 1.3 (ND) | 41% | 33 | **2-F,3-$CH_3$** | **0** | **0.57 (0.35-0.96)** | **> 60** | **9%** |
| 15 | 2-$CH_2CH_3$ | 0 | 3.1 (2.6-3.7) | > 60 | 21% | 34 | **2-F,5-$CH_3$** | **0** | **0.54 (0.30-0.84)** | **3.4 (ND)** | **17%** |
| 16 | **3-$CH_2CH_3$** | **0** | **0.44 (0.24-0.69)** | **3.5 (ND)** | **12%** | 35 | **3,4-di-$CH_3$** | **0** | **0.29 (0.20-0.43)** | **0.71 (ND)** | **27%** |
| 17 | **4-$CH_2CH_3$** | **0** | **0.39 (0.10-0.65)** | **6.6 (ND)** | **19%** | 36 | **3,5-di-$CH_3$** | **0** | **0.52 (0.32-0.82)** | **1.7 (ND)** | **27%** |
| 18 | **3-$CH(CH_3)_2$** | **0** | **0.36 (0.24-0.53)** | **2.6 (ND)** | **28%** | | | | | | |
| 19 | 3-cyclopropyl | 0 | 0.81 (0.67-0.97) | 1.9 (0.87-19.7) | 17% | | | | | | |

[a] Indicated groups were benzene ring replacements. [b] AC $IC_{50}$s calculated from concentration-response curves with inhibitor versus 3 μM A23187-stimulated cAMP accumulation in HEK AC1 or AC8 3/6 KO cell lines (n=3+). The 95% confidence interval for each analog provided in parentheses. [c] The % inhibition of AC8 was calculated by subtracting the % activity of AC8 at the AC1 $IC_{90}$ concentration from 100% (n=2+). All tabular AC1 $IC_{90}$ data is provided in Table S1. ND = not determined by Prism software due to wide variance or not enough data points at higher concentrations.

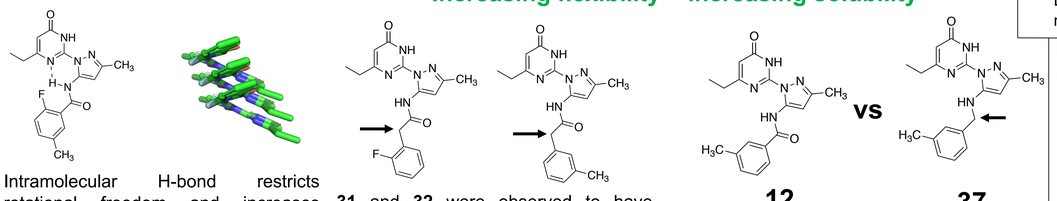
**Table 2. Predicted and experimentally determined physicochemical and pharmacokinetic properties**

| Cpd | MW (g/mol) | QPLogPo/w[a] | PSA (Å²) | CNS[b] | QPPMDCK (nm/sec)[c] | Solubility (μM)[d] | $P_{app}$ (nm/sec)[e] |
|---|---|---|---|---|---|---|---|
| **Desired Ranges** | < 400 | 2 - 5 | < 90 | 0 – 2 | > 500 | 10x AC1 $IC_{50}$ | - |
| **12** | 337 | 2.32 | 100.5 | -2 | 141 | 19.5 | nt |
| **16** | 351 | 2.65 | 100.5 | -2 | 141 | 10.8 | nt |
| **34** | 355 | 2.88 | 101.4 | 0 | 632 | 33.6 | 22.1 |
| **35** | 351 | 2.57 | 100.5 | -2 | 140 | 7.6 | nt |

[a] QikProp metric for predicted octanol/water partition coefficient. [b] QikProp multiparameter-based predictive metric for molecules CNS activity. Ranges from -2 (inactive) to +2 (active). [C] QikProp predictive metric for apparent MDCK cell permeability. [d] Thermodynamic solubility in PBS at pH 7.4 and 25 °C determined according to previously described protocol. [e] Experimental apparent MDCK permeability.

## Is solubility limiting the efficacy *in vivo*?

**Increasing flexibility = increasing solubility**



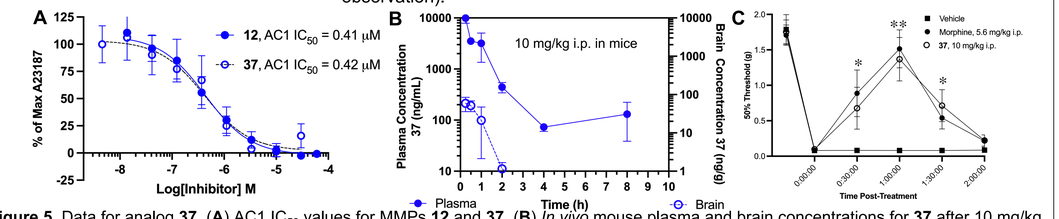




**Figure 5.** Data for analog **37**. (**A**) AC1 $IC_{50}$ values for MMPs **12** and **37**. (**B**) *In vivo* mouse plasma and brain concentrations for **37** after 10 mg/kg dose i.p. (**C**) CFA mechanical allodynia model. Morphine (positive control) and **37** were assessed for anti-allodynia properties using von Frey filaments. Values represent mean ± SEM of the 50% mechanical threshold ($n$ = 7). Molecules dosed via i.p. route. Statistical analysis performed using nonparametric one-way ANOVA (Friedman test), Dunn's post-hoc correction. * $p < 0.05$, ** $p < 0.001$ all vs. vehicle at each time point.

## AC Isoform selectivity

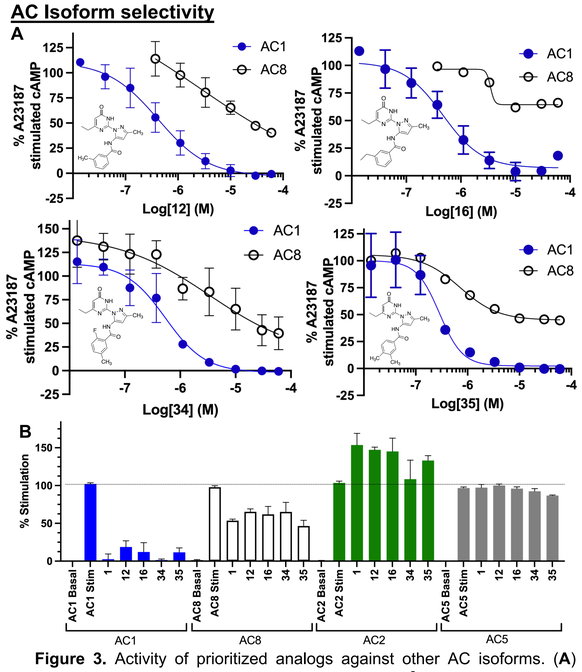



**Figure 3.** Activity of prioritized analogs against other AC isoforms. (**A**) Dose-response curves for analogs against the $Ca^{2+}$/CaM regulated AC1 and AC8. (**B**) Inhibition of representative AC isoforms from Group 1 (AC1 and AC8; $Ca^{2+}$/CaM regulated), Group 2 (AC2, conditionally activated by $G_{\beta\gamma}$ subunits), and Group 3 (AC5, negatively modulated by $Ca^{2+}$)

## *In vivo* mechanical allodynia model

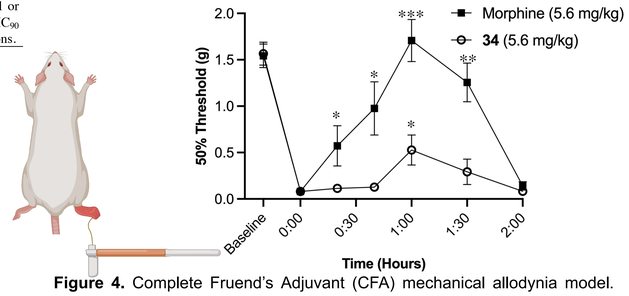



**Figure 4.** Complete Fruend's Adjuvant (CFA) mechanical allodynia model. Morphine (positive control) and **34** were assessed for anti-allodynia properties using von Frey filaments. Values represent mean ± SEM of the 50% mechanical threshold ($n$ = 6). Molecules dosed via i.p. route. Statistical analysis performed using nonparametric one-way ANOVA (Friedman test), Dunn's post-hoc correction. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.001$ all vs. 0 min time-point.

**Conclusion:** AC1 is a genetically validated therapeutic target to treat chronic pain conditions. A HTS in a cell-based model identified the pyrimidinone scaffold as selective and potent inhibitors of $Ca^{2+}$/CaM stimulated cAMP production from AC1. SAR optimization yielded the current lead **37** possessing mid-nanomolar cellular AC1 potency, selectivity over AC8, ability to traverse the BBB and provide anti-allodynia efficacy in a CFA mouse model comparable to morphine. The data suggest pharmacologic inhibition of AC1 is a viable treatment strategy for allodynia, chronic and inflammatory pain that bypasses the MOR and side effects associated with opioid therapies.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NIH National Institute of Neurological Disorders and Stroke
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Poster & References

Paper on this scaffold in the *Journal of Medicinal Chemistry*