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Identification of a selective small molecule inhibitor of type 1 adenylyl cyclase activity with analgesic properties

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Abstract

Adenylyl cyclase 1 (AC1) belongs to a group of adenylyl cyclases (ACs) that are stimulated by calcium in a calmodulin-dependent manner. Studies with AC1 knockout mice suggest that inhibitors of AC1 may be useful for treating pain and opioid dependence. However, non-selective inhibition of AC isoforms could result in substantial adverse effects. We used chemical library screening to identify a selective AC1 inhibitor with a chromone core structure that may represent a new analgesic agent. After demonstrating that the compound (ST034307) inhibited Ca²⁺-stimulated adenosine 5',3'-monophosphate (cAMP) accumulation in HEK cells stably transfected with AC1 (HEK-AC1 cells), we confirmed selectivity for AC1 by testing against all isoforms of membrane-bound ACs. ST034307 also inhibited AC1 activity stimulated by forskolin- and G_{αs}-coupled receptors in HEK-AC1 cells and showed inhibitory activity in multiple AC1-containing membrane preparations and mouse hippocampal homogenates. ST034307 enhanced μ -opioid receptor (MOR)-mediated inhibition of AC1 in short-term inhibition assays in HEK-AC1 cells stably transfected with MOR; however, the compound blocked heterologous sensitization of AC1 caused by chronic MOR activation in those cells. ST034307 reduced pain responses in a mouse model of inflammatory pain. Our data indicated that ST034307 is a selective small molecule inhibitor of AC1 and suggest that selective AC1 inhibitors may be useful for managing pain.

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Introduction

Adenylyl cyclases (ACs) are integrators of signaling through G protein-coupled receptors (GPCRs). ACs catalyze the production of cAMP (cyclic adenosine monophosphate) from ATP (adenosine triphosphate) (1). GPCRs coupled to inhibitory G proteins ($G_{i/o}$) generally inhibit ACs, whereas GPCRs coupled to stimulatory G proteins (G_{α_s}) activate ACs. Moreover, due to their regulatory properties, AC isoforms can also be stimulated or inhibited by G_{α_q} -coupled receptors and ion channels (1, 2). There are nine isoforms of membrane-bound ACs in humans, each of which exhibits different regulatory properties and expression patterns (3). All membrane-bound ACs are activated by G_{α_s} , however, based on their additional regulatory properties these ACs can be divided into four different groups (1, 3). Group 1 ACs are AC1, AC3, and AC8, which can be activated by calcium/calmodulin; group 2 ACs are AC2, AC4, and AC7, which are conditionally activated by $G\beta\gamma$ subunits; group 3 ACs are AC5 and AC6, which are inhibited by calcium; and the group 4 AC is AC9, which is relatively insensitive to the small molecule AC-activator forskolin (1–3).

The different regulatory properties and tissue distribution of the membrane-bound ACs suggest that each isoform may serve distinct purposes. The transcripts for the calcium-stimulated ACs, AC1 and AC8, are highly expressed in the hippocampus, which is a brain region associated with learning and memory (3, 4). Behavioral assays with mice lacking both AC1 and AC8 revealed impaired long-term memory in both passive avoidance and contextual learning assays, whereas mice lacking only AC1 or AC8 displayed wild-type-like behaviors (5). However, AC1 knockout mice lose remote contextual memories faster than wild-type mice (6). These studies suggest that AC1 and AC8 have redundant and specific roles in memory.

Other studies with mice lacking AC1 suggest that inhibitors of AC1 may be useful for treating neuropathic and inflammatory pain and opioid dependence (7–9). Besides the hippocampus, mRNA for AC1 is also expressed in regions of the central nervous system (CNS) associated with pain and nociception, such as the anterior cingulate cortex (ACC) and dorsal horn neurons of the spinal cord (10–12). The development of chronic pain may share some cellular mechanistic features with memory formation and maintenance, that is, strengthening of synapses through LTP (long-term potentiation) (4, 10). Indeed, the behavioral responses of AC1 knockout mice to inflammatory and neuropathic pain are largely inhibited compared to wild-type animals (8, 10).

Consistent with those animal studies, the small molecule inhibitor of AC1 activity NB001 has analgesic properties in both inflammatory and neuropathic rodent models of pain (8, 13). NB001 also inhibited LTP in neurons from the ACC and spinal cord, but not the hippocampus (13). Despite the promising effects of NB001, it has a few potential drawbacks including limited selectivity for AC1 versus AC8 and that the actions of NB001 appear to be mediated by indirect inhibition of AC (13, 14). The present study used a screening platform to identify a new selective AC1 inhibitor from a commercially-available natural products-derived small molecule library. The compound identified (ST034307), a chromone derivative, was highly selective for AC1 inhibition when assayed against other AC isoforms. We also investigated the effects of ST034307 on signaling events mediated by the μ -opioid

receptor (MOR), including AC inhibition, β -arrestin recruitment, and heterologous sensitization of AC. Moreover, we found that ST034307 exerted analgesic effects in a mouse model of inflammatory pain. In summary, we have identified and characterized a potent AC1 inhibitor that has activity in a mouse model of inflammatory pain.

Results

Screening of the NDL-3000 Natural Derivatives library

To find new AC1 inhibitors, we developed a screening platform based on a previous study from our group that identified small molecule inhibitors of AC2 (15). We also identified W400 as a non-selective AC1 inhibitor from the AC2 inhibitor screen (Fig. 1). W400 served as a positive control for inhibition in our screening platform, producing ~10-fold reduction in cAMP accumulation in HEK cells stably expressing AC1 (HEK-AC1) when stimulated with the calcium ionophore A23187 and the AC activator forskolin. A23187 is a calcium ionophore that activates the overexpressed AC1 in this system (15). We assessed the robustness of our screening platform by conducting a Z' factor analysis using 3 μ M A23187 + 30 nM forskolin as the maximal response and 30 μ M W400 (in the presence of 3 μ M A23187 + 30 nM forskolin) as the minimum response (16). Our screening resulted in a Z' factor equal to 0.55 ± 0.02 ($n = 10$), confirming that our platform was amenable to drug library screening (17).

We used the NDL-3000 Natural Derivatives library (TimTec), which is composed of 3040 natural or natural product derivative compounds with diverse chemical structures. Because forskolin, the most widely used compound to interact with ACs, is a natural compound (18), we anticipated that the NDL-3000 Natural Derivatives library would contain other regulators of AC activity. We screened the library for compounds that inhibit 3 μ M A23187 + 30 nM forskolin-stimulated cAMP accumulation in HEK-AC1 cells. With the screen, we identified ST034307 and ST072383 (Fig. 1) as inhibitors of AC1. In an assay for cell toxicity, neither compound reduced viability below ~85% compared with vehicle-treated cells (Table 1).

Selectivity of test compounds against closely related AC1 and AC8 isoforms

We conducted dose-response curves to determine the potencies and maximal effects of the compounds for inhibiting A23187-stimulated cAMP accumulation in HEK cells stably expressing either AC1 or AC8 (Fig. 2A, 2B, and Table 1). ST072383 was the more potent of the two inhibitors with an IC_{50} value of 0.35 μ M, however, ST072383 also inhibited AC8 (Fig. 2B). Unlike ST072383, ST034307 revealed selective inhibition of AC1. Indeed, ST034307 actually potentiated AC8 activity to a non-significant small extent. We also tested the previously reported AC1 inhibitor NB001 (Fig. 1) in our cell-based AC1 assay and failed to observe any significant inhibition at concentrations up to 30 μ M (fig. S1). We further tested ST072383 and ST034307 for AC1 inhibition using a second method to measure cAMP accumulation, which produced similar results (table S1).

Selectivity of test compounds against additional AC isoforms

We tested the selectivity of ST034307 and ST072383 using HEK cells transiently transfected with each of the nine different isoforms of membrane-bound ACs (Fig. 2C and

2D). Stimulation of the AC-transfected cells caused a significant increase in cAMP accumulation when compared to the same stimulation paradigms in the venus-transfected control cells (Fig. 2C). These results indicated that each treatment paradigm provided selective stimulation of the AC isoform that was transfected. ST034307 was selective for inhibition of AC1 in comparison to all other isoforms of membrane-bound ACs (Fig. 2D). We also found that ST034307 potentiated phorbol 12-myristate 13-acetate (PMA)-stimulated cAMP production by AC2 (Fig. 2D). A small potentiation of forskolin-stimulated AC5 and AC6 was also observed. In contrast, ST072383 inhibited all ACs, except for AC5 under the conditions tested. The P-site inhibitor NKY80 significantly inhibited the activity of AC1, AC2, AC3, AC4, AC5, AC6, and AC7, as well as the endogenous ACs in the venus-transfected cells.

Mechanistic insights on ST034307

We explored the AC inhibition by ST034307 in various cellular and in vitro contexts. The first set of assays conducted included inhibition of AC1 activation by forskolin and G_{α_s} -coupled receptors. To induce stimulation of AC1 through G_{α_s} -coupled receptors, we activated the endogenous β -adrenergic receptors in HEK cells with isoproterenol. ST034307 significantly inhibited the forskolin- or the isoproterenol-stimulated AC1 activity in HEK cells stably expressing AC1 (Fig. 3A). In contrast, ST034307 had no significant effects in the wild-type HEK cells (Fig. 3A). Although wild-type HEK cells endogenously express mRNA for AC1, no Ca^{2+} -stimulated cAMP response is observed (19, 20). Thus, the G_{α_s} - or forskolin-stimulated cAMP in the wild-type cells reflected the activation of the other endogenous AC isoforms that include AC3, AC5, AC6, AC7, and AC9 (20). These observations further support that the actions of ST034307 involve signaling through AC1, rather than indirect interference with receptor-mediated signaling events upstream of AC. To confirm that ST034307 was not inhibiting AC1 through effects on $G\beta\gamma$ or $G_{\alpha_{i/o}}$, we tested the inhibition of AC1 activity in HEK-AC1 cells also expressing β ark-CT, which is the C-terminal portion of GRK2 (G protein-coupled receptor kinase 2) and sequesters $G\beta\gamma$ (21), or treated with pertussis toxin to block $G_{\alpha_{i/o}}$ signaling. Neither sequestering $G\beta\gamma$ subunits with β ark-CT nor inactivating $G_{\alpha_{i/o}}$ signaling with pertussis toxin affected ST034307-mediated AC1 inhibition (fig. S2). Together, these observations further support that ST034307 inhibited AC1 directly.

To confirm that the inhibitor was not interfering with a downstream process and required membrane-bound proteins for activity, we also performed in vitro assays for AC1 inhibition using cellular membranes isolated from HEK-AC1 cells. We stimulated AC1 activity in membranes with forskolin or purified calmodulin and used the nonselective AC inhibitor, NKY80 (Fig. 1), as a control (14, 15). ST034307 modestly, but significantly, inhibited both the calmodulin- and the forskolin-stimulated AC1 activity in the membranes (Fig. 3B). Further support for AC as the direct target of ST034307 was provided by testing for inhibition of AC activity in membranes from Sf9 insect cells expressing recombinant AC1, AC2, or AC5 (14, 15). As in the cell-based assays, ST034307 inhibited AC1 activity (~30%), suggesting a direct mode of inhibition (Fig. 3C). As we observed for the HEK cells, ST034307 also potentiated AC2. The compound was inactive at AC5 even at 100 μ M.

P-site inhibitors (a class of non-competitive and uncompetitive AC inhibitors) display higher inhibitory activity when the AC is activated (the higher the activity of the AC, the more robust the AC inhibition by P-site inhibitors) (3, 22, 23). Therefore, we measured inhibition of AC1 activity by ST034307 in response to increasing concentrations of AC1 activators, A23187 and forskolin (fig. S3). Increases in the concentrations of A23187 resulted in an increase in the efficacy of inhibition of AC1 by the P-site inhibitor NKY80 (Fig. 4A). In contrast, as the concentration of AC1 activators increased, there was no change (A23187) or a decrease (forskolin) in the efficacy of inhibition by ST034307 (Fig. 4), suggesting that ST034307 is not a P-site inhibitor.

We assessed the activity of ST034307 in a more physiologically relevant system, the hippocampus. We stimulated hippocampal homogenates prepared from C57BL/6 mice with purified calmodulin in the presence of 10 μ M free Ca^{2+} . The addition of Ca^{2+} /calmodulin caused a significant increase in cAMP accumulation in these neuronal homogenates, supporting the presence of calcium-stimulated ACs (Fig. 5A). ST034307 significantly inhibited the Ca^{2+} /calmodulin-stimulated cAMP accumulation in the hippocampal homogenates (Fig. 5B). The presence of AC1, AC8, and other AC isoforms in the hippocampus (3, 4), likely explains the more robust inhibition of Ca^{2+} /calmodulin-stimulated cAMP accumulation by the non-selective AC inhibitor, NKY80 (14) compared to inhibition by the AC1-selective inhibitor ST034307.

Effects of ST034307 on μ -opioid receptor signaling

Inhibition of ACs may play an important role in the analgesic effects of agonists of the MOR, which is a $\text{G}\alpha_{i/o}$ -coupled receptor that is present with AC1 in areas of the nervous system that are linked to pain and nociception (the dorsal horn of the spinal cord and ACC) (10–12, 24–27). Thus, we investigated the effects of ST034307 on MOR signaling in HEK-AC1 and Chinese hamster ovary (CHO) cells stably expressing MOR. Upon agonist binding, MOR activates G proteins, which inhibit ACs (including AC1), and also recruits β -arrestins, which can lead to additional signaling events or signal termination (28). We exposed HEK-AC1/MOR cells to the MOR agonist DAMGO, ST034307, or both compounds for 1 h. Exposure to ST034307 enhanced MOR-mediated inhibition of AC1 in HEK-AC1/MOR cells (Fig. 6A). To assess potential effects of ST034307 on β -arrestin recruitment to MOR, we used a commercial assay platform from DiscoverX that is available in genetically engineered CHO cells expressing recombinant MOR and β -arrestin 2 fused to enzyme complementation partners enabling the detection of interactions between receptor and arrestin. Exposing cells to ST034307 for 30 min before DAMGO or vehicle addition had no effects on recruitment of β -arrestin to MOR in the CHO-MOR β -arrestin cells (Fig. 6B).

Prolonged activation of the MOR leads to a cellular adaptive response termed heterologous sensitization, which results in superactivation of AC and has been linked to opioid dependence (29–33). A 2-h exposure to DAMGO produced a 250% enhancement of A23187-stimulated cAMP accumulation in HEK-AC1/MOR cells when compared to vehicle-treated cells (Fig. 6C). We examined the effects of ST034307 on MOR-mediated heterologous sensitization of AC1 in two different paradigms. To determine the effects of ST034307 on the development of heterologous sensitization, we added ST034307 before the

initial activation of the MOR (Fig. 6D). To study the effects of ST034307 on the maintenance of heterologous sensitization, we added ST034307 after persistent activation of the MOR (Fig. 6D). ST034307 dose-dependently inhibited both the development and the maintenance of MOR-mediated sensitization of AC1 (Fig. 6D).

Analgesic properties of ST034307 in mice

Although we did not test for an effect on ACC neurons or spinal cord neurons in culture, we assayed if ST034307 has analgesic properties using a mouse model of inflammatory pain. We injected complete Freund's adjuvant (CFA) into the hind paw of mice to induce inflammation and measured pain as hypersensitivity to touch by Von Frey filaments (24, 34). Intrathecal injections (into the spinal canal) with ST034307 at doses as low as 0.25 μ g caused a significant relief of CFA-induced inflammatory pain, as indicated by the increase in the amount of pressure the mouse tolerated before removing the paw (Fig. 7). As a control pain reliever, we administered DAMGO at 50 ng per mouse (34), which provided a similar reduction in the hypersensitivity response (Fig. 7A). Limited dose-response experiments with ST034307 in this mouse pain model suggested an estimated ED₅₀ value for analgesia of 0.28 μ g (Fig. 7B).

To show that an increase in cAMP contributed to the CFA-induced hypersensitive, we used the nonselective AC activator forskolin. Because forskolin activates all isoforms of AC (except for AC9), we anticipated that forskolin treatment would increase cAMP accumulation at the site of action of ST034307 and, therefore, prevent or impair its analgesic actions. Indeed, co-injection of forskolin with ST034307 completely inhibited the analgesic response caused by ST034307 in our inflammatory pain model (Fig. 7C).

Discussion

Several studies have uncovered the unique physiological functions of AC1, as well as informed on the clinical potential of compounds targeting this protein (7–9, 13, 35, 36). Remarkably, in mice lacking AC1 acute pain responses such as mechanical pressure and noxious heating, were unchanged, whereas behaviors associated with chronic and inflammatory pain were largely inhibited (36). Inspired by these studies, Wang and colleagues identified NB001 as a small molecule that inhibits AC1 activity with approximately 10-fold selectivity over AC8 (13).

To identify additional selective inhibitors, we designed and performed a chemical library screen to identify potential AC1 inhibitors. Our efforts resulted in the discovery of a potent and selective AC1 inhibitor (ST034307 – IC₅₀ = 2.3 μ M, 95% CI = 1.2 to 4.5 μ M). ST034307 is a chromone-derived small molecule with central nervous system drug-like physicochemical properties (37): molecular weight = 298; CLogP = 4.07; TPSA = 26; 4 hydrogen bond acceptors and 0 hydrogen bond donors. Multiple types of models (HEK cells expressing AC1–AC9, HEK-AC1, and Sf9-AC1 cell membranes, and mouse hippocampal homogenates) and stimulation paradigms (A23187, calmodulin, forskolin, G α_s , and G α_s -coupled receptor) were employed to confirm the activity and selectivity of ST034307. We also examined the effects of ST034307 on acute and chronic MOR signaling in

heterologously expressing cells, as well as confirmed its ability to induce analgesia in a mouse inflammatory pain model.

The molecular mechanisms involved in the analgesic properties of AC1 inhibition have been associated with the requirement of cAMP for synaptic plastic changes like long-term potentiation (LTP) (4, 10, 13). The development of chronic pain has been linked to strengthening of synapses through LTP in the ACC as well as spinal cord (4, 10). It has been hypothesized that NMDA (N-methyl-D-aspartate) receptor-mediated Ca^{2+} influx in neurons from these regions of the nervous system causes AC1 activation, which provides the cAMP required for LTP (10). NB001-mediated inhibition of LTP in the ACC and spinal cord, but not hippocampus, is consistent with this hypothesis and also with the AC1 knockout models that point to analgesia without notable memory impairments (5, 6, 8, 9, 11, 13)

Of concern for any AC1 inhibitor is specificity, because double knockout mice lacking both AC1 and AC8 present behaviors consistent with severe memory impairments (5, 10). Therefore, one main requirement for an AC1 inhibitor to use in the treatment of pain is selectivity for AC1 versus AC8. ST034307 did not display any inhibition of AC8 at doses up to 30 μM revealing remarkable selectivity for inhibition of AC1 versus AC8. Further, ST034307 did not inhibit any of the other isoforms of AC. The robust potentiation of PMA-stimulated AC2 activity and the modest potentiation of AC5 and AC6 were somewhat surprising and may reflect a conserved binding site, such as the forskolin binding site at which forskolin analogs can have either stimulatory or inhibitory activity at individual AC isoforms (38). Because there are no genetic knockout rodent models for AC2, it is difficult to predict the physiological consequences of potentiating PMA-mediated AC2 activity (15, 39). PMA activates AC2 through protein kinase C (PKC) and AC2 transcripts are expressed in skeletal muscle, for which increased cAMP is linked to muscle hypertrophy, repair, and regeneration (15, 40, 41). Efforts to identify ST034307 analogs with greater overall selectivity are currently underway. The activity of ST034307 was not associated with $\text{G}\beta\gamma$ subunits signaling, because sequestering $\text{G}\beta\gamma$ subunits with $\beta\text{ark-CT}$ failed to alter its effects on AC1. Similarly, $\text{G}\alpha_{i/o}$ activity was ruled out, because ST034307-mediated AC1 inhibition was not altered by overnight pertussis toxin pre-treatment. These observations and the selectivity of AC1 inhibition versus AC2-AC9 suggested that the actions are unique to AC1 and did not involve a more general inhibition of AC activity through G protein subunits or through modulation of cAMP accumulation or degradation.

One important caveat with the previously described AC1 inhibitor, NB001, is that the compound does not inhibit AC1 in in vitro assays performed with cellular membranes (14). Those studies indicated that NB001 is not a direct AC1 inhibitor and may act through a distinct mechanism to inhibit AC1 activity in intact cells (14). The present study revealed that, unlike NB001, ST034307 had modest, but significant, inhibitory effect in studies using cellular membranes from HEK or Sf9 cells expressing AC1. ST034307 showed a similar degree of inhibition ($\sim 30\%$) to forskolin-, $\text{G}\alpha_s$ -, and calmodulin-stimulated AC1 activity. These observations suggested that ST034307 acts, in part, through a mechanism that targets membrane-associated AC1 activity, which is consistent with a direct interaction of ST034307 with AC1. Subsequent cell-based assays employing increasing concentrations of AC1 activators suggested that ST034307 is not a P-site inhibitor and that it apparently acts

through a distinct mechanism to inhibit AC1, which may partly explain the high selectivity of the compound.

MOR, which has long been targeted for analgesia and antinociception, is also present in the dorsal horn of the spinal cord and ACC (10–12, 24–27). Similar to numerous GPCRs, there are several different signaling pathways activated by MOR (42, 43). MORs couple to $G\alpha_{i/o}$ to inhibit ACs and can also lead to signaling events through $G\beta\gamma$ subunits, as well as through β -arrestins (42, 44, 45). Biased agonists selectively activate specific signaling pathways downstream of a GPCR (46–49). Notably, several studies have suggested that activation of G proteins by the MOR is linked to the therapeutic analgesia of opioids, whereas activation of β -arrestins is associated with many of the undesired side effects of these drugs, including tolerance, constipation, and respiratory depression (29, 50–52). Even though $G\beta\gamma$ -mediated activation of GIRK (G protein-coupled inwardly-rectifying potassium) channels has been associated with MOR-induced antinociception, it is intriguing and encouraging that both $G\alpha_{i/o}$ and $G\beta\gamma$ subunits can inhibit AC1 (1, 44). Here, we hypothesized that a type of functional selectivity (or pathway specificity) could be achieved by inhibiting AC1 to mimic the outcome of G protein activation by MOR. As hypothesized, ST034307 and DAMGO displayed additive effects for inhibition of AC1 when combined at submaximal concentrations. Moreover, β -arrestin recruitment to MOR was similar in the presence or absence of ST034307. These data suggested that ST034307 mimics (at least in part) the effects of $G\alpha_{i/o}$ protein activation by MOR.

Activation of G proteins by MOR is also linked to opioid dependence (24, 29, 31). Chronic activation of the MOR leads to a cellular adaptive response termed heterologous sensitization (32). Heterologous sensitization is linked to opioid dependence and is characterized by a marked increase in AC activity (29, 31–33). AC1 knockout mice lack many of the behaviors associated with morphine dependence (7, 9). That ST034307 inhibited both the development and the maintenance of MOR-mediated heterologous sensitization of AC1 suggested that AC1 inhibitors can prevent and suppress this adaptive response to chronic activation of the MOR that has been linked to opioid dependence. This idea is consistent with studies examining the effects of NB001 on naltrexone-mediated heterologous sensitization and hyperalgesia (24).

The promising *in vitro* profile of ST034307 led us to explore the activity of ST034307 in an *in vivo* assay to investigate its potential analgesic properties. Previous studies with NB001 suggested that AC1 inhibitors are efficacious in inflammatory pain models (13). Thus, we used a mouse inflammatory pain model in which intraplantar injection of CFA to the mouse's hindpaw induces an intense inflammatory reaction. Similar to DAMGO, intrathecal injections of ST034307 caused significant relief of CFA-mediated inflammatory pain. The response was dose-dependent and returned the mechanical threshold to baseline levels. We carried out this same experimental paradigm in the presence of the non-selective AC activator forskolin. We hypothesized that the addition of forskolin would activate other AC isoforms that are present with AC1 at the site of action of ST034307 and cause an increase in cAMP. This increase in cAMP should overwhelm the AC1 inhibition caused by ST034307 and prevent its analgesic activity. As expected, forskolin prevented ST034307-mediated relief of inflammatory pain, suggesting that the analgesia observed was due to

inhibition of cAMP accumulation by AC1. These results provide additional support indicating that inhibitors of AC1 may be useful for treating inflammatory pain and potentially chronic pain, either as independent agents or in combination with opioids. When used in combination with opioids, it may be possible to reduce the amount of opioid required to achieve pain relief and prevent cellular changes associated with opioid dependence and thus extend the usefulness of this class of analgesic agents.

Although previous studies suggest that selective inhibition of AC1 would not cause serious memory impairments (5, 6, 13), additional behavioral assays to examine the effects of small molecule inhibitors of AC1 on learning and memory should be conducted. The potential of AC1 inhibitors for treating opioid dependence is another interesting avenue that could be explored. Our discovery of a selective AC1 inhibitor (ST034307) should fuel and provide part of the necessary tools for such efforts. The studies conducted herein corroborate the utility of ACs as potential drug targets. We identified a potent small molecule inhibitor of AC1, and this compound displayed analgesic properties in a model of inflammatory pain.

Materials and Methods

Compounds and other chemicals used

Forskolin and phorbol 12-myristate 13-acetate (PMA) were purchased from Tocris (Ellisville, MO). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Pittsburg, PA). NKY80 was purchased from EMD Millipore (Temecula, CA). Isoproterenol, A23187, adenosine monophosphate (ATP), ethyleneglycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 3-isobutyl-1-methylxanthine (IBMX), 5'-guanylyl-imidodiphosphoate (GppNHp), TWEEN 20, MgCl₂, and 2-Amino-2-(hydroxymethyl)-1,3-propanediol (TRIS) were purchased from Sigma-Aldrich (St. Louis, MO). 2-bromo-1-(1-phenyl-4,5-dihydro-1H-benzo[d]azepin-3(2H)-yl)ethanone (W400), 6-chloro-2-(trichloromethyl)-4H-chromen-4-one (ST034307), and 4-chloro-1-methyl-3-nitroquinolin-2(1H)-one (ST072383) were purchased from TimTec (Newark, DE) and the structures of the compounds were confirmed using NMR.

Cell culture

Human embryonic kidney (HEK) cells stably expressing AC1, AC8, or AC1 with the MOR were cultured in high-glucose (4500 mg/L) Dulbecco's Modified Eagle Medium containing L-glutamine and sodium pyruvate (Life Technologies, Grand Island, NY) supplemented with 5% bovine calf serum (Hyclone, Logan, UT), 5% fetal clone I (Hyclone), Antibiotic-Antimycotic (Life Technologies), and G418 (Invivogen, San Diego, CA) (HEK-AC1), or hygromycin B (Fisher Scientific, Pittsburg, PA) (HEK-AC8), or G418 and puromycin (Sigma-Aldrich) (HEK-AC1/MOR). Chinese hamster ovary (CHO) cells expressing the MOR (CHO-MOR) in the PathHunter® β -Arrestin GPCR assay platform were purchased from DiscoverX (Freemont, CA). Cells were grown in Ham's F12 media supplemented with 1 mM L-glutamine (Thermo Scientific, West Palm Beach – FL), 10% fetal bovine serum (Hyclone), 50 U/ml penicillin, 50 μ g/ml streptomycin (Life Technologies), G418 and hygromycin B. Cells were grown and frozen as previously described (53).

Transient transfections

HEK cells were plated in 15 cm dishes at a confluence of 9.0×10^6 cells/dish and incubated at 37°C in a humidified incubator overnight. On the following day, a 6 ml solution containing 9 µg of AC plasmid or venus fluorescent protein (venus) control plasmid, and 60 µl lipofectamine 2000 (Life Technologies) in optiMEM (Life Technologies) was prepared and incubated at room temperature for 45 min. The solution was added dropwise to the cells, and transfection was carried out for 48 h. Cells were harvested, and cryopreserved as described above. For AC7 and AC9, HEK cells were plated in 10 cm dishes (at confluences of 3.0×10^6 or 3.5×10^6 cells/dish, respectively) and incubated at 37°C in a humidified incubator overnight. On the following day, a 3 ml solution containing AC7 plasmid (10 µg), AC9 plasmid (3 µg), or venus plasmid; plus $G\alpha_s$ plasmid (0.5 µg for AC7 and 0.3 µg for AC9) or venus plasmid; and Lipofectamine 2000 (48 µl for AC7 and 24 µl for AC9) in optiMEM was prepared and incubated at room temperature for 45 min. The solution was added dropwise to the cells, transfection was carried out for 48 h, and cells were harvested and cryopreserved.

cAMP accumulation in cells

We measured cAMP accumulation as previously described (46). Briefly, cryopreserved cells were thawed, resuspended in optiMEM (Life Technologies), and plated in white, flat bottom, low-volume, tissue culture-treated 384 well plates (PerkinElmer, Shelton, CT). Plates with cells were incubated in a 37°C humidified incubator for 1 h. Inhibitors were added and plates were incubated at room temperature for 30 min followed by the addition of AC stimulants in the presence of 500 µM 3-isobutyl-1-methylxanthine (IBMX – phosphodiesterase inhibitor). Cells were incubated at room temperature for 1 h and cAMP accumulation was measured using Cisbio's cAMP kit (Cisbio Bioassays, Bedford, MA) according to the manufacturer's instructions. For the pertussis toxin experiment, HEK-AC1 cells were incubated for 16 hours in the absence or presence of 25 ng/mL of pertussis toxin (Sigma). The cells were then incubated with 30 µM ST034307 for 30 minutes at room temperature, followed by the AC1 stimulation and cAMP quantification. For the CD8-βARK-CT experiment, HEK cells were plated in a 6-well plate at a cell density of 5.5×10^5 cells per well. After 24 hours of plating, the cells were transiently transfected for 48 hours with AC1 plus venus (control) or CD8-βARK-CT in a 1:2 (AC1 plasmid: Venus or CD8-βARK-CT plasmid) ratio. The cells were harvested, cryopreserved, and assayed as described above. Additional assays measured cAMP accumulation using the HitHunter® cAMP Assay Platform from DiscoverX according to the manufacturer's instructions. Luminescence (HitHunter® cAMP Assay) and fluorescence (Cisbio's dynamic 2 kit) counts were measured using a Synergy 4 (BioTek, Winooski, VT).

Compound screening

Cryopreserved HEK-AC1 cells were thawed, washed, resuspended in optiMEM and plated into white, flat bottom, tissue culture-treated 384 well plates (PerkinElmer) at 15 µl/well using a MultiFlo dispenser (BioTek). Cells were incubated in a 37°C humidified incubator for 1 h. Next, test compounds (3.5 mg/l final assay concentration) from the NDL-3000 Natural Derivatives library (TimTec) were added (70 nl/well) using a MultiPette-mounted

384 well pin tool and incubated at room temperature for 30 min. Following the incubation with test compounds, 5 μ l/well of 3 μ M A23187 in the presence of 30 nM forskolin and 500 μ M IBMX (final concentrations) was added to the cells using a MultiFlo dispenser. Cells were incubated at room temperature for 1 h and cAMP accumulation was measured as described above using a MultiFlo dispenser to sequentially add 10 μ l/well of cAMP-d2 and anti-cAMP cryptate conjugate working solutions (Cisbio Bioassays) to the cells. Average maximum cAMP values in the presence of the stimulants A23187 and forskolin were 187 ± 2.2 nM and minimum values (stimulants in the presence of W400) were 19.4 ± 0.3 nM during the screening campaign. Test compounds were screened in singlet and a Z' factor of 0.55 ± 0.22 ($n = 10$) was obtained using 30 μ M W400 as a positive control (15, 16).

Cell viability assays

Cell viability assays were conducted with HEK-AC1 cells following plating and compound incubation protocols identical to the procedures described above in “*cAMP accumulation in cells*”. Cell viability was measured as a percentage of vehicle using 2% Triton X-100 (Sigma-Aldrich) as a control. The CellTiter-Glo® Luminescent Cell Viability Assay kit from Promega (Madison, WI) was employed to assess cell viability according to the manufacturer’s instructions. Luminescence counts were measured using a Synergy 4.

cAMP accumulation in cellular membranes from HEK cells

Cellular membranes from HEK-AC1 cells were isolated and frozen as previously described in the presence of 1 mM EGTA (46). On the assay day, membranes were thawed on ice and resuspended in membrane buffer (33 mM HEPES, 0.1% TWEEN 20, 1 mM EGTA, pH 7.4). Protein concentration was measured using the Pierce BCA Protein Assay kit (Thermo Scientific) and 2.0 – 3.5 μ g/well was plated in a white, flat bottom, tissue culture-treated 384 well plate. Inhibitors (diluted in a 33 mM HEPES, 0.1% TWEEN 20 solution) were added and incubated for 20 min at room temperature. Next, 3 μ M calmodulin or 30 μ M forskolin (final concentrations) was added in stimulation buffer (33 mM HEPES, 0.1% TWEEN 20, 1.5 mM $MgCl_2$, 250 μ M ATP, 1 μ M GppNHp, 500 μ M IBMX, and 500 μ M $CaCl_2$ – 10 μ M free Ca^{2+}) and incubated at room temperature for 45 min. cAMP accumulation was measured using Cisbio’s dynamic 2 kit according to the manufacturer’s instructions.

Adenylyl cyclase assays in cellular membranes from Sf9 cells

Membranes from Sf9 cells expressing AC1, AC2, or AC5 were prepared as previously described (54). All activity assays were performed for 10 min at 30°C in a final volume of 50 μ l. The final concentration of $MgCl_2$ and Mg-ATP in the reaction was 10 mM and 200 μ M, respectively. AC-containing membranes (10–20 μ g) were premixed with $G\alpha_s$ (50 nM final). Inhibitors were solubilized in DMSO and incubated with AC-containing membranes for 10 min on ice before the start of the reaction. The final concentration of DMSO in the reaction did not exceed 3% for either vehicle or inhibitors. Reactions were initiated upon addition of a reaction mix containing [α - ^{32}P]ATP. The reactions were terminated with stop solution (2.5% SDS, 50 mM ATP, and 1.75 mM cAMP) and the products were separated by sequential chromatography on Dowex-50 and Al_2O_3 (54).

Assays with hippocampal homogenates

C57BL/6 mice (13 weeks old) were decapitated, their brains were quickly removed, and 2-mm slices encompassing the hippocampus were collected on ice. The hippocampal region was dissected and immediately frozen in a -80°C freezer, where they were stored until the assay day. Dissected hippocampal tissue was thawed on ice, weighed, and homogenized in membrane buffer (2 ml/mg – wet weight) with ten manual strokes using a Wheaton-Teflon glass homogenizer. Homogenates were added to a white, flat bottom, tissue culture-treated 384 well plate and inhibitors (diluted in a 33 mM HEPES, 0.1% TWEEN 20 solution) were added and incubated for 20 min at room temperature. Next, 3 μM calmodulin (final concentration) was added in stimulation buffer (same as assays in cellular membranes from HEK cells) and incubated at room temperature for 45 min. Cyclic AMP accumulation was measured using Cisbio's dynamic 2 kit according to the manufacturer's instructions.

β -arrestin recruitment assay

Recruitment of β -arrestin2 to the MOR was measured as previously described (46). Briefly, CHO-MOR cells (DiscoverX) were plated in white, flat bottom, low-volume, tissue culture-treated 384 well plates. Plates with cells were incubated in a 37°C humidified incubator overnight. Following the incubation, AC1 inhibitors or vehicle was added to the cells, which were incubated at room temperature for 30 min. Next, DAMGO or vehicle was added to cells, which were then incubated in a 37°C humidified incubator for 1.5 h. β -arrestin 2 recruitment to the MOR was assessed using the PathHunter® assay (DiscoverX) according to the manufacturer's instructions. Luminescence counts were measured using a Synergy 4.

Heterologous sensitization assays

Heterologous sensitization assays were conducted as previously described (55). Briefly, HEK-AC1/MOR cells were thawed and plated in white, flat bottom, tissue culture-treated 384 well plates. Plates with cells were incubated in a 37°C humidified incubator for 1 h. For inhibition of the development of sensitization, inhibitors were added and plates were incubated at room temperature for 30 min, followed by addition of DAMGO and incubation at 37°C for 2 h (to achieve sensitization). For the assays to measure inhibition of the maintenance of sensitization the order of DAMGO and ST034307 additions were reversed (DAMGO sensitization before AC1 inhibition). Next, cells were treated with 3 μM A23187 in the presence of 500 μM IBMX and 1 μM naloxone (final concentrations) and incubated at room temperature for 1 h. Cyclic AMP accumulation was measured using Cisbio's dynamic 2 kit according to the manufacturer's instructions.

Animals and housing

Wild-type C57BL/6 mice, were obtained from Taconic (Cambridge City, IN). Male mice age 5 weeks (18–23gr) were grouped and housed in single grommet ventilated plexiglass cages at ambient temperature (21°C) in a room maintained on a reversed 12L:12D cycle (lights off at 10:00, lights on at 22:00) in our animal facility that has Association for Assessment and Accreditation of Laboratory Animal Care approval. Food and water were provided *ad libitum*. The mice were given ~7 days to acclimatize to the housing conditions and reverse light cycle before the start of the experiments. Mice were then habituated to the containment

boxes for the Von Frey assay. All animal procedures were pre-approved by our Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Mice were not deprived of food or water at any time.

Inflammatory pain behavioral assays

C57BL/6 mice were placed in suspended rectangular plastic chambers on a wire mesh grid to habituate for 1 h. Next, a baseline measurement of mechanical sensitivity to Von Frey filaments was performed as previously described (24, 34). Immediately after baseline measurements, the mice were injected with complete Freund's adjuvant (CFA – 10 µl, non-diluted) into the intraplantar surface of the left hindpaw to induce inflammation (24). On the following day, inflammatory hypersensitivity was measured using Von Frey filaments. Next, drugs were injected intrathecally as previously described (34). Drug-induced analgesia was measured 10 min after intrathecal injections using Von Frey filaments. Data are represented as a percentage of the average baseline response.

Data and statistical analyses

All data and statistical analyses were carried out using GraphPad Prism 6 (GraphPad Software, San Diego, CA). Statistical analyses (one sample *t* tests, one-way ANOVAs, or two-way ANOVAs) are described in text or figure legends where appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Chemical structures of AC1 inhibitors.

Fig. 2. Inhibitory activity of ST034307 and ST072383 for AC isoforms

(A) Inhibition of 3 μ M A23187-stimulated cAMP accumulation in stably transfected HEK-AC1 cells. (B) Inhibition of 3 μ M A23187-stimulated cAMP accumulation in stably transfected HEK-AC8 cells. (C) Stimulation of HEK cells transiently transfected with venus control plasmid or AC isoforms. AC1- and AC8-transfected cells were stimulated with 3 μ M A23187; AC2-transfected cells were stimulated with 1 μ M PMA; AC3-transfected cells were stimulated with 30 μ M forskolin; AC4-transfected cells were stimulated with 10 μ M isoproterenol; AC5- and AC6-transfected cells were stimulated with 1 μ M forskolin; AC7-transfected cells were stimulated with a combination of 1 μ M PMA plus 1 μ M forskolin in

the presence of transfected $G\alpha_s$; AC9-transfected cells were stimulated with 100 nM isoproterenol in the presence of transfected $G\alpha_s$. The y-axis represents the cAMP accumulation when compared to the same transfection and stimulation condition in matched venus-transfected control cells. One sample *t* test with Bonferroni correction was carried out for statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ compared to the same transfection and stimulation condition in matched venus-transfected control cells ($n = 3-4$). **(D)** Inhibition of cAMP accumulation in the transiently transfected HEK cells using the indicated concentration of inhibitor. Cells were transfected and stimulated as described in (C). The “venus”-transfected HEK cells were stimulated with 30 μ M forskolin. One sample *t* test with Bonferroni correction was carried out for statistical analyses. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to vehicle-treated cells (in the absence of inhibitor, $n = 3-4$). In A, B, and D data was normalized by defining the cAMP levels from activator treatment (without inhibitor) as 100% and baseline cAMP levels as 0%. All data shown represent the average and S.E.M. of at least three independent experiments conducted in duplicate or triplicate.

Fig. 3. Specificity and activity of ST034307 in cells and membrane preparations

(A) HEK-AC1 and nontransfected HEK cells (HEK-WT) were treated with 30 μM ST034307 and stimulated with 300 nM forskolin or 10 μM isoproterenol. (B) Cellular membranes from HEK-AC1 cells were isolated and cAMP production was stimulated with either 30 μM forskolin or 3 μM calmodulin in the presence of 10 μM free Ca²⁺ with or without either of the indicated AC inhibitors. (C) Cellular membranes from Sf9 cells expressing AC1, AC2, or AC5 were isolated and cAMP accumulation was stimulated with 50 nM Gα_s in the presence of 100 μM ST034307. The data was normalized by defining the cAMP levels from activator treatment (without inhibitor) as 100% and baseline cAMP levels

as 0%.. All data shown represent the average and S.E.M. of at least three independent experiments conducted in duplicate or triplicate. One sample t test with Bonferroni correction was carried out for statistical analyses. * $p < 0.05$, ** $p < 0.01$ all compared to vehicle-treated (in the absence of inhibitor).

Fig. 4. Mechanistic insights regarding ST034307

(A) Inhibition of A23187-stimulated cAMP accumulation in HEK-AC1 cells. (B) Inhibition of forskolin-stimulated cAMP accumulation in HEK-AC1 cells. The data was normalized by defining the cAMP levels from activator treatment (without inhibitor) as 100% and baseline cAMP levels as 0%. All data shown represent the average and S.E.M. of at least three independent experiments conducted in triplicate. Data were analyzed using two-way ANOVA followed by Tukey's multiple comparisons test. * $p < 0.05$, ** $p < 0.01$ between indicated concentrations.

Fig. 5. Inhibition of Ca²⁺/calmodulin-stimulated cAMP accumulation in hippocampal homogenates

(A) Mouse hippocampal homogenates were stimulated with 3 μM calmodulin in the presence of 10 μM free Ca²⁺ with basal cAMP activity in the absence of Ca²⁺/calmodulin set at 1. (B) Hippocampal homogenates were stimulated Ca²⁺/calmodulin in the presence of ST034307 or NKY80. Data are normalized to the response in the absence of the inhibitors (3 μM calmodulin plus 10 μM free Ca²⁺ was defined as 100% and baseline cAMP levels was defined as 0%) and represent the average and S.E.M. of six independent experiments conducted in triplicate. One sample *t* test for (A) and one sample *t* test with Bonferroni

correction for (B), ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared to basal or vehicle-treated (in the absence of inhibitor).

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Fig. 6. Effects of ST034307 on MOR signaling

(A) Inhibition of A23187-stimulated cAMP accumulation in HEK-AC1/MOR cells. Approximate EC₂₀ concentrations of ST034307 (0.5 μM) and DAMGO (4 nM) or EC₅₀ concentrations of ST034307 (7.5 μM) and DAMGO (15 nM) were added alone or in combination as indicated and cAMP accumulation measured. Data was normalized by defining the inhibitory response to 1 μM DAMGO in the presence of 3 μM A23187 as 100% and the cAMP response to 3 μM A23187 as 0%. (B) β-arrestin 2 recruitment in CHO-MOR cells that were treated with 10 μM DAMGO, 30 μM ST034307, or DAMGO + ST034307. (C) Heterologous sensitization of AC1 by the MOR was achieved by pre-treating the cells with 1 μM DAMGO for 2 h and subsequently stimulating cAMP accumulation with A23187 (3 μM) in the presence of naloxone (1 μM). (D) Effects of ST034307 on the development and maintenance of DAMGO-stimulated heterologous sensitization. Timeline of the drug treatments conducted for determining the effects of ST034307 on the development (top) and maintenance (bottom) of DAMGO-induced heterologous sensitization of AC1 (shown in graph below). Following drug pretreatment, heterologous sensitization was triggered by the addition of A23187 (3 μM) in the presence of naloxone (1 μM). The data in C and D were

normalized to vehicle-treated cells under basal conditions (0%) or stimulated with A23187 in the presence of naloxone (100%). The data shown represent the average and S.E.M. of at least three independent experiments conducted in duplicate. One sample *t* test with Bonferroni correction was carried out for statistical analyses in (A), (B), and (C). For (A) **p*<0.05, ***p*<0.01, and ****p*<0.001 compared to respective DAMGO + ST034307 column; for (B) and (C) * *p*<0.05 and *** *p*<0.001 compared to vehicle-treated cells (in the absence of any drugs).

Fig. 7. Analgesic properties of ST034307 in a mouse model of inflammatory pain

(A) On day 0, baseline (BL) measurements of mechanical sensitivity of C57BL/6 mice to von Frey filaments were recorded and inflammatory hypersensitivity was induced by injection of CFA to the hind paw. On day 1, inflammatory hypersensitivity was measured by von Frey test. Animals received intrathecal injections with saline (n=10), 50 ng MOR-selective agonist DAMGO (n=11), or 0.5 μg ST034307 (n=11) and inflammatory hypersensitivity was measured again. *p<0.05, **p<0.01 compared to saline injection at 10 min, analyzed by one-way ANOVA. (B) Dose-response experiments with ST034307 (n=6 for each condition). Estimated ED₅₀ value for analgesia of 0.28 μg (95% CI = 0.13 – 0.43; n = 6). (C) At 1 day after CFA-induced inflammatory pain, animals received intrathecal injections with saline (n=9), 10 μg forskolin (n=8), 0.5 μg ST034307 (n=9), or 10 μg forskolin + 0.5 μg ST034307 (n=8) and mechanical hypersensitivity was measured by von Frey test 10 min after injections. *p<0.05, **p<0.01 compared to each corresponding treatment, analyzed by one-way ANOVA with Tukey's test. The data shown represent the average and S.E.M. of each group of measurements to stimulation of the ipsilateral hind paw of the mice. All data was normalized by defining the baseline measurements as 100% and the CFA-induced hypersensitivity as 0%.

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Table 1

Inhibition of A23187-stimulated AC1 or AC8 activity and cell toxicity of hit compounds. Compounds were tested for inhibition of 3 μ M A23187-stimulated cAMP accumulation in HEK-AC1 or HEK-AC8 cells. IC₅₀ values are presented in μ M with 95% confidence intervals (C.I.) and percent inhibition (with 100% set to the basal cAMP) with S.E.M. are reported. We also report the cell toxicity results as a percentage of the vehicle-treated cells. The data in the table represent the average of at least three independent experiments conducted in duplicate. NIO (no inhibition observed).

Compound	HEK-AC1		HEK-AC8		Cell viability	
	IC ₅₀ @ (95% C.I.)	% inhibition @ (SEM)	IC ₅₀ @ (95% C.I.)	% inhibition @ (SEM)	% vehicle @ (SEM)	
ST072383	0.3 (0.2 – 0.4)	113 ($\pm$ 4)	1.6 (0.5 – 5.7)	55 ($\pm$ 9)	97 ($\pm$ 3)	
ST034307	2.3 (1.2 – 4.5)	108 ($\pm$ 10)	NIO	-25 ($\pm$ 13)*	85 ($\pm$ 6)	

* This value was defined as the bottom of the dose-response curve for antagonism, resulting in a small non-significant potentiation (negative value).