Bithionol Potently Inhibits Human Soluble Adenylyl Cyclase through Binding to the Allosteric Activator Site*

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The universal second messenger cAMP regulates various cellular processes such as cell adhesion, gene expression, and energy metabolism (1, 2). In mammals, cAMP formation from ATP is catalyzed by two types of adenylyl cyclases (ACs),3 nine transmembrane AC (tmAC) enzymes (AC1–9) and one soluble AC (sAC; AC10) (3). Mammalian sAC and tmAC both belong to nucleotidyl cyclase Class III, the largest of the six evolutionary distinct classes (2), and thus share a common catalytic core architecture (3). However, these ACs differ in their biological function and regulation. tmACs reside in the plasma membrane and are regulated by heterotrimeric G proteins in response to signals acting via G protein-coupled receptors. They thereby mediate cellular responses to extracellular signals. The nine tmAC isoforms show distinct tissue expression patterns and contribute to different physiological processes such as learning (AC1) and cardiac myocyte function (AC5) (4). sAC, in contrast, resides inside cells where it acts as an intracellular sensor for bicarbonate (HCO₃⁻), ATP, and calcium (3). sAC is expressed in many tissues and can be found throughout the cytoplasm, inside the nucleus, and in mitochondria (3, 5). It contributes to functions such as sperm activation (6, 7), acid-base regulation (5, 8), astrocyte/neuron communication (9, 10), and pressure regulation in the eye (11).

Inside cells, cAMP levels are determined by the opposing actions of ACs and cyclic nucleotide-degrading phosphodiesterases. Although phosphodiesterases are widely used as therapeutic targets (12), only a little progress has been made in developing AC-targeting drugs (13). Most of the few AC modulators that are available lack potency, specificity, or pharmacologically favorable properties (3, 14). Among the challenges hindering development of selective AC modulators are the similarities among the catalytic cores of different isoforms and a dearth of structural data (3). At present, only crystal structures for catalytic cores of an artificial AC5/AC2 heterodimer and, more recently, human sAC are available (3, 15, 16). These structures confirmed the general Class III catalytic core architecture with a dimer of identical or structurally closely related domains and the catalytic site formed at the dimer interface (3).

The signaling molecule cAMP regulates functions ranging from bacterial transcription to mammalian memory. In mammals, cAMP is synthesized by nine transmembrane adenylyl cyclases (ACs) and one soluble AC (sAC). Despite similarities in their catalytic domains, these ACs differ in regulation. Transmembrane ACs respond to G proteins, whereas sAC is uniquely activated by bicarbonate. Via bicarbonate regulation, sAC acts as a physiological sensor for pH/bicarbonate/CO₂, and it has been implicated as a therapeutic target, e.g. for diabetes, glaucoma, and a male contraceptive. Here we identify the bisphenols bithionol and hexachlorophene as potent, sAC-specific inhibitors. Inhibition appears mostly non-competitive with the substrate ATP, indicating that they act via an allosteric site. To analyze the interaction details, we solved a crystal structure of an sAC-bithionol complex. The structure reveals that the compounds are selective for sAC because they bind to the sAC-specific, allosteric binding site for the physiological activator bicarbonate. Structural comparison of the bithionol complex with apo-sAC and other sAC-ligand complexes along with mutagenesis experiments reveals an allosteric mechanism of inhibition; the compound induces rearrangements of substrate binding residues and of Arg179, a trigger between the active site and allosteric site. Our results thus provide 1) novel insights into the communication between allosteric regulatory and active sites, 2) a novel mechanism for sAC inhibition, and 3) pharmacological compounds targeting this allosteric site and utilizing this mode of inhibition. These studies provide support for the future development of sAC-modulating drugs.

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3 The abbreviations used are: AC, adenylyl cyclase; tmAC, transmembrane adenylyl cyclase; sAC, soluble adenylyl cyclase; HCP, hexachlorophene; DIDS, 4,4’-disothiocyano-2,2’-stilbenedisulfonic acid; ApCpp, α,β-methyl-ene-ATP; ASI-8, (4-amino furyran-3-yl)-[3-(1H-benzimidazol-2-yl)ethoxy]phenyl[methanone; BBS, bicarbonate binding site; sAC-cat, sAC catalytic domains; r.m.s.d., root mean square deviation.
karyotes, many Class III ACs are active as homodimers of identical domains featuring two symmetric active sites. In contrast, in mammalian tmACs and sAC, two related but non-identical catalytic domains are found in tandem on a single polypeptide chain, resulting in two asymmetric sites (4). The active site features a set of conserved Class III catalytic residues, whereas the second site is degenerate and appears to have evolved into a regulatory pocket (3). In sAC, this site mediates specific activation by bicarbonate, whereas the corresponding site in tmAC allows stimulation by the plant terpene forskolin (4, 17). As a regulatory site for physiological and pharmacological activators, it is a particularly interesting target site for drug development.

Despite the attractiveness of the pseudosymmetric regulatory site, most molecularly characterized AC inhibitors bind to the active site. A widely used class of inhibitors related to the substrate ATP, referred to as P-site inhibitors, exhibits low isoform specificity and likely also affects other nucleotide-binding proteins. The same applies to other compounds based on nucleosides or analogs such as adenine linked to ion chelators and fluorophore-modified nucleotides (3). Compounds binding to a largely hydrophobic pocket next to the active site exhibit some selectivity toward sAC relative to tmACs (18, 19), but the available compounds targeting this site are not suitable as pharmacological drugs. To date, only two classes of compounds are known to modulate ACs via the pseudosymmetric regulatory site: forskolin-related compounds, which modulate tmACs and exhibit some isoform selectivity (14), and the sAC inhibitor (4-aminofurazan-3-yl)\-[3-(1H-benzoimidazol-2-ylmethoxy)phenyl]methanone (ASI-8), which was recently developed by fragment-based screening (20). ASI-8 has a reported \( IC_{50} \) for sAC in the low micromolar range, and it extends from the regulatory site toward the active site where it would clash with substrate (3), suggesting a competitive inhibition mechanism. However, ASI-8 remains to be characterized with respect to kinetic mechanism, isoform selectivity, and suitability for use in cellular systems.

The organochlorine hexachlorophene (HCP) has a wide range of effects such as bacteriostatic activity and inhibition of the Wnt/β-catenin pathway (21). It was also reported to inhibit a mammalian AC activity (22), but conflicting results reported in another AC study (23) suggested potential isoform-specific effects. In an effort to identify novel scaffolds for the development of isoform-specific AC inhibitors, we tested whether HCP and bithionol (see Fig. 1A), a closely related bioactive organochlorine, might inhibit sAC. We found that both HCP and bithionol are potent inhibitors of human sAC. Biochemical characterization and crystal structure analysis show that bithionol binds to the sAC bicarbonate binding site, revealing new insights in allosteric AC regulation and identifying new leads for drug development.

**Experimental Procedures**

**Chemicals**—All chemicals were obtained from Sigma unless otherwise stated.

**Protein Production and Purification**—A protein construct comprising the human sAC catalytic domains (sAC-cat; residues 1–469) and a C-terminal His\(_6\) tag was expressed in Hi5 insect cells as described (24). The protein was purified through nickel affinity chromatography followed by anion exchange and size exclusion chromatography as described in detail (24). The sAC-cat-R176A variant and a corresponding wild-type protein were expressed as GST fusions in Hi5 cells as described (15).

**Crystal Structure Determination of sAC Complexes**—Human sAC-cat was crystallized in apo form in hanging drops at 4 °C as described (15). After 5 days, crystals were soaked in drops of cryosolution containing 100 mM sodium acetate, pH 4.8, 200 mM trisodium citrate, 18% (w/v) PEG 4000, 20% (v/v) glycerol, and 20 mM ligand for 2 h at 4 °C. Subsequently, crystals were flash frozen in liquid nitrogen.

Complete data sets for the crystals with space group P6\(_3\) were collected at 100 K at the Berlin Electron Storage Ring Society for Synchrotron Radiation beamline 14.1 (BESSY BL14.1) operated by Helmholtz-Zentrum Berlin (25). All diffraction data were processed with XDSAPP (26). The resolution limit of the data set was automatically set by XDSAPP based on CC\(_{\text{sym}}\) (27). Molecular replacement was done with Molrep (28) using the apo structure of sAC (Protein Data Bank code 4CLF) as a search model. Manual model building was done in Coot (29), and refinement was done in Refmac (30). Model quality was analyzed in Coot, and structure figures were generated in PyMOL (Schrödinger, LLC, New York).

**Binding Measurements**—The bithionol binding affinity was determined by measuring microscale thermophoresis on a Monolith NT. LabelFree instrument (Nanotemper Technologies, Munich, Germany) with 25% UV-light-emitting diode setting and 40% IR laser power. A concentration of 0.4 \( \mu \text{M} \) sAC-cat protein was used in 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, and 15 mM CaCl\(_2\). The \( K_p \) value was determined twice in independent experiments. Binding transitions were fitted with a single site equation in GraFit (Erithacus Software, East Grinstead, UK).

**Activity Measurements**—Activity assays were done with 100 ng of purified sAC-cat protein in 50 mM Tris/HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl\(_2\), 10 mM CaCl\(_2\), and 5 mM ATP at 37 °C. Reactions were stopped by flash freezing. Protein was denatured by heating to 95 °C or by addition of 2% SDS. cAMP produced was measured either as \( [3^2\text{P}] \) cAMP as described previously (31), via RapidFire (Agilent, Santa Clara, CA) mass spectrometry or by reversed phase chromatography. For reversed phase chromatography, samples diluted 1:1 with 40 mM ammonium acetate, pH 4.5, were analyzed on a UPLC system (Waters, Milford, MA) with a C\(_{18}\) column (Kinetex EVO, Phenomenex; 5 \( \mu \text{m}, 2.1 \times 150 \text{ mm} \) in 97% 20 mM ammonium acetate, pH 4.5, and 3% acetonitrile. cAMP eluted after 1.2 min at a flow rate of 1.0 ml/min. Signal areas were integrated with Empower software (Waters). Measurements were performed in duplicate, and data shown are representatives of at least two repetitions. For fitting of inhibition data at varying inhibitor concentrations, we used a mixed inhibition model where inhibitor binds to apoenzyme with affinity \( K_i \) and to a substrate complex with \( \alpha \times K_i \) and with both binding events causing complete inhibition. Fitting was done in GraphPad Prism (GraphPad Software, La Jolla, CA) using the following equation.
Bithionol Is an Allosteric sAC Inhibitor

A

B

C

D

E

F

\[
v = \frac{v_{\text{max}} \times [S]}{[S] + \left(\frac{[I]}{K_d} \times \frac{1}{\alpha \times K_i} + K_m \times \left(\frac{1}{[I]} + \frac{1}{K_i}\right)\right)} \quad (\text{Eq. 1})
\]

cAMP accumulation assays in cultured cells were performed as described (32). Briefly, cellular cAMP was quantitated using the Correlate-EIA Direct cAMP assay (Enzo Life Sciences, Farmingdale, NY) after cellular phosphodiesterases were inhibited with isobutylmethylxanthine (500 μM) for the indicated time.

Results

Bithionol and HCP Are Potent sAC Inhibitors—Because of its variability at inhibiting AC activity in cell lysates (22), we hypothesized that HCP might be an isoform-selective AC inhibitor. We thus tested HCP and the closely related bithionol (Fig. 1A) for effects on the activity of purified human sAC-cat protein (residues 1–469). Both substances potently inhibited sAC, and dose-response experiments yielded IC_{50} values of 1.6 ± 0.1 μM for HCP and 4.0 ± 0.2 μM for bithionol (Fig. 1B). Thus, these compounds inhibit sAC with similar potencies as ASI-8, the most potent sAC inhibitor identified to date, and KH7, a widely used, highly sAC-specific compound (3).

Inhibition Is Mostly Non-competitive with ATP—Because HCP is an aggressive substance with pleiotropic effects, potentially including membrane disruption and protein denaturation, we tested whether concentrations of HCP capable of fully inhibiting sAC affected the structural integrity of the enzyme. Thermal denaturation of sAC-cat in the presence of 1 or 10 μM HCP did not differ significantly from denaturation of the enzyme in the absence of the compound (Fig. 1C). Thus, HCP was not inhibiting sAC by disrupting its structural integrity. Nevertheless, because it has less aggressive chemical properties, we used bithionol to further investigate the mechanism of sAC inhibition. Analyzing the binding of bithionol to apo-sAC through thermophoresis measurements yielded a K_{d} of 0.43 ± 0.06 μM (Fig. 1D), revealing a binding affinity 10-fold stronger than its inhibition potency in activity assays (see above). To further analyze this potential substrate competition, we performed ATP titrations at varying inhibitor concentrations. They revealed a significant decrease of the apparent v_{\text{max}} with increasing bithionol concentrations and a weaker increasing effect on the substrate K_{m} (v_{\text{max}}^0 = 130 (0 μM bithionol), 52.2 and 0.39 (1.95 μM), 42.8 and 0.64 (7.8 μM), and 6.0 and 0.21 (31.25 μM)); the K_{m} at 31.25 μM bithionol is inaccurate due to the low activity. Thus, as also illustrated by the Lineweaver-Burk transformation of these data (Fig. 1E), bithionol acts as a mixed-type inhibitor on sAC. Directly fitting the inhibition data with a mixed model (i.e., where inhibitor binds to apoenzyme with affinity K_{d} and to enzyme-substrate complex with α × K_{i}) agreed well with the measured data (Fig. 1F) and yielded an α factor of 1.7. Thus, bithionol and substrate can bind concomitantly, but they will decrease each other’s affinity for sAC. This decreased affinity for bithionol in the presence of ATP likely contributes to the discrepancy between K_{d} (Fig. 1D) and IC_{50} (Fig. 1B; the 4.0 μM IC_{50} then corresponds to a K_{d} of 2.3 μM). The remaining discrepancy indicates an incomplete inhibition model; as described below, bithionol affinity for sAC can also be diminished by other components of the activity assay (i.e., bicarbonate). However, our data reveal that although bithionol has a small competitive effect it inhibits sAC mostly through a non-competitive mechanism, indicating that it exploits an allosteric site.

Bithionol Blocks the Activator Binding Site and Its Entrance—To identify the molecular details of this allosteric binding site and inhibition mechanism, we solved a crystal structure of an sAC/inhibitor complex. In soaking experiments with HCP, sAC apo crystals cracked and dissolved possibly due to a compound effect on the protein conformation (see “Discussion”). In contrast, after soaking sAC apo crystals with bithionol, we were able to collect diffraction data to 2.24-Å resolution (Table 1).
TABLE 1

Diffraction and refinement statistics

Values in parentheses refer to the highest resolution shell. CC, cross-correlation.

| sAC-bithionol | sAC-apo |
|---------------|---------|
| Data collection | Data collection |
| Space group   | P6₁     | P6₁     |
| Unit cell constants | a = b = 99.4 Å, c = 100.0 Å; α = β = 90°, γ = 120° | a = b = 99.4 Å, c = 100.0 Å; α = β = 90°, γ = 120° |
| Wavelength (Å) | 0.918   | 0.918   |
| Resolution (Å) | 86.23–2.24 | 86.23–2.24 |
| Total reflections | 137,147 (22,232) | 137,147 (22,232) |
| Unique reflections | 27,051 (4,321) | 27,051 (4,321) |
| Multiplicity | 5.1 (5.1) | 5.1 (5.1) |
| R₁ for F > 0 | 11.9 (1.7) | 11.9 (1.7) |
| Completeness (%) | 100 (99) | 100 (99) |
| CC₁/₂ | 100 (56) | 100 (56) |
| R_meas (%) | 15.4 (99.6) | 15.4 (99.6) |

Refinement

Refinement resolution (Å) 2.34 2.34
Reflections 25,668 25,668
R₁/R_meas (%) 17.7/24.4 17.7/24.4
Protein atoms 3,668 3,668
Solvent atoms 233 233
Ligand atoms 19 19
r.m.s.d.
Bond lengths (Å) 0.02 0.02
Bond angles (°) 1.97 1.97
Ramachandran plot
Preferred region (%) 95.8 95.8
Additionally allowed region (%) 4.2 4.2
Disallowed region (%) 0 0
Average B-factor (Å²) 36.5 36.5
Protein (Å²) 36.2 36.2
Solvent (Å²) 39.2 39.2
Ligand (Å²) 42.0 42.0

* R_meas = (∑ᵢ|Fᵢ(obs) − Fᵢ(calc)|)/ (∑ᵢFᵢ(obs)) where Fᵢ is the intensity of an individual measurement, (̅) is the corresponding mean value, and k and n are the indices and redundancies of the reflections.

Comparing the sAC-bithionol complex with apo-sAC and other sAC-ligand complexes revealed conformational differences that suggest a mechanism for the inhibitory effect of this ligand. Phe₃₃₈ and Phe₃₃₆ are moved slightly closer to the ATP adenosine binding site in the bithionol complex (Fig. 3A). This movement seems to tighten the substrate binding site. Striking changes in the bithionol complex are also found in and around the catalytic β₂-β₃ loop. Although the β₂-β₃ loop from the second catalytic domain (C2), which contributes to the BBS, is in a position similar to its position in the sAC-ApCpp complex, the catalytic β₂-β₃ loop of the first catalytic domain (C1) is in an inactive conformation. The density for the loop residue Asp²⁹⁹, which is one of the two conserved Asp residues binding the two catalytically essential divalent ions, is not well defined. This lack of order appears to be due to the bithionol ligand. Bicarbonate-dependent sAC activation involves a “flipping” away of Arg₁₇₆ from Asp⁹⁹. In the apo state, Arg₁₇₆ forms a salt bridge with Asp⁹⁹, whereas in the bicarbonate-activated state, Arg₁₇₆ assumes an “activation position” that frees Asp⁹⁹ to be appropriately positioned with Asp⁹⁹ to form two binding sites for the divalent ions in the substrate complex (15). In the sAC-bithionol structure, Arg₁₇₆ is oriented similarly to the sAC-bicarbonate complex but shifted away from the BBS to provide space for the inhibitor (Fig. 3B). In fact, the Arg₁₇₆ side chain interacts significantly with the inhibitor and contributes to its binding affinity (see above). Because of the altered Arg₁₇₆ orientation and associated smaller active site rearrangements, Asp⁹⁹ appears to be flexible but positioned too close to Asp⁹⁹ for ion site formation. The Asp⁹⁹-Asp⁹⁹ distance is 2.8 Å due to a shift of the region spanning from Asp⁴⁷ to Ala⁵₃. In particular,
**Bithionol Is an Allosteric sAC Inhibitor**

**FIGURE 2. Crystal structure of an sAC-bithionol complex.**

A, overall structure of the sAC-bithionol complex in backbone representation with bithionol displayed as sticks. B, bithionol in stick representation overlaid with Fo – Fc omit electron density (green) contoured at 2.5σ. Several interacting residues are shown as sticks colored according to atom type. C, surface of active site and inhibitor-containing BBS of the sAC-bithionol complex overlaid with bicarbonate from an sAC-bicarbonate complex and ApCpp from an sAC-ApCpp complex. Protein is shown as gray surface, and all ligands are in stick representation colored according to atom type. D, interaction scheme for the sAC-bithionol complex. Interactions to side chains are indicated by black dots, backbone interactions are indicated with dashed lines, and interactions with the aromatic ring systems are indicated with green broken lines. E, dose-response relationships for bithionol inhibition of wild-type human sAC (blue circles) and an sAC-R176A mutant (red squares) assayed in the presence of 1 mM ATP, 5 mM MgCl2, 5 mM CaCl2, and 30 mM NaHCO3. Data points are normalized to the activity in the absence of bithionol (error bars, S.E.; n = 3; absolute basal activity: wild type, 44.1 nmol/min; mutant, 8.6 nmol/min). F, bithionol inhibition appears to be competitive with bicarbonate. Dose-response relationships for bithionol inhibition of wild-type human sAC assayed in the presence of 1 mM ATP, 5 mM MgCl2, 5 mM CaCl2, and no NaHCO3 (magenta circles; absolute basal activity, 13.6 nmol/min) or in the presence of 20 mM (blue triangles; absolute basal activity, 55.4 nmol/min) or 40 mM (orange inverted triangles; absolute basal activity, 63.1 nmol/min) NaHCO3 (error bars, S.E.; n = 3) are shown. Data points are normalized to the respective basal activity in the absence of bithionol, and each titration was fitted individually with a standard inhibitor binding model (IC50 values, 6 (no bicarbonate), 9 (20 mM bicarbonate), and 11 (40 mM bicarbonate)).

**FIGURE 3. Comparison of the sAC-bithionol complex with other sAC conformations and analysis of cellular bithionol effects and selectivity.**

A, overlay of the sAC-bithionol complex (gray) with an sAC-ApCpp complex (blue; r.m.s.d., 0.6 Å for 407 Ca atoms). ApCpp, bithionol, and relevant amino acid side chains are shown in stick representation and colored according to atom type (carbon atoms colored as the corresponding protein). Ca2+ is shown as a yellow sphere. B, overlay of the sAC-bithionol complex with an sAC-bicarbonate complex structure (cyan; r.m.s.d., 0.4 Å for 356 Ca atoms). Bithionol, bicarbonate, and relevant amino acid side chains are shown in stick representation and colored according to atom type. C, time course of cAMP accumulation in sAC-overexpressing 4T1 cells in the absence of inhibitor (black circles) or in the presence of 50 μM bithionol (cyan triangles), 100 μM bithionol (blue squares), or 30 μM KT7 (red inverted triangles; error bars, S.E.; n = 3). D, time course of cAMP accumulation in sAC KO mouse embryonic fibroblasts in the presence of 50 μM forskolin and no inhibitor (black circles), 100 μM bithionol (blue squares), or 50 μM 2′,5′-dideoxyadenosine (magenta diamonds; error bars, S.E.; n = 3).
Thr^{52}, which usually interacts with the γ-phosphate of the substrate ATP, is shifted and rotates ~3 Å away. These active site rearrangements induced by bithionol binding to the BBS likely hinder formation of a productive sAC complex with catalytic ions and ATP.

**Bithionol Is Active in Cellular Systems and Specific for sAC Relative to tmACs**—We analyzed adenylyl cyclase activity in cellular systems by inhibiting phosphodiesterase activity and allowing cAMP to accumulate. To test whether our allosteric inhibitor bithionol can modulate sAC activity in vivo, we tested its effect on cAMP accumulation in cultured 4-4 cells, which stably overexpress sAC (33). As we showed previously (32, 33), the majority of cAMP accumulating in these 4-4 cells is produced by sAC. Similarly to the widely used sAC-specific inhibitor KH7, bithionol caused a dose-dependent decrease in cAMP accumulation in these 4-4 cells (Fig. 3C). Because bithionol exploits the unique bicarbonate site of sAC, we predicted it would be specific for sAC relative to tmACs. To analyze its effects on tmAC-dependent cAMP formation, we tested whether bithionol inhibited forskolin-stimulated cAMP accumulation in sAC KO mouse embryonic fibroblasts, which exclusively reflects tmAC activity (32). As expected, forskolin-stimulated cAMP accumulation in sAC KO mouse embryonic fibroblasts was inhibited by the tmAC-specific P-site inhibitor 2',5'-dideoxyadenosine (32), whereas bithionol did not inhibit this tmAC-dependent cAMP accumulation at concentrations up to 100 μM (Fig. 3D). Thus, bithionol is an sAC-specific AC inhibitor in cellular contexts.

**Discussion**

Few potent inhibitors are currently available for sAC, a potential target, e.g. for contraception (34) and for therapy of diabetes (35), ocular hypotony, and glaucoma (11). We describe here that the bisphenol bithionol inhibits human sAC potently and specifically by exploiting an allosteric mechanism via the bicarbonate regulation site of the enzyme. The corresponding site in mammalian tmACs serves as the binding site for the non-physiological, tmAC-specific activator forskolin (3, 36). Comparing the sAC-bithionol and tmAC-forskolin (Protein Data Bank code 1CJT (36) complexes illustrates the molecular differences causing the sAC specificity of bithionol (Fig. 4A).
sAC, the inhibitor fills the BBS and its entrance almost completely. In contrast, the tmAC forskolin site is significantly extended compared with the BBS; it would fail to provide an appropriately sized binding pocket for bithionol, resulting in insufficient interactions for high affinity binding. In fact, the forskolin and bithionol binding sites hardly overlap (Fig. 4A). The extended β2-β3 loop in sAC tightens the BBS and prevents access to most of the region corresponding to the tmAC forskolin site. This tightening of the regulatory site in sAC prevents forskolin binding to sAC and provides binding interactions for bithionol, which make the inhibitor sAC-specific.

Bithionol is the first known sAC inhibitor that acts through the BBS and via a mostly non-competitive, allosteric mechanism. Allosteric activation of tmACs by forskolin binding to the analogous regulatory site appears to be mediated via stabilization of an active orientation of the catalytic domains (C1 and C2) (37). Bithionol does not seem to have a significant effect on the relative orientation of sAC C1 and C2 domains, and such a domain reorientation also does not appear to be relevant for the bicarbonate-dependent sAC activation mediated by this site (15). Instead, binding of bicarbonate and of bithionol involves major rearrangements of residues relevant for productive substrate binding and for communication between active and regulatory sites. A key residue for this communication is Arg176, which assumes a unique conformation in the sAC-bithionol complex. Arg176 forms a salt bridge with the catalytic Asp99 in the inactive sAC apo state, reorients toward the ATP 2'-OH in a sAC-substrate complex, and turns away from the active site for interaction with bicarbonate in the BBS in the stimulated sAC state (Fig. 3, A and B) (15, 38). Although the mechanistic role of Arg176 is not fully understood, its rearrangements are clearly implicated in sAC catalysis and, in particular, in sAC activation (15). And shown here, Arg176 is involved in bithionol inhibition. The Arg176/bithionol interaction appears to provide binding energy and causes a unique side-chain conformation oriented away from the active site and displaced from its activation position (Fig. 3B). Together with the associated rearrangements of the catalytic Asp residues, which prevent binding of the catalytic metal ions, the reorientation of Arg176 appears to contribute to the allosteric mechanism leading to sAC inhibition. However, an sAC-R176A mutant is inhibited by bithionol, albeit with lower potency, and inhibition also appears to have a competitive component. The lower potency likely reflects the loss of interaction energy between the Arg and bithionol. The competition could stem from a direct clash with the substrate ribose, although the exact position of the ATP sugar in a productive substrate complex can only be estimated based on the available structures (15, 38). Another contribution to a lower substrate affinity in the presence of bithionol could come from the restriction of the adenosine binding site caused by the bithionol-induced reorientation of Phe336.

A previously described sAC inhibitor occupying the BBS is ASI-8, the compound with the most potent effect on sAC (IC50 ~ 0.4 μM) described thus far (20). ASI-8 is a longer molecule than bithionol, however, and it extends out of the BBS (and the connecting channel) into the substrate binding site (Fig. 4B). No data are available for the specificity or mechanism of ASI-8 inhibition, but comparisons of the respective crystal structures predict that ASI-8 would severely clash, and thus compete, with the substrate ATP. ASI-8 was developed by fusing smaller fragments, and a subset of the smaller precursors showed BBS binding and very weak sAC inhibition. Because these smaller fragments do not block the substrate site, they may be allosteric inhibitors similar to bithionol. An inhibitor class using a related mechanism but mostly another binding site than bithionol are catechol estrogens (19). They bind to a central surface of the catalytic dimer and orient their catechol moiety toward the catalytic estrogens and the substrate phosphates. Through a direct, strong interaction with ion A, they interfere with proper ion positioning and with the ATP conformation required for turnover (19).

Bithionol and HCP appear to affect a number of targets and have pleiotropic biological effects from antiviral and antibacterial activity to inhibition of cancer cell growth. In fact, bithionol is used as an antiparasitic agent in animals and was used as an antibacterial agent in cosmetics until this application was prohibited by the Food and Drug Administration because of its photosensitizing effect. Bithionol also showed little specificity in its cytotoxic effect on a panel of ovarian cancer cell lines (39), and we found general cytotoxic effects starting around 10 μM (data not shown), i.e. at concentrations that would be required for significant sAC inhibition in vivo. Therefore, bithionol and HCP themselves appear unsuitable as drugs. However, the binding site, inhibition mechanism, and chemical scaffold can serve as starting points for the development of more specific sAC inhibitors. Another sAC inhibitor binding to a bithionol-related region is 4,4′-diphenylol (39), DIDS is known as a blocker of bicarbonate channels and inhibits sAC weakly. It binds to the entrance shared by the sAC active site and its BBS, blocking access for substrate as well as activator (Fig. 4C) (15). Its binding site overlaps partially with the bithionol site, exploiting the same hydrophobic patch formed by Phe45/336/338. Being smaller than DIDS, bithionol is positioned more deeply in the BBS, whereas DIDS extends further to the active site entrance of the catalytic core (Fig. 4C). Although DIDS shows limited sAC affinity, it illustrates the possibility to extend BBS ligands toward the active site entrance for increased interaction interfaces, affinity, and potency. Together, these observations suggest that adding moieties that mimic the interactions observed in the sAC complexes with ASI-8 (20) and DIDS (15) may improve HCP/bithionol derivatives.

Understanding why HCP was more potent than the closely related bithionol might also be helpful for future development of sAC inhibitors based on this scaffold. Because no experimental sAC:HCP complex structure could be obtained, we modeled the HCP complex based on the sAC-bithionol structure (Fig. 4D). One of the additional chlorines found in HCP would point into the pocket between Phe45 (2.9 Å) and the side chain of Arg176 (δ carbon position, 3.0 Å). The second chlorine atom would point in the direction of Leu102 (102 Å) and Lys95 (δ carbon position, 3.4 Å). Although we cannot exclude that bithionol and HCP have different inhibition mechanisms as described previously for another target (40), it is tempting to hypothesize that the additional interactions due to the chlorines might cause the slightly higher HCP affinity and potency. The sensitive
response to small compound variations suggests that further modifications of the bithionol/HCP scaffold might enable improving it into a specific sAC inhibitor.

Our work identified HCP/bithionol as potent sAC inhibitors that exploit the unique activator binding site in sAC for an allosteric mechanism of sAC inhibition. The BBS thus serves as a general allosteric sAC regulation site, enabling activation as well as inhibition. Whether there are physiological sAC ligands that exploit this site for inhibition remains to be seen. However, analyzing the allosteric mechanisms of sAC activation and inhibition through BBS ligands helps understanding sAC regulation and provides novel approaches for pharmacological sAC modulation. Our structural analysis of sAC inhibition by bithionol reveals contributing conformational changes and binding details, which provide a rational basis for further development of BBS ligands as potent and specific sAC modulators.

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**Bithionol Is an Allosteric sAC Inhibitor**

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