Inhibition of Kinesin Motor Proteins by Adociasulfate-2

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Kinesin motor proteins are involved in cell division and intracellular transport of vesicles and organelles, and as such, they play a role in neurological disease, cancer, and developmental disorders. Inhibitors of kinesin would be valuable as probes of cell physiology and as potential therapeutics. Adociasulfate-2 (AS-2) is the only known natural product inhibitor of kinesins, but its mechanism of action is unknown. We utilized kinetic studies, dynamic light scattering, and transmission electron microscopy to investigate the inhibitory action of AS-2. Our data suggest that AS-2 is not a classical 1:1 inhibitor. Instead, a rodlike aggregate that mimics microtubules is complexed with kinesin and inhibits its ATPase activity. An intriguing implication of this hypothesis is that aggregates of a chiral natural product can have interesting and biologically relevant properties. This mode of action might represent one way in which a small molecule can disrupt a protein−protein interaction.

Introduction

The kinesins[1−3] are a large superfamily of motor proteins that are involved in cell division and intracellular transport of vesicles and organelles. The general kinesin structure includes a motor domain (∼320 amino acids), a coiled-coil region that facilitates dimerization, and a tail domain that attaches to cellular cargoes for transport. Through their motor domain, kinesins hydrolyze ATP and move along microtubules (MTs), which are cylindrical tubulin polymers 25−30 nm in diameter and found throughout the cytoplasm. Kinesins perform diverse functions in the cell, and they play a role in neurological disease, cancer, and developmental disorders. As such, inhibitors of kinesins would be valuable tools for studying cell biology and possibly as therapeutic leads for cancer and other diseases. Understanding the mechanism of their inhibitory action will enable the discovery of other inhibitors. A few inhibitors of kinesins exist, such as monastrol,[4] terpendole E,[5] and HR22C16.[6] They specifically inhibit Eg5, a member of the kinesin-5 family, but are not known to act on other kinesins.

Adociasulfates 1−6 and 10, sulfated hydroquinones isolated from the *Haliclona* species of marine sponges, are the only non-nucleotide natural products to inhibit the MT-stimulated ATPase activity of kinesin.[7−9] Adociasulfate-2 (AS-2, Figure 1) arrests nuclear divisions in *Drosophila*,[7] and it has been used to study kinesin-based motility in green algae.[10,11] AS-2 competes with MTs for binding to the motor domain of kinesin;[7] however, its inhibition mechanism is unknown. Interestingly, it does not compete with ATP for binding to the motor protein.[7] These findings suggested to the discoverers that AS-2 acted as a microtubule mimic.[7]

Hopkins et al.[12] reported that rose bengal lactone (RBL, Figure 1) specifically inhibits the MT-stimulated ATPase activity of human kinesin ($IC_{50} = 7.5 \pm 2.5\ \mu M$). Like AS-2, RBL is competitive with MTs but not competitive with ATP. Recently, McGovern et al.[13] have shown that RBL forms aggregates that nonspecifically inhibit several unrelated enzymes, which casts doubt on the idea that RBL is a specific inhibitor of kinesin.

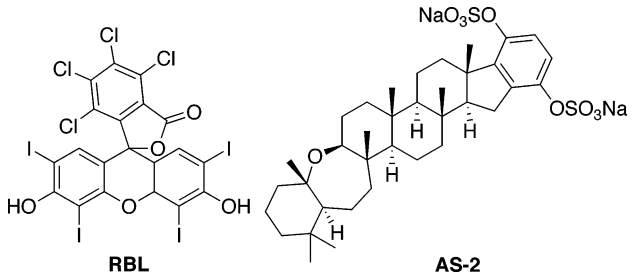

Figure 1. Structures of rose bengal lactone (RBL) and adociasulfate-2 (AS-2).

We wondered if the action of AS-2 might also be the result of nonspecific inhibition. AS-2 is an amphiphilic molecule with the potential to aggregate in solution. Aggregate formers that are known to nonspecifically interact with proteins and cause inhibition are called "promiscuous;"[13−15] however, AS-2 does not inhibit other ATPases, such as rabbit kidney ATPase, apyrase, myosin II, and pyruvate kinase,[7] making this designation inappropriate. AS-2 inhibits several kinesins[7] probably because the MT interaction surface is highly conserved.[16]

We investigated the formation of inhibitory aggregates of AS-2 by studying the kinetics of AS-2 inhibition of kinesin, the dynamic light scattering (DLS) of AS-2 in solution, and the structure of AS-2 aggregates by transmission electron microscopy (TEM). We show that AS-2 is not a classical 1:1 inhibitor. Interestingly, the shape of the AS-2 aggregates is rodlike in buffer with magnesium ions but globular in water only. Our results suggest that the structure of AS-2 responsible for the inhibitory action on kinesin is a rodlike aggregate, probably with the sulfate groups exposed to the aqueous environment, mimicking the negatively charged surface of MTs[16] and resulting in a microtubule-like complex with kinesin.

Results and Discussion

The efficacy of reversible specific inhibitors of proteins is typically independent of preincubation of the enzyme with the inhibitor.[13] The ATPase activity of kinesin with and without a 5 min preincubation was monitored using a colorimetric (360 nm), enzyme-linked inorganic phosphate detection assay.[12,17] Each assay contained 15 mM PIPES, 5.0 mM $MgCl_2$, 20 μM paclitaxel (Taxol), 0.4 mM ATP, 0.1 unit purine nucleoside

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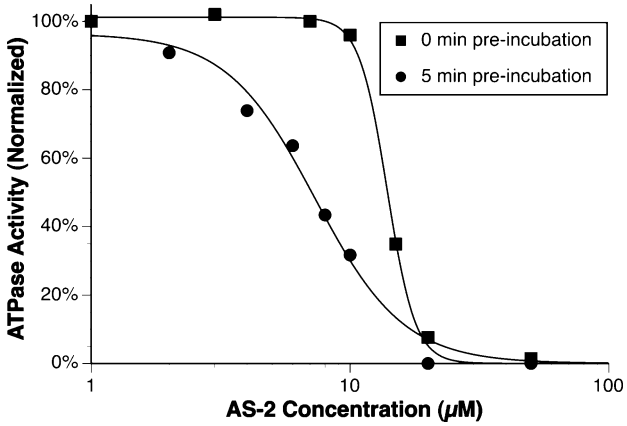

Figure 2. Increased inhibitory effect of AS-2 on kinesin ATPase activity with (●) and without (■) a 5 min preincubation. Data points represent the kinesin ATPase activity as determined from the initial linear rate of reaction (see Supporting Information) normalized to the initial rate in the absence of AS-2. The data are fit to a four-parameter logistic function.

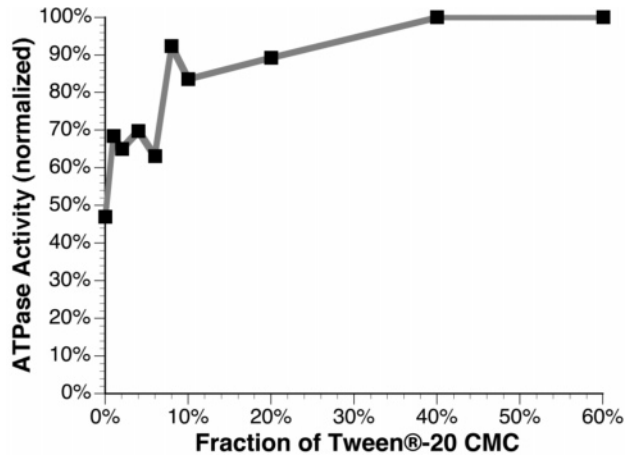

Figure 3. Decreased inhibitory effect of AS-2 in the presence of Tween-20. Data points represent the kinesin ATPase activity as determined from the initial linear rate of reaction normalized to the initial rate in the absence of AS-2 and Tween-20. Experiments were carried out using 6 μM AS-2 at each concentration of detergent.

phosphorylase, 0.2 mM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 20 nM kinesin (K560[12]), 200 nM microtubules, and 0−50 μM AS-2 to a final volume of 150 μL. The initial linear rate of reaction, normalized to the initial rate in the absence of AS-2, was plotted against the concentration of AS-2 (Figure 2). We observed an improvement in the inhibition of kinesin by AS-2 after a 5 min preincubation time (IC_{50} = 7.3 μM) compared to no preincubation (IC_{50} = 13.9 μM). Additionally, the shape of the IC_{50} curve in the absence of preincubation is quite steep, which is a characteristic property of some "promiscuous" inhibitors.[18] This result suggests a nonspecific interaction between AS-2 and kinesin.

The presence of detergents should not affect a classical 1:1 enzyme inhibitor, but the efficacy of an aggregated inhibitor will diminish in the presence of detergents, which break up colloidal aggregates.[19,20] In the phosphate detection assay, addition of Tween-20 to 40% of its critical micellar concentration (cmc) restored kinesin's ATPase activity in the presence of 6 μM AS-2 relative to the activity of the enzyme in the absence of detergent and AS-2 (Figure 3). In the absence of Tween-20, AS-2 lowers the activity to 48%. These results indicate that the active form of AS-2 is an aggregate.

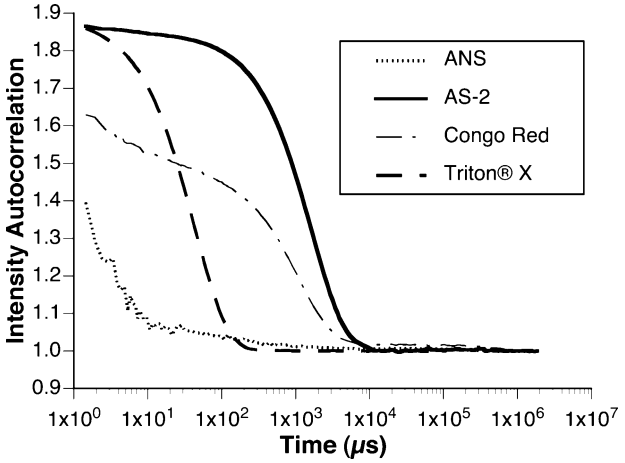

Figure 4. Autocorrelation function obtained for ANS (1 mM), AS-2 (1 mM), Congo Red (750 μM), and Triton X (1%) by DLS.

AS-2 was analyzed by DLS, which can detect the presence of aggregates in solution. These experiments, carried out on concentrations of up to 1 mM AS-2 in buffer (15 mM PIPES and 5.0 mM $MgCl_2$ at pH 6.8), clearly indicated the presence of aggregated molecules of AS-2 in solution after centrifugation at 10000*g* for 30 min. Strong, well-defined autocorrelation functions were obtained (Figure 4). The buffer included magnesium ions because they are also present in the ATPase activity assay and are known to have an effect on the cmc of aggregate formers. Congo Red (750 μM) and Triton X (1%), known aggregators, were used as positive controls, and 8-anilino-1-naphthalenesulfonic acid (ANS, 1 mM) was used as a negative control in experiments conducted at comparable laser powers. By use of a Rayleigh sphere model, the radii of the Congo Red, Triton X, and AS-2 aggregates were estimated to be 83.6, 4.1, and 165.4 nm, respectively. Because the shapes of the aggregates are not known, these values represent crude estimates of the particle sizes.

"Promiscuous" inhibitors usually form spherical aggregates that interact in a nonspecific manner with proteins and result in inhibition.[13,14] We used TEM to view the AS-2 aggregates formed (Figure 5 and Supporting Information). AS-2 (100 μM) in water gave the expected micellar structures, but to our surprise, the structures observed for 50 μM AS-2 in 15 mM PIPES and 5.0 mM $MgCl_2$ at pH 6.8 were not spherical but rodlike and ~300 nm wide. These structures did not appear in micrographs acquired from water or the buffer in the absence of AS-2 (data not shown).

Conclusions

Taken together, our data and the data of Faulkner and Goldstein[7] suggest that the AS-2 aggregates behave like microtubule mimics. In the presence of MTs, the kinesin ATPase rate rises ~1000-fold because of an increased rate of ADP release, which is the rate-limiting step of the enzymatic process.[21] AS-2 is not competitive with ATP binding,[7] but addition of AS-2 in the absence of MTs results in an increase in the kinesin ATPase rate from ~0.01 to ~0.06 s^{-1} (a 6-fold increase)[7] and a release of ADP that correlates with AS-2 concentration.[7] Our results suggest that the structure of AS-2 responsible for the inhibitory action on kinesin is a rodlike aggregate, probably with the sulfate groups exposed to the aqueous environment, mimicking the negatively charged surfaces of MTs[16] and resulting in a microtubule-like complex with kinesin. We hypothesize that the AS-2 aggregate competes with

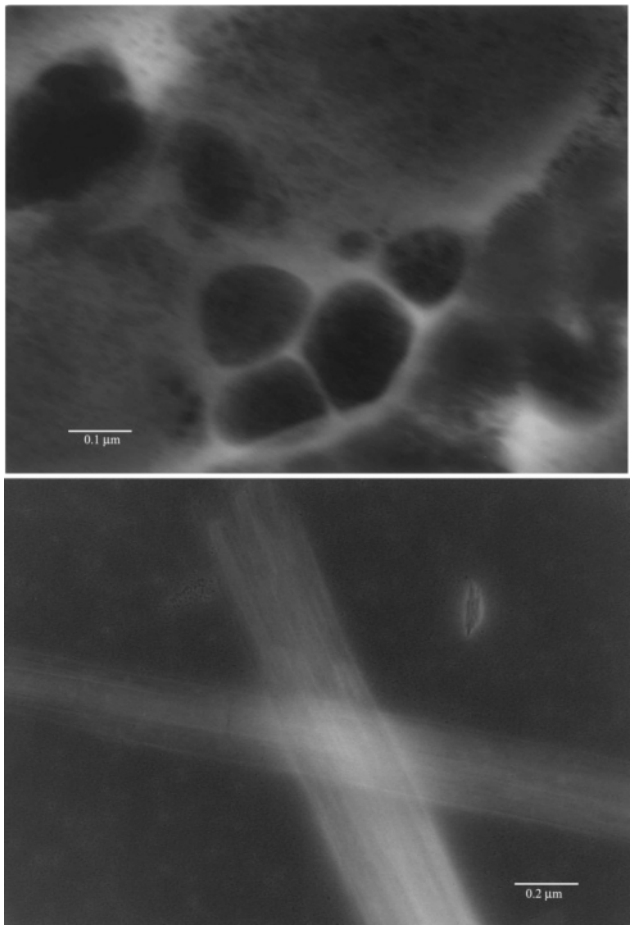

Figure 5. TEM images of AS-2 aggregates in (top) water (scale bar = 100 nm) and (bottom) 15 mM PIPES buffer and 5.0 mM $MgCl_2$ at pH 6.8 (scale bar = 200 nm).

MTs for binding to kinesin, but AS-2 induces significantly lower kinesin ATPase activity, resulting in an observed inhibition of the enzyme.

The findings are interesting because the biological activity of natural products is typically assumed to be the result of classical 1:1 binding. AS-2 is a chiral natural product containing multiple stereocenters, which makes it unusual among classical aggregators, and its inhibitory action as an aggregate is contrary to the assumptions of the papers on its effects.[7,10,11] The ordered structure of the AS-2 in buffer is also unusual. It implies that aggregates of small molecules can have interesting and biologically relevant properties, despite being a serious problem for high-throughput screening. Because AS-2 competes with MTs for binding to kinesin, it is also an example of a small molecule disrupting a protein−protein interaction.

Experimental Section

Reagents. 2-Amino-6-mercapto-7-methylpurine riboside (MESG) and purine nucleoside phosphorylase (PNP) were obtained as components of the EnzChek kit from Molecular Probes (Eugene, OR) or ATPase ELIPA kit from Cytoskeleton, Inc. (Boulder, CO). PNP, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), ATP, and 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma-Aldrich (St. Louis, MO). $MgCl_2$ was purchased from J. T. Baker (Phillipsburg, NJ). Paclitaxel (Taxol) was purchased from BIOMOL (Plymouth Meeting, PA). Tubulin was purchased from Cytoskeleton, Inc. (Denver, CO) and polymerized prior to use. The kinesin construct (K560) was obtained from Ronald Vale's lab. DMSO, Tween-20, and Triton X were purchased from Fisher Scientific (Fair Lawn, NJ). Congo Red was obtained from Matheson, Coleman and Bell (East Rutherford, NJ). Adociasulfate-2 (AS-2) was a gift from D. John Faulkner. Stock solutions of AS-2 and Congo Red were prepared in DMSO prior to dilution in PIPES buffer.

Kinesin K560 Subcloning, Expression, and Purification. Human conventional kinesin construct, K560/GFP/6His obtained from the Vale Labs, was subcloned to produce a K560/6His (64.63 kDa) construct. The polymerase chain reaction (PCR) was used to amplify DNA that included the K560 portion of the construct (1−560 amino acids) from the K560/GFP/6His construct. The design of primers included the XhoI and XbaI restriction sites to enable T4 ligation of the K560 construct into a pET23a expression vector (Novagen, San Diego, CA), which contains a 3′-6His tag for protein purification. PCR primers are as follows:

K560 forward:
5′-GAGAGACCACAACGGTTTCCCTCTAGAAAT-3′

K560 reverse:
5′-GTTCACTCGAGACCTTTTAGTAAAGATGCC-3′

After restriction digest of the PCR product and the pET23a vector with XhoI and XbaI, the two were ligated with T4 DNA ligase for 16 h at room temperature to create a vector containing K560 between the two restriction sites. This vector was amplified in DH5α (Invitrogen, Carlsbad, CA) cells by standard procedures. Restriction analysis with PvuII and sequence comparison with authentic K560 (Vale lab) confirmed successful subcloning.

Chemically competent *E. coli* BL21 cells (Novagen, San Diego, CA) were transformed with the K560/6His pET23a vector, plated onto Luria broth containing ampicillin, and incubated overnight at 37 °C. From a single colony, a culture was grown at 37 °C in 1 L of TPM medium (20 g/L tryptone, 15 g/L yeast extract, 8 g/L NaCl, 10 mM glucose, 2 g/L Na_2HPO_4, 1 g/L KH_2PO_4, and 100 μg/mL ampicillin) to OD_{600} = 1. The flask was cooled on ice until the culture was below 20 °C, and then it was induced with 100 μM IPTG. Growth was continued at 20 °C overnight, after which the cells were harvested and lysed. The lysate was loaded onto a Ni^{2+} affinity chromatograph (His Trap HP, Amersham Biosciences) and washed with buffer containing 20 mM imidazole (pH 6.0). K560/6His was eluted with 500 mM imidazole (pH 7.2). Buffers were supplemented with ATP (0.1 mM) and βME (10 mM) before use. Further purification was performed by High Trap-Q (Amersham Biosciences) in buffers containing 25 mM PIPES, 2 mM $MgCl_2$, and 1 mM EGTA, using NaCl as a salt gradient. K560 was eluted at 250 mM NaCl. NIH Image was used to estimate the purity of K560/6His, using known concentrations of BSA as standards. Typically, K560/6His of 40−50% purity was obtained at a concentration of 0.5 mg/mL. After purification, K560/6His was dialyzed in BRB12, divided into 50 μL aliquots, and flash-frozen (10% sucrose added) and stored at −80 °C.

Further purification of K560/6His was carried out using a microtubule bind-release assay. K560 (100 μL) was mixed with a 3× concentration of microtubules in binding buffer (25 mM PIPES, pH 6.8, 70 mM NaCl, 1 mM EGTA, 2 mM $MgCl_2$, 5% sucrose, 1 mM DTT, 50 μM ATP, and 20 μM paclitaxel) and incubated at room temperature for 30 min. The mixture was then spun at 80 000 rpm for 10 min over 100 μL of cushion (60% glycerol and 40% binding buffer made without salt or paclitaxel). A small microtubule pellet was observed after centrifugation. The pellet was washed twice with 100 μL of wash buffer (binding buffer without salt or ATP) to remove denatured protein and then resuspended in 100 μL of release buffer (binding buffer with 250 mM NaCl and 1 mM ATP). The vials were incubated at room temperature for 5 min followed by centrifugation for 5 min at 100 000 rpm. The supernatant containing active protein was removed and analyzed by SDS−PAGE.

The ATPase activity of K560/6His was confirmed using the EnzChek or ATPase ELIPA assay. Our K560 showed an initial hydrolysis rate of 8156 nmol of P_i/min/mg of kinesin or 8 ATP/s/head using 20 nM kinesin and 200 nM MT. These values agree with results obtained by others: 9360 nmol of P_i/min/mg of kinesin

using 20 nM kinesin and 0.5 μM MT[17] and $k_{cat} = 27 \pm 7$ ATP/s/motor domain,[16] which is consistent when one considers that this value was obtained at V_{max}. The $K_m = 2.0 \pm 0.5$ μM for microtubule-dependent ATPase activation is consistent with literature values: $K_m = 1.1 \pm 0.35$ μM[16] and $K_m = 1.2$ μM.[12]

Enzyme-Linked Inorganic Phosphate Assay. Reactions were set up in 96-well plates (Corning Costar No. 3596). Each reaction was carried out in triplicate and contained 15 mM PIPES, 5.0 mM $MgCl_2$, 20 μM paclitaxel, 0.4 mM ATP, 0.1 unit of PNP, 0.2 mM MESG, 20 nM kinesin (K560), and 200 nM microtubules in a final volume of 150 μL. All assays contained 5% DMSO. Inhibition assays contained 0−50 μM AS-2. For 5 min preincubation inhibition assays, all of the reagents except K560 and ATP were added to the well. K560 was then mixed into each well and allowed to incubate for 5 min before ATP was added to initiate the reaction. The absorbance at 360 nm was measured every 30 s for 30 min using a spectrophotometer (SpectraMax 340 PC, Molecular Devices). Inhibition studies without preincubation were done similarly except that K560 was added last to initiate the reaction. The protocol for the detergent experiments involved the addition of Tween-20 (0−60% cmc) with the other reagents. ATP was added to initiate the reaction.

Dynamic Light Scattering. Solutions of Congo Red (750 μM), ANS (1 mM), Triton X (1%), and AS-2 (1 mM) were made in buffer (15 mM PIPES and 5.0 mM $MgCl_2$ at pH 6.8). The solutions (100 μL) were centrifuged at 10 000 rpm for 30 min, and the supernatant (15 μL) was placed in a cuvette for analysis. The cuvette was allowed to equilibrate in the instrument at 25 °C for 5 min before the readings (25 acquisitions) were taken using a DYNAPro 99 (Protein Solutions, Piscataway, NJ) instrument at 30% laser power and analyzed using DYNAMICS version 6.0 software from Protein Solutions. Two independent experiments for each solution were carried out.

Transmission Electron Microscopy (TEM). Solutions of AS-2 (50 and 100 μM) and Congo Red (50 μM) were made in 15 mM PIPES buffer (pH 6.8). At room temperature and in duplicate, a 5 μL aliquot of each sample was placed on a carbon-coated grid. The samples were incubated for 5 min under a Petri dish with a damp filter paper for 5 min. The grids were then dried with the tip of a triangular-shaped filter paper and stained afterward with 1% uranyl acetate. The grids were inverted in a drop of the stain and allowed to incubate for 30 min. After careful removal from the drop, each grid was blotted dry using the tip of a filter paper. After drying, each grid was viewed and images were obtained using a JEOL 100 CX transmission electron microscope (JEOL USA, Peabody, MA) at 80 kV. Micrographs were recorded up to 100000× magnification.

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Supporting Information Available: Kinetic curves, ATPase assay standard curves for P_i, and additional TEM images. This material is available free of charge via the Internet at http://pubs.acs.org.

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