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A Chemoproteomics Approach to Determine the Mechanism of Testicular Toxicity for the Bruton's Tyrosine Kinase Inhibitor CC-292

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ABSTRACT

During drug development, potential safety issues can occur at any time. Understanding the cause of a toxicity can help with deciding on how to advance the drug development program. Chemoproteomics provides a way to help understand the cause of a toxicity wherein the affected tissue is accessible and can be probed with a covalently binding compound that is analogous to the offending drug. In this case, *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide (CC-292), a covalently binding Bruton's tyrosine kinase inhibitor, had produced testicular toxicity in rodents. Experiments were conducted using a CC-292 analog that could be chemically modified with biotin to probe rodent testes homogenates for potential binding sites that were subsequently recovered with avidin beads. These biotin-tagged proteins undergo trypsin digest on the avidin beads to yield peptides that are identified using mass spectrometry. Two proteins were identified from the testicular homogenates of both rats and mice, namely retinal dehydrogenase 1 (ALDH1A1) and retinal dehydrogenase 2 (ALDH1A2). Literature confirmed a link between inhibition of these enzymes and testicular toxicity. Subsequently, molecular modeling was used to demonstrate that CC-292 can be docked into both the nicotinamide adenine dinucleotide and retinal binding pockets of the analogous human ALDH1A2 enzyme. These data suggest that the off-target binding site for CC-292 on retinal dehydrogenase enzymes may provide a mechanistic explanation to the testicular toxicity observed in rodents and that there may be a potential concern for human male fertility.

SIGNIFICANCE STATEMENT

Biotinylated covalently binding drug analogues are used to enrich bound proteins from tissue homogenates wherein toxicity was observed in rodents. Bound proteins were subsequently identified by mass spectroscopy. Competition of the analog binding with the parent inhibitor itself and three-dimensional molecular modeling were used to establish a likely link between the off-targets of CC-292, ALDH1A1, and ALDH1A2 with potential testicular toxicity.

Introduction

The attrition rate of promising therapeutics due to potential safety concerns in early drug development is high (Waring et al., 2015). Issues can occur at any time as both nonclinical and clinical studies test new doses and typically longer duration dosing regimens. When a potential safety issue is identified, there is a re-evaluation of the risk/benefit profile of the compound, and decisions are needed on how to advance the drug development program. Questions arise, such as is the toxicity related to the target itself, off-target binding sites, the specific chemotype, or a combination of these factors? Can the issue be safely managed, or is it better to halt development and look to an alternative compound/chemotype? Therefore, it is highly advantageous to try to understand the underlying cause of an identified hazard within a given target organ to assist with decision making for the therapeutic.

Determining the cause of toxicity can be challenging if previously unidentified off-target interactions are suspected. Chemoproteomics presents an opportunity to address this challenge by measuring drug-protein interactions on an unbiased proteome-wide scale (Schirle and Jenkins, 2016; Drewes and Knapp, 2018). This technique can be readily applied to nonclinical studies wherein the tissues and cells are accessible in which the toxicity was observed using molecules that bind irreversibly or, in some cases, those that have very high target affinity and low dissociation rates. Although not all off-target sites will bind the selectable analog covalently, those that do present an irreversible modification that allows their identification using proteomic approaches.

In this case, an investigation into rodent testicular toxicity caused by a covalently binding small-molecule inhibitor of Bruton's tyrosine kinase (BTK), *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide

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ABBREVIATIONS: ALDH, retinal dehydrogenase; ALDH1A1, retinal dehydrogenase 1; ALDH1A2, retinal dehydrogenase 2; BTK, Bruton's tyrosine kinase; CC-292, *N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide; MS, mass spectrometry; NAD, nicotinamide adenine dinucleotide; PDB, Protein Data Bank.

(CC-292), is described. CC-292 caused dose-dependent testicular toxicity in mice and rats after daily oral dosing for 1 and 3 months, respectively. This compound was in clinical trials and intended for use in hematologic cancers and anti-inflammatory indications (Evans et al., 2013). Experiments were conducted wherein the covalently binding compound 292TC, a CC-292 analog that may be easily and efficiently modified with biotin with click chemistry (Hein et al., 2008; Fig. 1), was used to probe rodent testes homogenates for potential binding targets. In this application, "click chemistry" specifically means the high yielding reaction between the azide moiety of an azido-derivatized biotin with an alkyne-derivatized probe. Proteins bound to 292TC/biotin are then enriched by extraction onto avidin beads and then digested with trypsin on the beads yielding peptides that are identified using mass spectrometry. Two proteins were identified in the testes of both mice and rats that bound to 292TC, retinal dehydrogenase 1 (ALDH1A1), and retinal dehydrogenase 2 (ALDH1A2). These enzymes are critical in catalyzing the chemical reaction converting retinal to retinoic acid (Fig. 2). Literature confirmed that inhibition of these enzymes was linked with testicular toxicity through suppression of spermatogenesis (Paik et al., 2014; Arnold et al., 2015; Kent et al., 2016). Molecular modeling was then used to demonstrate that CC-292 may fit into the NAD and retinal

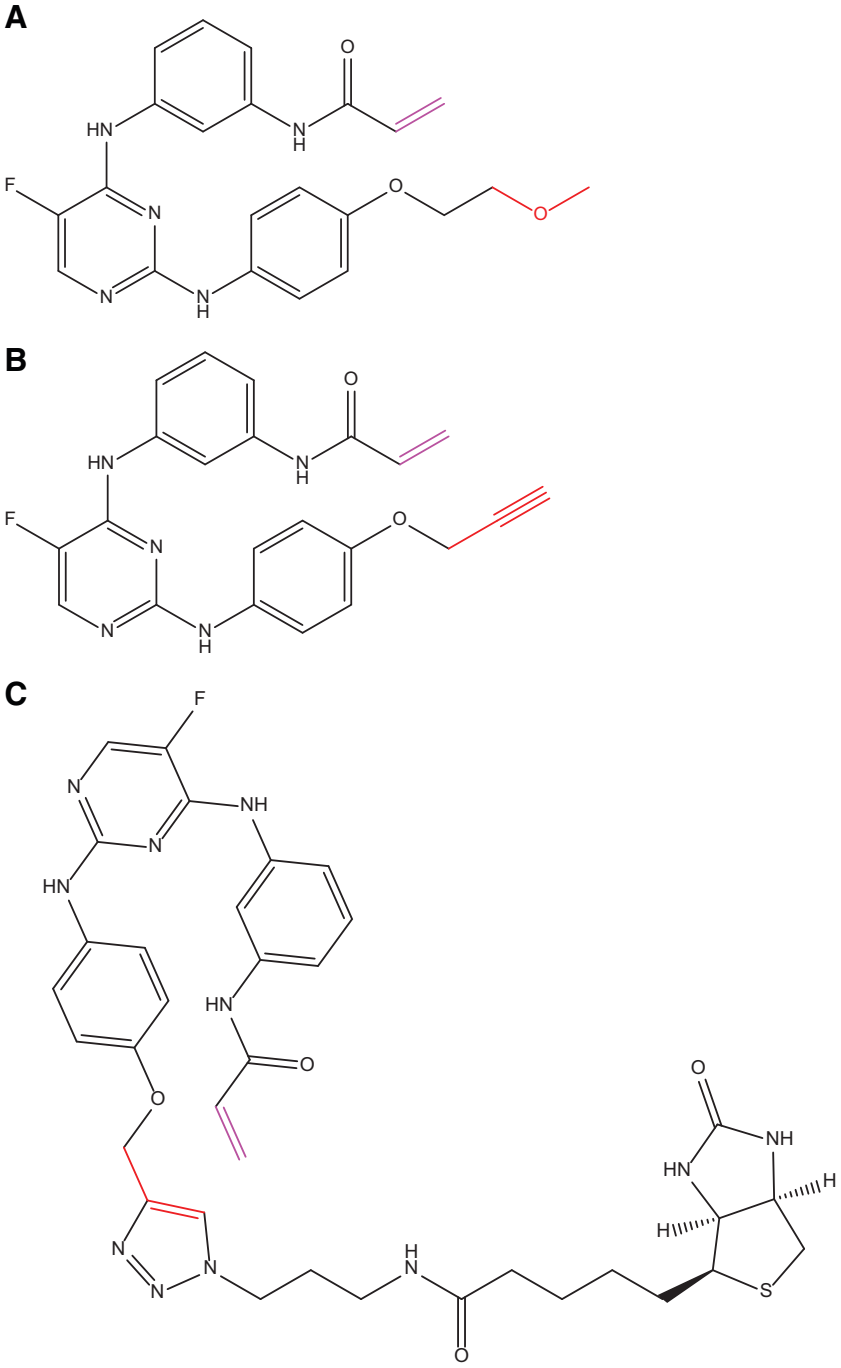

Fig. 1. Chemical structures for CC-292, 292TC, and the 292TC/biotin conjugate. (A) CC-292 (*N*-(3-(5-fluoro-2-(4-(2-methoxyethoxy)phenylamino)pyrimidin-4-ylamino)phenyl)acrylamide) has an aryl acrylamide group (magenta) that can irreversibly react with an active site cysteine residue in BTK or an off-target binding site. CC-292 contains a methoxy group (red) that is replaced with an alkyne moiety in its analog 292TC. (B) 292TC retains the aryl acrylamide group (magenta) and has an alkyne moiety (red) that can react with biotin azide using click chemistry. (C) 292TC conjugated with biotin azide containing the aryl acrylamide portion (magenta). Biotin can be used to immobilize the conjugate with any bound proteins on to streptavidin beads. Bound proteins may then be identified by mass spectrometry.

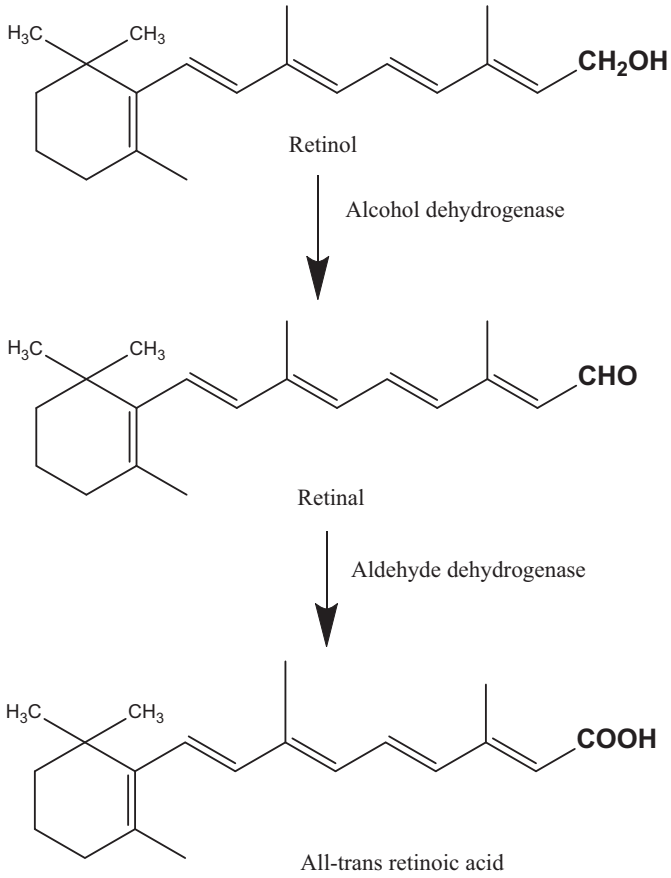

Fig. 2. The role of ALDH1A enzymes in the conversion of retinol to all-*trans* retinoic acid. Schematic showing two steps in the enzyme catalyzed conversion of retinal to retinoic acid.

binding pockets of the analogous human ALDH1A2 enzyme, thus suggesting that this may be a concern for male human reproductive health. These proteomics data presented a hypothesis upon which to base a rational redesign of CC-292 to produce a BTK inhibitor that does not interact with ALDH1A1 or ALDH1A2.

Materials and Methods

Test Articles

Celgene compound 292 (CC-292) (Fig. 1A) and its structural analog 292TC (Fig. 1B) were synthesized and found to be of suitable quality for nonclinical experiments. For chemoproteomics, the alkyne group on 292TC can be reacted with the azido moiety of a biotin azide. This allowed the formation of a 292TC/biotin conjugate (Fig. 1C) that can be captured by streptavidin beads, thereby enabling the enrichment of proteins binding to the probe from complex mixtures, such as tissue homogenates.

Animal Care and Use

All in vivo experiments were performed in strict accordance with the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All protocols were approved by the Institutional Animal Care and Use Committees at Covance, Inc. or Huntingdon Life Sciences, and appropriate efforts were made to reduce animal suffering. Toxicology studies were conducted according to U.S. Food and Drug Administration Good Laboratory Practice regulations, 21 CFR part 58.

C57BL/6 (wild-type) male mice (10 per group) were obtained from Taconic Farms (Germantown, NY). Male mice were individually housed in solid-bottom, polycarbonate cages with contact bedding. Mice were randomized into dose groups based on body weight and were administered test article by oral gavage at dosage levels of 0 [vehicle: 1% (w/v) methylcellulose, 0.1% (v/v) Tween 80 in deionized water], 100, and 300 mg/kg/day for a period of at least 28 days. The mice were 7–8 weeks old at dosing initiation ranging from 17.4 to 25.0 g in weight.

Sprague-Dawley (Crl:CD[SD]) male rats (10 per group) were obtained from Charles River Laboratories (Stone Ridge, NY). Animals were pair-housed in solid-bottom, polycarbonate cages with contact bedding. Rats were randomized into dose groups based on body weight and were administered test article by oral gavage at dosage levels of 0 [vehicle: 1% (w/v) methylcellulose, 0.1% (v/v) Tween 80 in deionized water], 30, 75, or 200 mg/kg/day for at least 90 days. The rats were 7 weeks old at dosing initiation ranging from 180 to 240 g in weight.

All animals were kept at a constant room temperature and maintained on a controlled environment with a 12-hour light:12-hour dark cycle. Food and water were provided ad libitum. After 1 week of adaptation, the mice or rats were randomized into the different dose groups according to body weight.

Macroscopic and Histopathologic Evaluations of Testes

For mice, animals were anesthetized with sodium pentobarbital, exsanguinated, and necropsied. For rats, animals were euthanized by CO_2 or isoflurane inhalation, and this was followed by exsanguination. All testes were weighed and then collected in modified Davidson's fixative (Latendresse et al., 2002) and stored in 10% neutral-buffered formalin before processing to slides for microscopic examination.

Procurement of Testes Homogenate

Mice or rats were euthanized by CO_2 inhalation, and testicles were removed, placed in a clean tube, and processed as follows. For mice, one full testis and, for rats, one quarter of a testis were placed in a beaded Precellys tube along with 1 ml of ice-cold lysis buffer [1% (v/v) Triton X-100 in Tris-buffered saline with protease inhibitors] (Roche, Pleasanton, CA). Samples were homogenized in the Precellys homogenizer (Bertin Instruments) with two 15-second pulses. The homogenized samples were then transferred to a clean centrifuge tube and centrifuged at 14k $\times$ g for 5 minutes at 4°C. The clarified homogenate was transferred to a clean tube and diluted to 1 mg/ml protein content with lysis buffer.

Compound Treatment of Testes Homogenate and Preparation of Peptides for Mass-Spectrometry Analysis

For identification of the 292TC adductome (discovery chemoproteomics), homogenates were incubated with DMSO or the covalently binding 292TC using 1 ml of homogenate at 1 mg/ml total protein and 10 μM of compound for 1 hour at room temperature. The probe 292TC, which differs slightly in structure from CC-292 in that it contains an alkyne group in place of a methoxy group (Fig. 1), allows the use of Cu(I)-catalyzed azide-alkyne-cycloaddition "click chemistry" (Rostovtsev et al., 2002) for conjugation with biotin. For biotinylating the covalently binding analog, the following stock solutions were prepared in water: 10 mM biotin azide, 23 mM Tris-hydroxypropyltriazolylmethylamine, 12.53 mM copper sulfate, and 100 mM sodium ascorbate. Biotinylation was achieved by adding 10 μl of each of these reagents to 400 μl of testes homogenate and incubating for 60 minutes at room temperature. After biotinylation, 50 μl of 100% trichloroacetic acid was added to precipitate the protein, and this was followed by 2 $\times$ 500 μl acetone washes. The pellet was resuspended in 250 μl of 50 mM phosphate buffer (pH 7.2) with 6 M urea. To this mixture, 2.5 μl of a 1 M dithiothreitol freshly prepared solution, and 70 μl of 10% SDS was added and then incubated at 80°C for 20 minutes. After cooling to room temperature, 20 μl of 0.5 M

iodoacetamide was added at room temperature for 30 minutes in the dark. The sample was diluted to a total volume of 3 ml in PBS. High-capacity streptavidin agarose beads (Thermo Fisher, 20357), 100 μl, were added to a 3-ml syringe with a porous polypropylene frit. The protein solution was pulled into the syringe and then incubated while rotating for 4 hours with gentle mixing. The beads were then washed with 3 ml of 1% SDS in PBS then six times with PBS alone. On-bead digestion was performed by adding 200 μl of trypsin (10 μg/ml) dissolved in 25 mM ammonium bicarbonate, 2 M urea, and 1 mM calcium chloride to the syringe and set to rotating overnight at 37°C. At the end of the digestion, the solution was pushed out into an Eppendorf LoBind tube. The syringe was then rinsed with 300 μl of water, and the rinse combined with the digested peptide sample. Subsequently, 5 μl of 5% trifluroacetic acid was added to quench the reaction. The peptides were purified using 200 μl C18 ziptips and the manufacturers protocol (Millipore, Burlington, MA). Peptides were eluted from the ziptip with 80% acetonitrile/0.1% trifluroacetic acid into a clean Eppendorf LoBind centrifuge tube and then dried in a vacuum centrifuge. The peptides were reconstituted in 20 μl of 0.1% formic acid, sonicated, vortexed, centrifuged, and transferred to a high-pressure liquid chromatography vial.

Competition experiments were conducted by pretreating testes homogenates first with CC-292, and this was followed by incubation with 292TC. A similar competition approach was used with ibrutinib, a marketed covalently binding BTK inhibitor, and a structural analog that can be modified for affinity enrichment similar to 292TC with biotin. Quantification of competition between the parent and analog molecules was performed in a targeted mass-spectrometer analysis measuring peptide intensities specific to ALDH1A2.

Mass-Spectrometry Methods

Discovery Mass Spectrometry. Peptides from the discovery experiment were prepared as described above, and 2 μl of the sample was injected, separated, and analyzed by nanospray liquid chromatography tandem mass spectrometry. A PicoChip analytical column (NewObjective, Woburn MA) packed with Reprosil-PUR C18-AQ, 3-μm beads was preceded by a 2-cm trap column (ThermoFisher). One microgram of total peptide was injected using a gradient of mobile phase A (water, 0.1% formic acid) and mobile phase B (80% acetonitrile, 0.1% formic acid) of 3%–20% B over 75 minutes, 20%–30% B over 15 minutes, 30%–50% B over 4 minutes, and 50%–98% B over 4 minutes at a flow rate of 300 nl/min (ThermoFisher Easy-nLC 1200 system).

Data are acquired using a high mass accuracy Orbitrap Q Exactive-HF. The instrument is operated in data-dependent mode to automatically switch between full-scan mass spectrometry (MS) and MS/MS acquisition. Full-scan MS spectra are acquired in the Orbitrap with 120,000 resolution, 3×106 target AGC value, and dynamic exclusion set to 45 seconds. The 20 most intense multiply charged ions ($2 \leq z \geq 8$) are subsequently fragmented in Orbitrap by HCD with a target value of 5×105 ions, maximum injection time of 100 ms, and 30,000 resolution. Source conditions are as follows: spray voltage of 2.2 kV, no sheath and auxiliary gas flow, heated capillary temperature 275°C, and normalized collision energy 27% with a 1.4-m/z isolation window.

Thermo .RAW data files are searched via ProteomeDiscoverer 1.4 using SequestHT search algorithm and Percolator for postsearch validation (Käll et al., 2007; Spivak et al., 2009). The search is performed against the SwissProt version of the appropriate organism database from UniProt (http://www.uniprot.org/). Search settings include the following: semitryptic digestion, MS1 tolerance of 10 ppm, MS2 tolerance of 0.02 Da, static modification of 57.021464 Da on cysteines representing carbamidomethylation from iodoacetamide treatment, and dynamic modifications of 15.995 Da on methionine representing oxidation.

Targeted Mass Spectrometry. Peptides (mouse testes) from the competition experiment were prepared as described above, and 1 μl of the sample was injected into a 10-cm PicoChip column filled with 3 μM Reprosil-PUR C18 (New Objective, Woburn, MA). The system was operated at 2.6 kV using an Easy-nLC 1200 (Thermo) high-pressure liquid chromatography system. The mobile phase buffer consists of 0.1% formic acid in water. The eluting buffer is 0.1% formic acid (buffer A) in 100% acetonitrile (buffer B). The liquid chromatography flow rate was 300 nl/min. The gradient was set as 0%–80% buffer B for 30 minutes. The sample was analyzed on a TSQ Quantiva Triple Stage Mass Spectrometer (Thermo, Germany). Each sample was analyzed under multiple reaction monitoring with an isolation width of ±0.7 Th for both Q1 and Q3. An unscheduled multiple reaction monitoring–MS method containing about six transitions per peptide was used for the data acquisition with an average peak width of 15 seconds at baseline and a minimum number of points per peak of 10, resulting in dwell times of 1.5 ms. Peak area, height, and FWHM = full width at halfmaximum were calculated from the most abundant transition for each peptide using Skyline. For mouse ALDH1A1, the sum of three peptides (^{421}ANNTTYGLAAGLFTK^{435}, and ^{398}EEIFGPVQQIMK^{410}, and ^{379}GFFVQPTVSNVTDEMR^{395}) were quantified using Skyline and plotted in Fig. 5. For mouse, ALDH1A2, the sum of four peptides (^{494}EMGEFGLR^{501}, ^{371}VLELIQSGVAEGAK^{384}, ^{283}VTLELGGK^{290}, and ^{259}IAFTGSTEVGK^{269}) was quantified using Skyline and plotted in Fig. 5.

Computational Methods

All the modeling described in this section was completed using the Schrodinger Suite version 2015-01. PyMOL was used as the visualization tool and for generation of images for publication.

CC-292 was prepared using LigPrep with default settings. CC-292 was then docked into the ALDH1A2 crystal structure (PDB 6ALJ) using GlideSP (Friesner et al., 2004; Halgren et al., 2004), which uses the OPLS3 force field. Reduced van der Waals radii were used to allow for some flexibility in the binding pocket. The predicted top-10 scoring poses according to GlideScore were collected. Finally, by applying molecular mechanics generalized Born surface area scoring to each of the predicted poses, they were rescored to identify the top likely binding pose. As an additional step, a covalent bond was manually imposed between the acrylamide reactive group and the thiol of Cys 320. This conjoined CC-292 and the surrounding binding site residues were minimized to observe any conformational changes resulting from the covalent bond formation. The same protocols were used for modeling CC-292 into the retinal binding site of ALDH1A2 as were used with the NAD binding site modeling.

Results

Pathologic Changes in the Male Reproductive Organs of Rodents. CC-292 was administered to male C57BL/6 mice at doses of 0, 100, and 300 mg/kg/day for up to 28 days. Microscopic evaluation of tissues from male mice at 100 and 300 mg/kg/day revealed the presence of testicular lesions. These manifested as minimal to moderate degeneration of the epithelial lining of the seminiferous tubules with prominent syncytial cell formation and a decrease/absence of elongated spermatids (Fig. 3). This correlated with a decrease in mean absolute testis weight of –40% at 300 mg/kg/day compared with concurrent controls.

CC-292 was administered to male Sprague-Dawley rats by oral gavage at doses of 0, 30, 75, and 200 mg/kg/day for up to 93 days. Minimal to severe tubular degeneration/atrophy in the testis and increased germ cell debris and/or reduced sperm in the epididymis were noted at ≥30 mg/kg/day, when severity increased with dose. This correlated with small and soft testis and epididymis with decreased weights in both organs at ≥75 mg/kg/day. Additionally, a decrease in motile sperm (sperm that are swimming) was observed at ≥75 mg/kg/day and

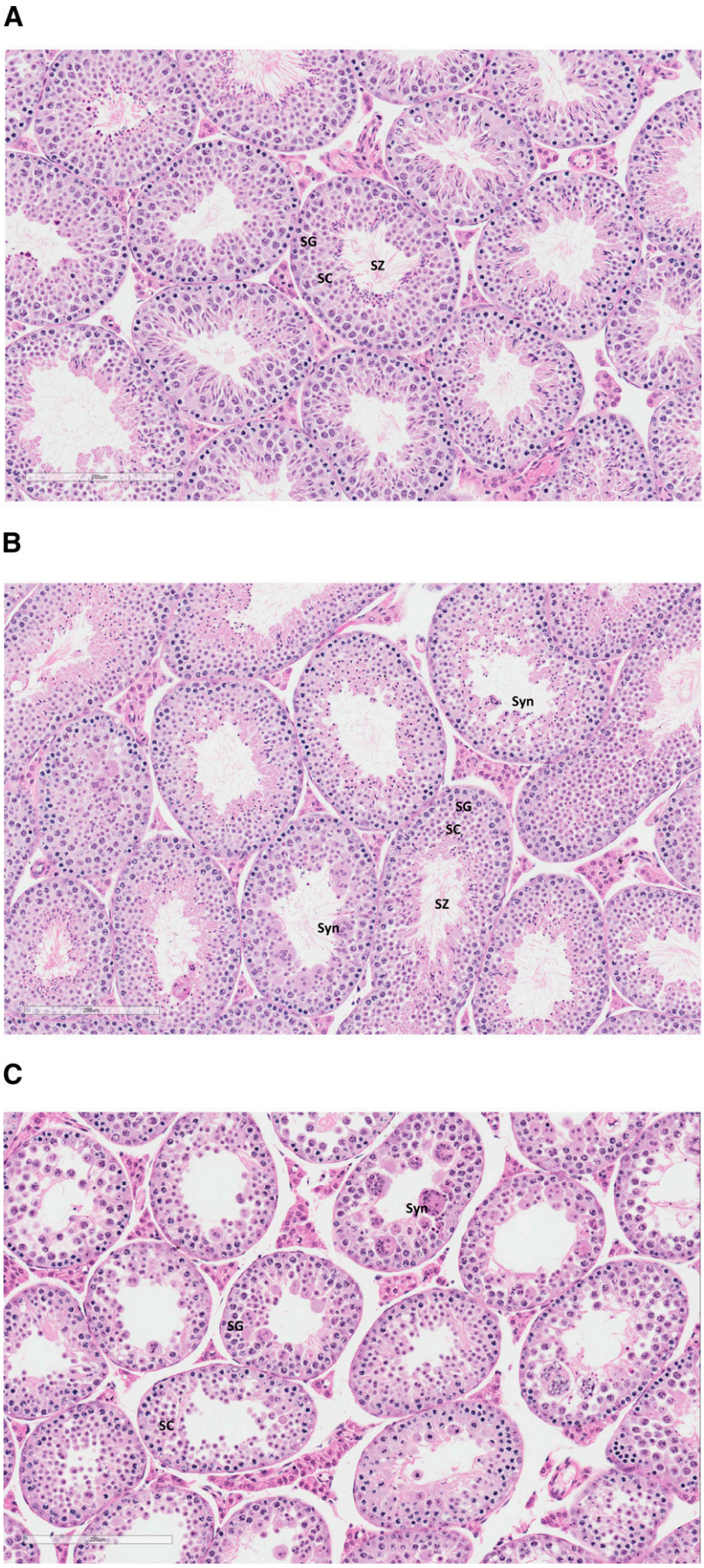

Fig. 3. Hematoxylin and eosin–stained sections of mouse testes after exposure of the mice to CC-292. Representative sections are displayed from mice dosed once daily for 28 days with either (A) vehicle, (B) 100 mg/kg/day CC-292, or (C) 300 mg/kg/day CC-292. Testicular lesions manifested as minimal to moderate degeneration of the epithelium lining of the seminiferous tubules with prominent syncytial cell (Syn) formation and a decrease (B) or absence (C) of elongated spermatozoa (SZ). Spermatocytes (SCs) and spermatogonia (SG) were largely unaffected.

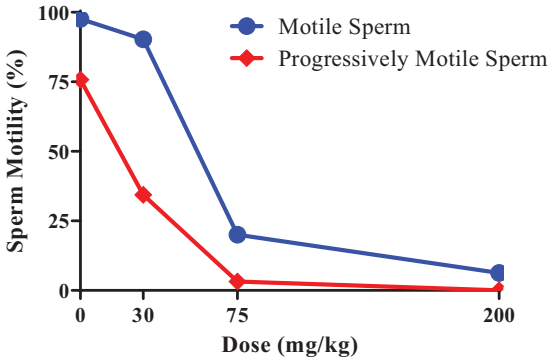

Fig. 4. Mean sperm motility endpoints in rats. Sperm analysis was conducted in rats. Representative data demonstrate a dose-dependent decrease in mean values for percentage of motile and progressively motile sperm in rats administered CC-292 for up to 3 months. This correlated with decreased absolute sperm counts, decreased testicular and epididymis weights, and increased severity of histopathology.

progressively motile sperm (sperm that are swimming in a mostly straight line or large circles) at ≥30 mg/kg/day (Fig. 4). There was also a decrease in sperm counts in the epididymis and testis at ≥75 mg/kg/day and increased number of decapitated sperm at ≥30 mg/kg/day.

Chemoproteomic Enrichment and Identification of Proteins Irreversibly Binding CC-292. An unbiased chemoproteomics approach was used to identify the CC-292 adductome or proteins that have formed a covalent bond with CC-292 in rat and mouse testes homogenates to gain insight into protein targets responsible for the observed testicular toxicity. For this purpose, a structurally close analog of CC-292 was designed as a probe molecule, 292TC (Fig. 1B), in which the methoxy group is replaced with an alkyne moiety. 292TC retains the aryl acrylamide that can irreversibly react with an active site cysteine in BTK or an off-target protein binding site. The alkyne moiety accommodates biotin attachment via click chemistry so that the irreversibly tagged protein can subsequently be immobilized on streptavidin beads for identification.

Testes homogenates were treated with DMSO (to determine the nonspecific proteins pulled down) or 292TC for 1 hour at room temperature. The analog was then biotinylated while protein bound to allow 292TC/protein complexes to be captured using streptavidin beads. After on-bead protein digestion with trypsin, liberated peptides were sequenced on an Thermo QE-HF mass spectrometer, and the derivative proteins identified. The samples treated with DMSO were used to differentiate nonspecific proteins from those actually covalently modified by 292TC. Two and seven proteins were uniquely identified as targets for 292TC in rat and mouse testes, respectively (Table 1). The two rat proteins, retinal dehydrogenases 1 and 2/ALDH1A1 and ALDH1A2, were among the seven identified in the mouse. Similarly, occupancy by CC-292 was demonstrated in mouse testes lysates by incubating the homogenates with either the 292TC alone or with CC-292 followed by 292TC, each for 1 hour, and the two results were compared. Figure 5 shows that, for both ALDH1A1 and ALDH1A2, CC-292 competed with 292TC by occupying ∼60% and 90% of the two proteins, respectively, as quantified by peptide intensity compared with 292TC alone. A similar experiment was performed using ibrutinib, a known covalent BTK inhibitor, and a corresponding analog that may be used similarly to 292TC, but no significant ALDH peptides were detected (Fig. 5).

Inhibition of aldehyde dehydrogenase 1A2 has been implicated in testicular toxicity. Specifically, studies have demonstrated that ALDH1A1 and ALDH1A2 catalyze the production of retinal to retinoic acid (Fig. 2). Retinoic acid is a key signaling molecule with several biologic roles, including spermatogenesis. Reports have shown that inhibition of the ALDH1A enzymes, usually with cysteine reactive covalent compounds that are targeted to this enzyme, results in impaired retinoic acid synthesis (Paik et al., 2014; Arnold et al., 2015; Chen et al., 2018) and testicular toxicity.

In Silico Modeling of CC-292 in Human ALDH1A2. In silico docking of CC-292 to ALDH1A2 was used as a means of predicting how it might bind and potentially translate to humans. CC-292 was modeled into both the NAD (Fig. 6A) and the retinal (Fig. 6B) binding sites using the published human crystal structure PDB 6ALJ. These two sites are independent and nonoverlapping and are on opposite sides of Cys 320. Both models predicted that the inhibitor could bind in productive low-energy conformations without imposing a covalent bond for either site. Both modes permit easy access to Cys 320 with the acrylamide reactive group proximal to the Cys 320 thiol. The two binding modes are both compatible with the two rotamer conformations of Cys 320 described in the published crystal structures. Based on modeling scores as well as observation, a stronger binding in the retinal binding site is predicted without ruling out binding in the NAD site.

TABLE 1

Rodent proteins that bound to the 292TC/biotin conjugate that were positively identified by mass spectrometry

Rodent testes/CC-292 adduction profile. A list of proteins that were identified by mass spectrometry after binding to the 292TC/biotin conjugate in testicular homogenates from rats and mice and enrichment on streptavidin beads. The database sequence for each protein that was positively identified is presented.

Species	Protein	Sequence Identifier
Rat	Retinal dehydrogenase 1	P51647
	Retinal dehydrogenase 2	Q63639
Mouse	Retinal dehydrogenase 1	P24549
	Retinal dehydrogenase 2	Q62148
	Testis-expressed sequence 101 protein	Q9JMI7
	Protein disulfide-isomerase A3	P27773
	Protein disulfide-isomerase A6	Q922R8
	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	Q8VC30
	Transitional endoplasmic reticulum ATPase	Q01853

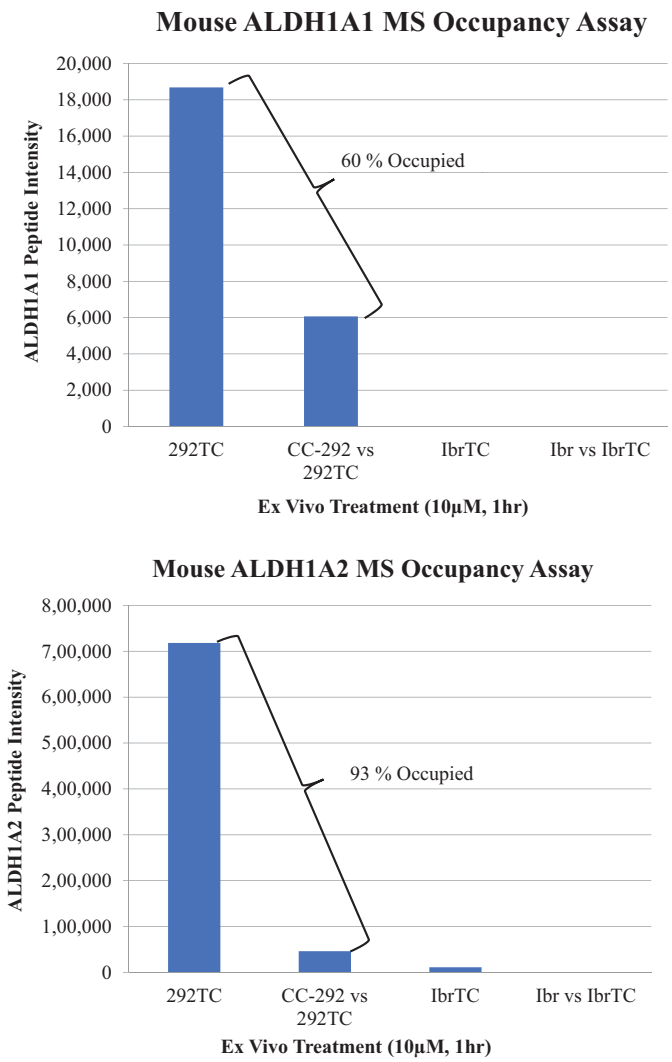

Fig. 5. CC-292 competes with 292TC for binding to ALDH1A1 and ALDH1A2. This experiment demonstrates the 292TC/biotin conjugate is binding to proteins that may also bind to CC-292. In similar experiments, the marketed BTK inhibitor Ibrutinib (Ibr) did not compete with its biotin conjugating analog (IbrTC) demonstrating that it does not bind to ALDH1A enzymes.

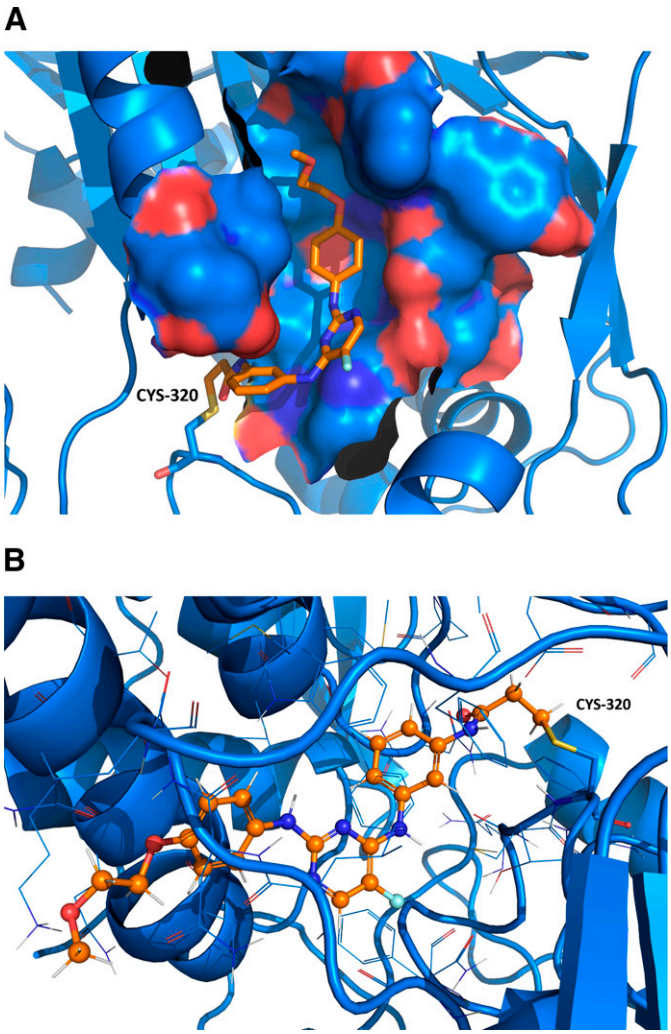

Fig. 6. A model of CC-292 docked into an ALDH1A2 crystal structure. Association of CC-292 from both the NAD binding site (A) and retinal binding site (B) show that it can covalently bind to Cys 320.

Imposing the covalent bond on either the NAD or retinal binding site modes does not add any additional torsional stress or constraints. Therefore, no conformational changes were necessary to accommodate the covalent bond. Chen et al. (2018) showed that WIN 18,446, a covalently binding inhibitor of ALDH1, binds to the retinal pocket and forms a covalent bond with Cys 320. The modeling scores suggest a higher binding affinity for CC-292 at this same site. In contrast to the S_N2 nucleophilic displacement reaction suggested between WIN 18,446 and the Cys 320 thiol, a CC-292 adduct would likely proceed via a Michael addition of the thiol nucleophile to the acrylamide β-carbon in CC-292.

The amino-acid sequence of ALDH1A2 is highly conserved, with >97% sequence identity across mouse, rat, and human. Cys 320 is conserved in all three. A similar argument can be made for ALDH1A1 even though the overall sequence identity is somewhat less. There is a strong conservation of sequence homology in the NAD and retinal binding sites on both ALDH1A1 and ALDH1A2 from mouse, rat, and human (Fig. 7). Therefore, it is possible that the testicular toxicity observed in rodents is translatable and of potential concern in humans.

Discussion

Understanding the molecular basis of toxicity can empower decision making on how to advance a drug candidate and what aspects may need refinement within back-up molecules as well as possibly justifying termination of development. It is challenging to determine specific causes for off-target binding site toxicity because of the vast complexity of potential interactors with drug candidates. Chemoproteomic approaches offer a useful tool for addressing this challenge. This approach was used here to identify ALDH1A2 as an off-target protein that interacted with the covalently binding BTK inhibitor CC-292, and that may have contributed to testicular toxicity observed in mice and rats. Note that covalent inhibition of the ALDH1A enzymes is not a general attribute of covalent BTK inhibitors but is compound- or chemotype-specific as

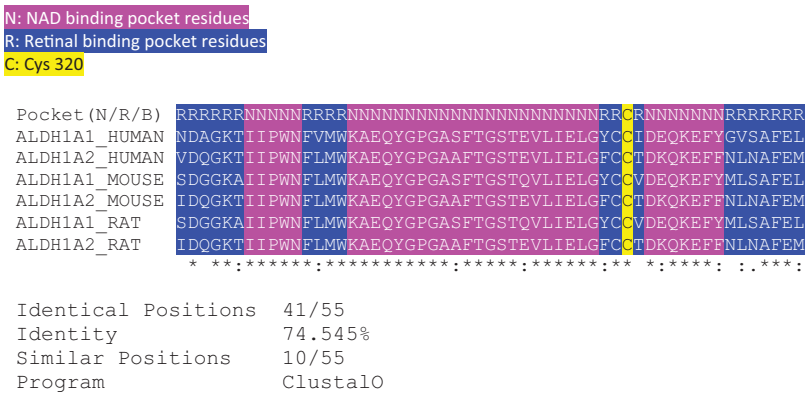

Fig. 7. Alignment of human, mouse, and rat ALDH1A1 and ALDH1A2 NAD and retinal binding Sites. A 50-ns molecular dynamics simulation of ALDH1A2 structure (PDB 6ALJ) was polled once every 1 ns to collect 50 individual structures. The residues forming the binding sites in each of these 50 individual structures were determined. A cumulative set of residues was determined from these 100 binding sites. This cumulative set of residues was used to define the NAD and retinal binding sites in the aligned protein sequences.

evident by comparing CC-292, which can bind irreversibly to the dehydrogenase, and ibrutinib, which does not. BTK itself is not likely to be involved in the maintenance of male fertility, as tissue expression is mostly restricted to lymphoid organs and certain immune cell populations (Human Protein Atlas available from http://www.proteinatlas.org). This explains how BTK itself was not detected in the rat or mouse adductomes described here. Conversely, ALDH1A enzymes are highly expressed in male reproductive tissues (Human Protein Atlas available from http://www.proteinatlas.org) and are critical in metabolizing retinal to all-*trans* retinoic acid, which activates the retinoic acid responsive element critical for spermatogenesis (Fig. 2). WIN 18,466, a potent pan-ALDH1A inhibitor, has been reported to inhibit spermatogenesis in mammals with morphology consistent with the testicular toxicity observed in the rodent toxicity studies using CC-292 (Paik et al., 2014). In silico docking models of CC-292 suggest two potential binding modes involving the NAD and retinal binding pockets that place the covalent acrylamide moiety of CC-292 adjacent to a conserved cysteine on ALDH1A2 (Fig. 6). When the cysteine is bonded to CC-292, the binding mode and protein conformation are unchanged, supporting the feasibility of these modes. Either covalent binding mode would irreversibly inhibit the enzyme and is likely to interfere with the enzyme's ability to convert retinal to retinoic acid. This chemoproteomics work demonstrates that CC-292 covalently binds to ALDH1A1. Both the human NAD and retinal binding sites of ALD1A1 and ALDH1A2 share significant homology between them and across mice, rats, and humans (Fig. 7). In the literature, ALDH1A2 is ascribed a major role in retinoic acid production, but in as much as ALDH1A1 is involved, this family member is also likely inhibited by CC-292.

Although it is well established that all-*trans* retinoic acid is essential for spermatogenesis, and the major enzymatic source for this is through ALDH1A enzymes, this may not be absolute. Beedle et al. (2019) used a conditional ALDH1A2 germ cell knockout, a tamoxifen-induced ALDH1A2 knockout model, and the pan-ALDH1A inhibitor WIN 18,446 to show that postnatal mice severely deficient in ALDH1A2 can under these controlled conditions proceed normally through spermatogenesis. The authors suggest an additional source of all-*trans* retinoic acid generation independent of the ALDH1A family may be possible. Nonetheless, they and others have consistently demonstrated that chronic inhibition of this family using WIN 18,466 does result in degenerative seminiferous tubules, and therefore these enzymes are certainly important for testicular health.

Given the high degree of conservation of the ALDH1A enzymes between rodents and humans, there is a possibility that CC-292 could affect male human fertility as a testicular toxicant. With this information, informed decisions on the future direction for development of CC-292 and the design of next generation molecules can be made. Target identification and chemoproteomics are most commonly associated with on-target mechanism of action discovery and phenotypic screening. Here we demonstrate that its role in determining potential causes of organ toxicity can be equally useful.

Authorship Contributions

Participated in research design: Labenski, Voortman, Medikonda, Sherratt.

Conducted experiments: Labenski, Voortman, Medikonda, Sherratt.

Contributed new reagents or analytic tools: Labenski, Voortman.

Performed data analysis: Labenski, Voortman, Medikonda, Sherratt, Corin.

Wrote or contributed to the writing of the manuscript: Labenski, Voortman, Medikonda, Sherratt, Corin.

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