

Casticin Inhibits AKR1C3 and Enhances Abiraterone Efficacy in Castration-Resistant Prostate Cancer

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Abstract

Castration-resistant prostate cancer (CRPC) remains a major therapeutic challenge due to the development of resistance to androgen deprivation and next-generation antiandrogens such as abiraterone (ABI). One of the key mechanisms underlying this resistance involves overexpression of aldo-keto reductase 1C3 (AKR1C3), an enzyme contributing to intratumoral androgen biosynthesis. In this study, casticin (CAS), a flavonoid derived from *Vitex agnus-castus*, was identified as a potent inhibitor of AKR1C3. CAS demonstrated inhibitory activity in enzymatic assays ($IC_{50} = 5.99 \mu M$), effectively reduced AKR1C3-dependent coumestrol metabolism in 22Rv1 prostate cancer cells, and exhibited cytotoxicity preferentially in AKR1C3-expressing 22Rv1 cells compared to AKR1C3-low LNCaP cells. Importantly, CAS enhanced the antitumor efficacy of ABI in 22Rv1 cells, showing a synergistic effect (Combination Index 0.31–0.71), while no synergy was observed in LNCaP cells or in combination with enzalutamide. Molecular docking and dynamics simulations revealed stable binding of CAS in the AKR1C3 active site, with key hydrogen bonding and aromatic interactions supporting its inhibitory mechanism. These findings position CAS as a promising chemosensitizing agent that targets AKR1C3 to overcome ABI resistance in CRPC.

Introduction

Prostate cancer is one of the most common malignancies among men worldwide and poses growing public health burden, particularly in developed countries. It accounts for a significant proportion of cancer diagnoses and deaths, with its incidence increasing due to aging populations and improved diagnostic techniques. Despite considerable advances in screening, early detection, and therapeutic approaches, prostate cancer remains a leading cause of cancer-related morbidity and mortality in men [1].

A major challenge in prostate cancer management is the development of resistance to conventional treatments. While androgen deprivation therapy is initially effective in most patients with advanced disease, tumors often progress to a more aggressive and lethal stage known as castration-resistant prostate cancer (CRPC) [2]. In recent years, new-generation antiandrogens such as enzalutamide (ENZ) and androgen biosynthesis inhibitors like abiraterone (ABI) have improved patient outcomes. However, resistance to these agents commonly arises, significantly limiting their long-term efficacy and emphasizing the urgent need for alternative therapeutic strategies [3].

One molecular target that has gained interest in the context of therapy resistance is aldo-keto reductase 1C3 (AKR1C3; 17 β -hydroxysteroid dehydrogenase type 5). This enzyme plays

a critical role in intratumoral androgen biosynthesis by catalyzing the NADPH-dependent reduction of 4-androstene-3,17-dione (androstenedione) to testosterone and of 5 α -androstane-3,17-dione to dihydrotestosterone (DHT). These reactions are key steps in the intracrine production of potent androgens that sustain androgen receptor (AR) signalling in castration-resistant prostate cancer (CRPC), even in the presence of systemic androgen deprivation [4, 5]. Additionally, AKR1C3 acts as an AR

coactivator and stabilizes AR by preventing its ubiquitination [6, 7]. High expression of AKR1C3 is associated with poor prognosis in prostate cancer, and its levels correlate with androgen receptor splice variants (e.g., AR-V7), which exhibit ligand-independent activity [7–9]. As a promising drug target to overcome resistance, inhibitors of this enzyme are being sought from natural sources and through rational drug design. ASP9521 was tested in clinical trials for prostate cancer, and currently, an IND for a novel AKR1C3 inhibitor is under evaluation [4, 10]. Interestingly, several natural flavonoids have been identified as AKR1C3 inhibitors, including genistein, liquiritigenin, and 2'-hydroxyflavanone [11–13].

Casticin (CAS), a major flavonoid derived from Chaste tree (*Vitex agnus-castus* L.; VAC) fruits, has demonstrated promising anticancer activity in several types of malignancies, including breast, lung, and prostate. Its ability to inhibit cell proliferation, induce apoptosis, and interfere with key oncogenic signalling pathways makes it a candidate of interest in the search for novel treatments [14]. Interestingly, while VAC is generally used in the treatment of premenstrual syndrome, mastalgia, and hyperprolactinemia, Historically, it served as an antiandrogenic agent.

VAC was traditionally used in ancient Rome as an anaphrodisiac, with seed and leaf preparations believed to suppress sexual desire. In Christian monasteries, it was known as "monks' pepper" for its role in supporting celibacy. Classical authors like Pliny the Elder, Dioscorides, and Theophrastus associated it with chastity and infertility. Its Latin name - *agnus castus* - reflects this symbolic and practical role in promoting sexual abstinence [15]. There are also some modern studies suggesting the potential of VAC in decreasing the serum level of testosterone [16, 17].

Based on the significant role of CAS in the pharmacological activity of VAC and historical reports of the plant's antiandrogenic effects, CAS, as a flavonoid constituent, was investigated as a potential inhibitor of AKR1C3. Given the enzyme's established role in resistance to current androgen-targeting therapies, identifying novel AKR1C3 inhibitors is of particular therapeutic relevance [10]. Moreover, the well-documented anticancer properties of CAS, including its ability to inhibit proliferation, induce apoptosis, and modulate key oncogenic pathways, make it a strong candidate for further evaluation in the context of treatment-resistant prostate cancer [14]. Additionally, CAS presents preferred physical-chemical and pharmacokinetics properties, including oral bioavailability in rats making the molecule a genuine drug candidate [18].

CAS was evaluated as an inhibitor in a kinetic study with recombinant AKR1C3. It also inhibited AKR1C3-dependent coumestrol reduction. CAS was also found to reverse resistance to ABI in castration resistant 22Rv1 AKR1C3-positive prostate cancer cell line, in contrast to LNCaP prostate cancer cell line which lacks AKR1C3 expression. The basis of CAS interactions with AKR1C3 was investigated using molecular docking and molecular dynamics simulations.

Materials and methods

Chemicals

CAS (98% purity) and ENZ (98%) were from Cayman Chemicals. Abiraterone acetate (99%) was from Thermo Scientific Chemicals. 9,10-Phenanthrenequinone (95%), indomethacin (IND, 98%) were from Sigma Aldrich. Coumestrolone (99%) and ASP9521 (98%) were from MedChem Express. Studied chemicals were dissolved in DMSO (Sigma Aldrich) to a concentration of 25 mM. Next they were diluted in buffers and media. Appropriate DMSO vehicle controls were prepared.

Recombinant AKR1C3

Expression of AKR1C3 recombinant protein was performed in *Escherichia coli* Rosetta (DE3) transformed with pET21b carrying an *E. coli*-codon-optimized sequence coding for hAKR1C3 introduced between NdeI/XhoI restriction sites (GenScript). Cells were grown in LB medium supplemented with ampicillin (100 µg/ml) at 37°C till the optical density at 600 nm reached the value of 0.6–0.8, then temperature was lowered to 22°C and the expression of recombinant protein was induced by IPTG (final concentration 1 mM), and the cells were cultivated for further 4 h. Then, bacteria were harvested by centrifugation (10,000 g, 15 min), resuspended in Lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 10 mM imidazole), and sonicated. The lysate was clarified by centrifugation (21,000 g, 20 min) and loaded on NiNTA resin. Subsequently, the column was washed with Lysis buffer, and AKR1C3 was eluted with Elution buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 500 mM imidazole). The protein was further purified by gel filtration in 20 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM DTT, 1 mM EDTA using HiLoad 26/600 Superdex 200 pg. The procedure resulted in a pure preparation of AKR1C3, as evidenced by SDS-PAGE.

AKR1C3 inhibition

A reaction mixture contained 0,1 M phosphate buffer (pH 7.4), recombinant AKR1C3 enzyme (0,2 µM), substrate (9,10-Phenanthrenequinone, 10 µM), and CAS or reference inhibitor in a concentration range. 200 µM NADPH was added to initiate the reactions, and NADPH oxidation process was determined by measuring the decrease in absorbance at 340 nm (SpectraMax® iD3, Molecular Devices) over 10 minutes. The velocity of the reaction was calculated in OriginPro, by a linear regression method. %decrease in reaction velocity was calculated against the control reaction (vehicle control). DMSO at used concentrations (0,7%) did not influence the enzyme activity.

Cell culture

22Rv1 (ATCC, CRL-2505) and LNCaP (ECACC, CRL 1740) prostate cancer cell lines were cultured in standard conditions (37°C, 5% CO₂) in phenol red-free RPMI 1640 medium (Gibco) supplemented with 10% FBS and 1% antibiotics (Gibco). For experiments (viability, coumestrolone reduction) RPMI 1640 was supplemented with 10% charcoal-treated FBS (Gibco) and 1% antibiotics (Gibco). Cells were tested for Mycoplasma contamination using MycoStrip (Invitrogen).

Coumestrolone reduction assay

22Rv1 cells were seeded into 96-well plates at a density of 20,000 cells per well and incubated overnight at 37°C. The following day, cells were pretreated with CAS or reference compounds for 1 hour, followed by the addition of coumestrol at a final concentration of 10 µM. After 24 hours of incubation, 100 µl of the culture medium was transferred to a black 96-well plate for fluorescence measurement. Fluorescence intensity was measured with an excitation wavelength of 385 nm and emission wavelength of 510 nm on a plate reader (Spectra Max iD3, Molecular Devices) and normalized to cell viability in SRB assay. Cells treated with coumestrol alone were used as a control, representing 100% coumestrol reduction. Data represent the mean of three independent experiments, each performed in triplicate.

Sulforhodamine B viability assay

Cells were seeded at a density of 10^4 cells (22Rv1) or 5×10^3 cells (LNCaP) per well in 96-well plates. After 24 h, solutions of CAS, reference agents were preincubated for 3 h, and next ABI, ENZ or vehicle were added. After 72 h incubation, cells were fixed with trichloroacetic acid (50% w/v) for 1 hour at 4°C. Cells were washed with water, and stained for 30 minutes with sulforhodamine B solution (0.4% in 1% acetic acid). Then cells were washed four times with 1% acetic acid, and the incorporated stain was solubilized in 10 mM Tris solution. Absorbance of the solution was measured at 565 nm on a plate reader (Spectra Max iD3, Molecular Devices). Viability was calculated by dividing the average absorbance of each experimental condition by the absorbance of the control, multiplied by 100 (%). Three replicates were used.

Combination Index

To quantify drug interaction, the Combination Index (CI) was estimated by the Chou-Talalay method using CompuSyn software (ComboSyn, Inc. NY, USA).

Statistics

Statistical significance was assessed using Student's t-test with a threshold of $p < 0.05$. For multiple comparisons, Bonferroni correction was applied. All analyses were performed using GraphPad Prism.

Molecular modeling

The presented molecular modelling studies were performed using the Small-Molecule Drug Discovery Suite 2024-4 (Schrödinger Inc.), on a workstation operated by a Linux Ubuntu 24.04 LTS system. The CAS molecule was prepared by LigPrep and docked using the Induced-Fit Docking protocol to an initial AKR1C3 model, which was based on the 4DBS experimental structure [19], downloaded from the RCSB PDB and processed using the Protein Preparation Workflow. The conformational model reflecting the best-scored complex was used for re-docking in the Glide SP procedure (with a constrained H-bond to His117), and re-scoring using the MM-GBSA minimisation algorithm.

The final complex was directed to MD simulations carried out by the Desmond GPU software (D.E. Shaw Research and Schrödinger Inc.). First, it was relaxed using a Brownian motion simulation for 1 ns. The production phase was carried out for 120 ns, at a temperature of 310 K, and a pressure of 1.01325 bar (NPT ensemble class), with a trajectory and energy recording interval of 100 ps (1200 frames in total). Three independent replicates with different random seeds and initial velocities were performed. Following each run, the Simulation Interaction Diagram was used for RMSD plotting and generation of interaction timelines.

Results

Recombinant AKR1C3 inhibition

CAS was found to inhibit AKR1C3 with a potency similar to reference selective inhibitor ASP9521. IC_{50} of CAS was 5.99 μ M, while ASP9521 IC_{50} was 3.32 μ M (Fig. 1).

AKR1C3 inhibition in 22Rv1 cells

Coumestrol is a substrate for AKR1C3-catalysed reaction, which results in the formation of coumestrol. While the product exhibits fluorescence with excitation and emission wavelengths of 385 nm and 510 nm, respectively, the substrate does not. Therefore increase of the fluorescence of a medium with coumestrol incubated with the cell culture is related to the reduction process. CAS was found to inhibit coumestrol reduction in a similar way to IND. ASP9521 possessed much higher activity; it inhibited coumestrol reduction in the highest percentage, even at the concentration of 2.5 μ M (Fig. 2).

Cytotoxicity assessment

CAS was evaluated as a cytotoxic and chemosensitizing agent in of 22Rv1 and LNCaP cell lines. While 22Rv1 cell line is characterized by high expression of AKR1C3, LNCaP cell line is known to present a lack or trace level of AKR1C3. Both cell lines express androgen receptor and are androgen-dependent. Therefore, comparison of different biological effects on these cell lines is used to estimate the contribution of AKR1C3 to the activity.

In the viability assay, CAS was incubated with 22Rv1 and LNCaP for 72 h, next SRB assay was performed. CAS was found to present cytotoxicity in both cell lines (22Rv1 IC_{50} = 6.54 μ M; LNCaP IC_{50} = 70.90 μ M). However, it was more potent in 22Rv1, than in LNCaP (Fig. 3)

Drug combinations

CAS was assessed in combination treatments with drugs ABI and ENZ, used to treat CRPC. In 22Rv1 cell line, CAS in concentrations of 0.5, 1, and 2 μ M was found to synergize with ABI in concentrations of 10 μ M and 25 μ M. In five of six combinations decrease in viability was statistically significant against both CAS alone and ABI alone, an appropriate concentration (Fig. 4A). Combination Index calculated by Chou-

Talalay method was used to measure the potency of observed interactions. Its values range from 0.71 – 0.31, indicating a robust potency of a synergy (Table 1).

No statistically significant decrease viability of CAS and ABI combination against drugs alone was found in AKR1C3-low expressing LNCaP cells (Fig. 5A). This may suggest the contribution of this enzyme to the effect observed in 22Rv1 cells. No interaction was also found between CAS and ENZ in both cell lines (Fig. 4B, Fig. 5B).

Table 1
Combination Index (CI) for CAS and ABI in 22Rv1 cell lines. CI < 0.3 indicates a strong, 0.3–0.7 a robust, 0.7–0.85 a moderate, and 0.85–0.9 a slight synergism.

CAS [μM]	ABI [μM]	CI
0.5	10	0.71
	25	0.59
1.0	10	0.49
	25	0.49
2.0	10	0.52
	25	0.31

Molecular docking

To elucidate the robust inhibitory activity of CAS against AKR1C3, molecular modelling tools were employed. CAS was docked into the structural model of AKR1C3, revealing a binding mode characteristic of known inhibitors within the orthosteric site, with a Glide gscore of – 9.84, and MM-GBSA score – 59.26 kcal/mol. The carbonyl group acted as a hydrogen bond acceptor, forming interactions with His117 and Tyr55, two key residues involved in catalysis. Additionally, the chromen-4-one ring was stabilised through extensive π-aromatic interactions with Tyr24, Trp227, and Phe306 (Fig. 6)

Molecular dynamics

To evaluate interaction stability and active site residence time, molecular dynamics (MD) simulations were performed. The system, based on the docking-predicted CAS-AKR1C3 complex, retained all key interactions during the initial 1 ns minimisation step. Subsequent 120 ns MD simulations, performed in triplicate, produced stable trajectories with protein RMSD values below 2 Å and ligand RMSD values within 4.0 Å (Fig. 7).

The above interactions collectively contributed to the stable retention of CAS within the orthosteric binding site, supporting the in vitro findings and suggesting a favourable residence time consistent with potent AKR1C3 inhibition [20]. Representative RMSD and interaction profiles from simulation replicate #1 are shown in Figs. 7 and 8, respectively. Results from replicates #2 and #3 are provided in the Supplementary Information.

Discussion

CAS was evaluated as an AKR1C3 inhibitor and consequences of inhibition were translated into ability to affect resistant prostate cancer cells. Its inhibitory activity was confirmed using a recombinant enzyme assay and a cellular assay with coumestrol. In both assays, CAS demonstrated significant activity, comparable to ASP9521 and IND.

CAS exhibited notable cytotoxicity against the 22Rv1 cell line, with an IC_{50} of 6.54 μ M, in contrast to its much weaker effect on the LNCaP cell line (IC_{50} = 70.90 μ M). Based on IC_{50} almost 10-fold difference was found. Both 22Rv1 and LNCaP are androgen receptor-positive and androgen-dependent cell lines, but they differ in their detailed characteristics. The 22Rv1 cell line is characterized by a high expression of AKR1C3, especially when cultured in castration-level androgen conditions using charcoal-treated FBS [9, 21]. In contrast, AKR1C3 is undetectable or present only at trace levels in LNCaP cells [7, 9, 22]. Additionally, 22Rv1 expresses splice variants of AR (such as AR-V7), which are specifically associated with resistance to standard treatments [7, 22].

Previously, CAS was tested on the androgen-independent DU145 prostate cancer cell line and showed low cytotoxicity (reducing viability to about 80% at 50 μ M after 48 hours), but also exhibited anti-migratory activity [23]. In other androgen-independent PC-3 prostate cancer cell line CAS was cytotoxic (IC_{50} = 28,8 μ M), induced apoptosis and cell cycle arrest at G2/M phase [24]. Furthermore, VAC extracts demonstrated efficacy in inducing cytotoxicity in prostate cancer cells both in vitro and in vivo [25, 26].

CAS was then examined for its chemosensitizing activity. ABI and ENZ, standard agents used in clinical oncology, were employed as reference drugs. For each cell line, three concentrations of CAS below the IC_{50} were selected. CAS demonstrated significant synergy with ABI in the 22Rv1 cell line. Combined treatment with CAS and ABI in 22Rv1 cells caused a significant reduction ($p < 0.05$) in cell viability compared to treatment with either agent alone.

To quantify the potency of this interaction, the Combination Index (CI) was used. The CI is a quantitative measure to evaluate the interaction between two or more drugs or treatments, indicating whether the combined effect is synergistic ($CI < 1$), additive ($CI = 1$), or antagonistic ($CI > 1$). The CI is commonly calculated using methods such as the Chou-Talalay method and is widely used in pharmacology and cancer research to optimize combination therapies [27]. The CI for the interaction between CAS and ABI ranged from 0.31 to 0.71, indicating a moderate level of synergy.

In contrast, no significant reduction in cell viability was observed in the AKR1C3-negative LNCaP cell line. Additionally, no interaction between CAS and ENZ was detected in either 22Rv1 or LNCaP cell lines.

Enhancement of ABI activity when combined with AKR1C3 inhibitors has been reported previously. For example, indomethacin, an AKR1C3 inhibitor, increased ABI efficacy in the resistant C4-2B cell line, and combined treatment significantly reduced tumor volume in SWR22Rv1 mouse xenografts [28]. More recently, a combination of IND and ABI has been investigated in clinical trials [29]. Derivatives of 3hydroxybenzoxazole enhanced ABI's cytotoxicity against 22Rv1 cells, suppressed PSA expression, and inhibited testosterone production [30]. Likewise, hydroxytriazole analogues not only potentiated ABI activity but also sensitized 22Rv1 cells to ENZ [31].

Molecular docking placed CAS in the SP2 hydrophobic pocket - critical for steroid stabilization - where it engages Trp227 and Phe306 via π - π stacking. Hydrogen bonds with catalytic tetrad residues Tyr55 (SP2) and His117 (SP3) were also predicted.

During 120ns molecular dynamics simulations, CAS maintained a hydrogen bond with His117 for 97–98% of the trajectory and remained anchored to at least one aromatic cluster residue (Tyr24, Trp227, Phe306, or Phe311) throughout, each contact occurring $\geq 15\%$ of the time. Particularly stable π - π interactions were observed between the chromen-4one core and Tyr24 (48%), Trp227 (25%), and Phe306 (43%). Notably, interactions involving the substituted phenol ring—absent in docking—emerged with Trp227 (28%) and Phe311 (19%), and an additional hydrogen bond with Ser129 persisted for 20–32% of the simulation (see Fig. 8).

In summary, CAS - a flavonoid from VAC - was confirmed as an AKR1C3 inhibitor through recombinant enzyme assays, cellular studies, and molecular modelling. It exhibited potent cytotoxicity against the resistant 22Rv1 line and synergized with ABI. These findings may underlie VAC's antiandrogenic effects and suggest therapeutic potential for CAS (and VAC) not only in castrationresistant prostate cancer but also in other AKR1C3-related conditions, such as certain cancers, polycystic ovary syndrome, ovarian dysfunction, and hyperandrogenism [10].

Declarations

Authors declare no conflict of interest

Funding Declaration

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Ethics and Consent to Participate declarations: not applicable

The study used commercially available human-derived cell lines (22Rv1 and LNCaP) that do not require ethical approval according to the institutional guidelines. All experimental procedures were conducted in

accordance with relevant institutional and international regulations.

Author Contribution

K.P. – conceptualization, experimental design, in vitro assays, manuscript writing (original draft and final version) M.Z. – experimental design, AKR1C3 inhibition assays A.B. – molecular docking and dynamics simulations, figure preparation, manuscript writing M.Š. – molecular docking and dynamics simulations, figure preparation P.K.A. – cytotoxicity studies B.W. – recombinant protein expression and purification M.K. – molecular docking and dynamics simulations, critical revision of the manuscript E.P. – critical revision of the manuscript, supervision

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Figures

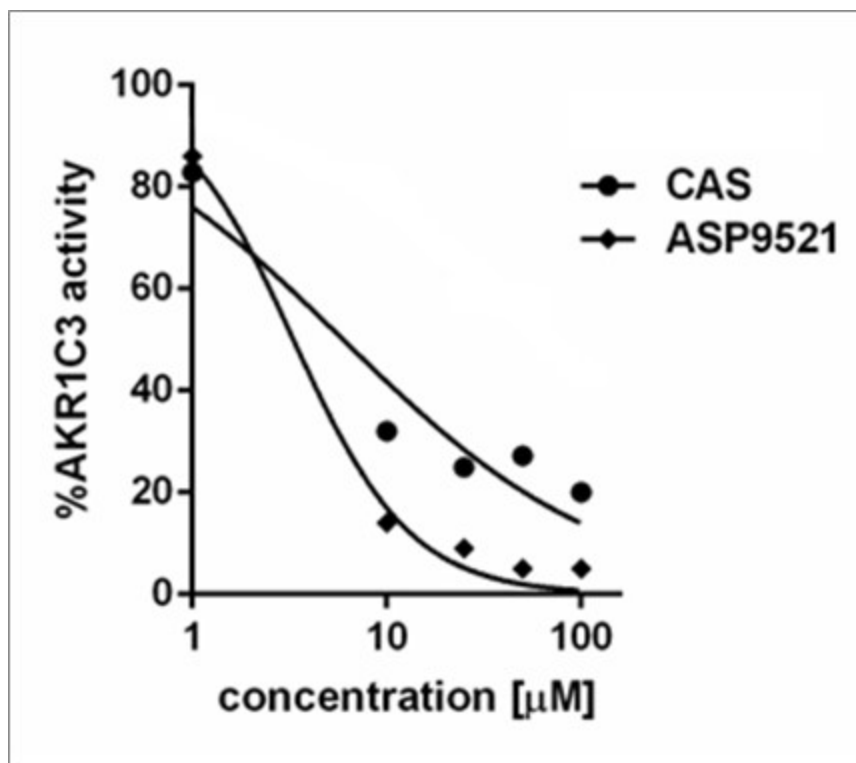


Figure 1

Activity of AKR1C3 with CAS and standard AKR1C3 inhibitor ASP9521.

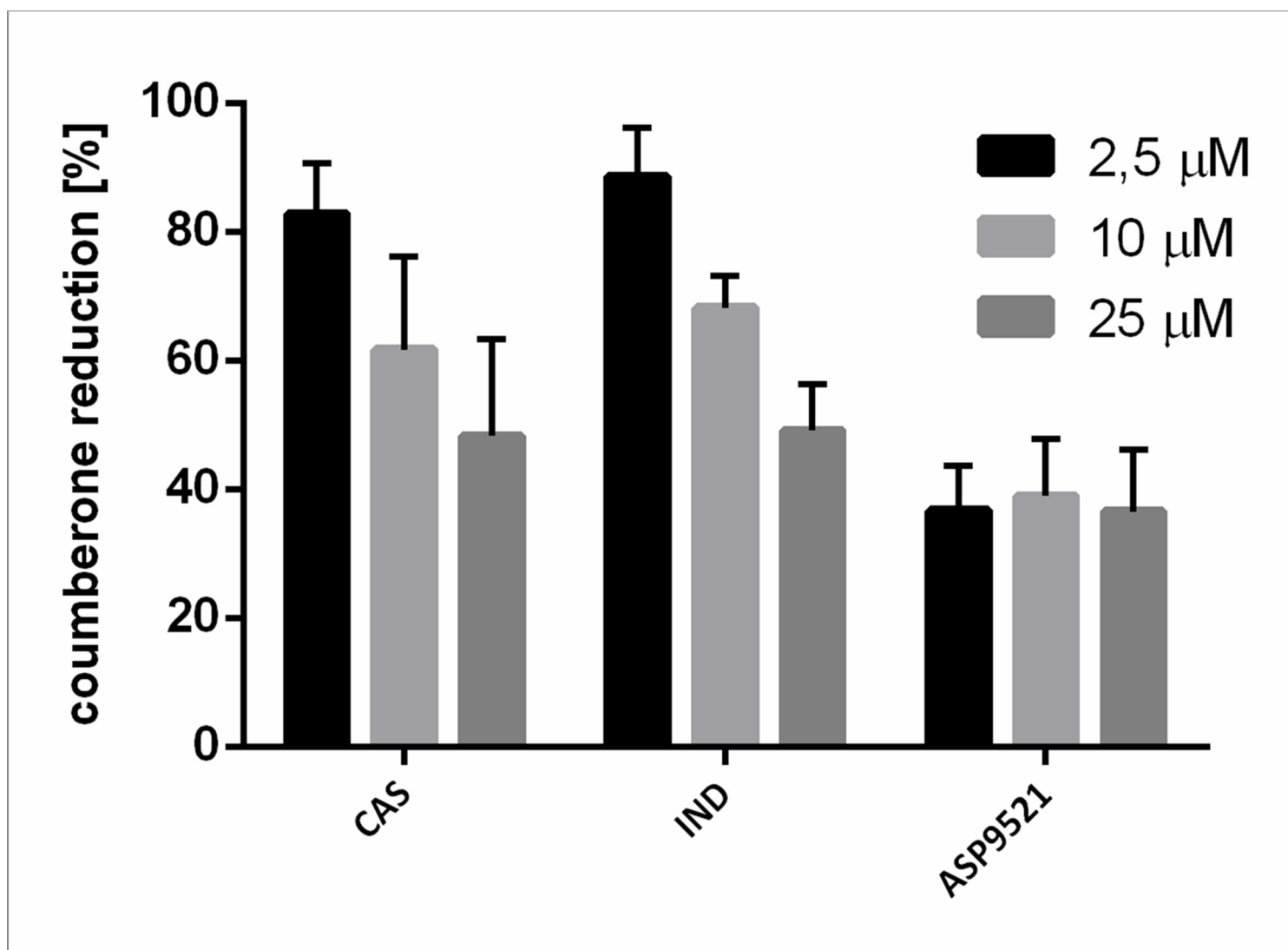


Figure 2

Coumestrol reduction in 22Rv1 cells was decreased by CAS and reference AKR1C3 inhibitors (IND, ASP9521). 100% coumestrol reduction is related to vehicle control.

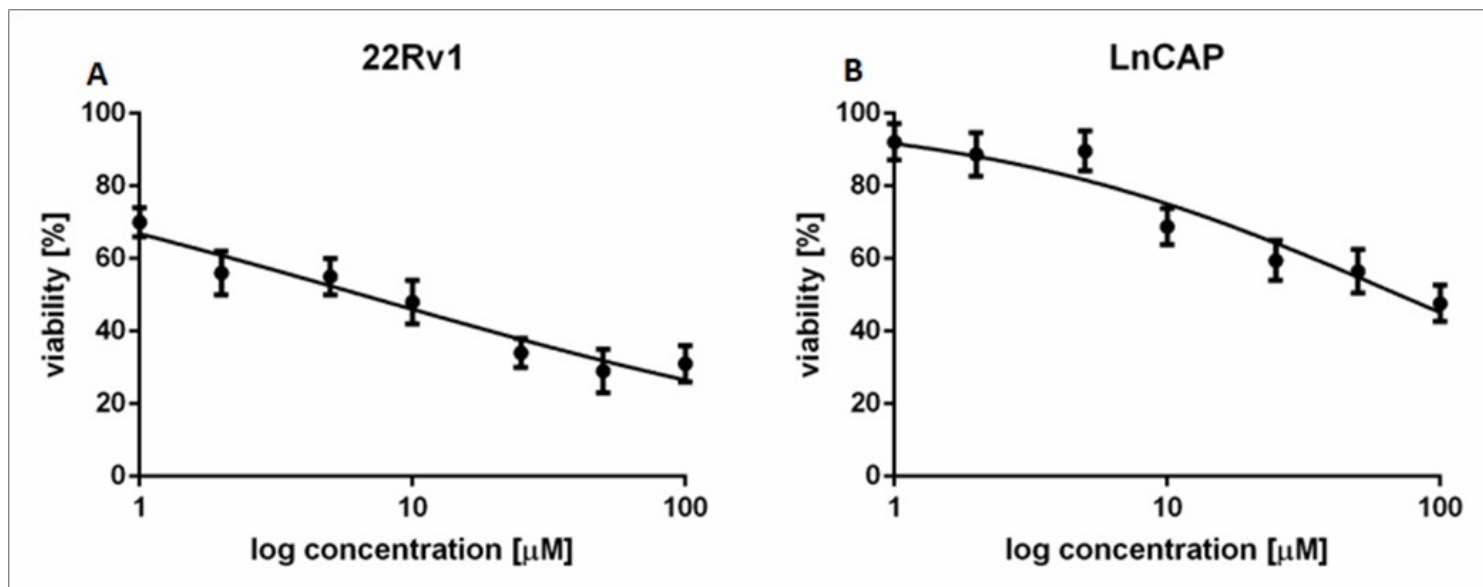


Figure 3

Viability of 22Rv1 (A) and LNCaP (B) cells incubated with CAS for 72 h in SRB assay.

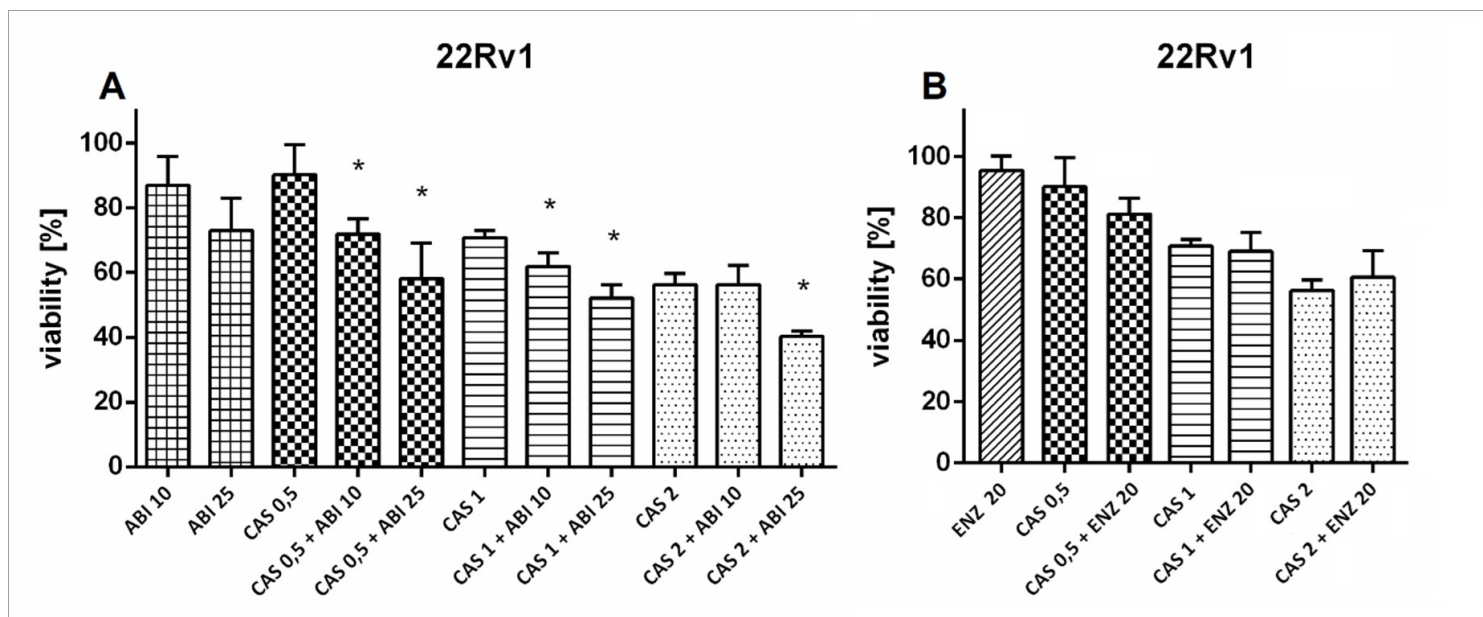


Figure 4

Drug combination assay in 22Rv1 cells. Cells were preincubated with CAS for 3 hours, after which ABI or ENZ was added. After 72-hour incubation SRB assay was performed. Numbers next to drugs' names represent μ M-concentrations. Statistical significance ($p < 0.05$) is indicated when the combined treatment condition differs significantly from both CAS alone and ABI or ENZ alone (t-Student test).

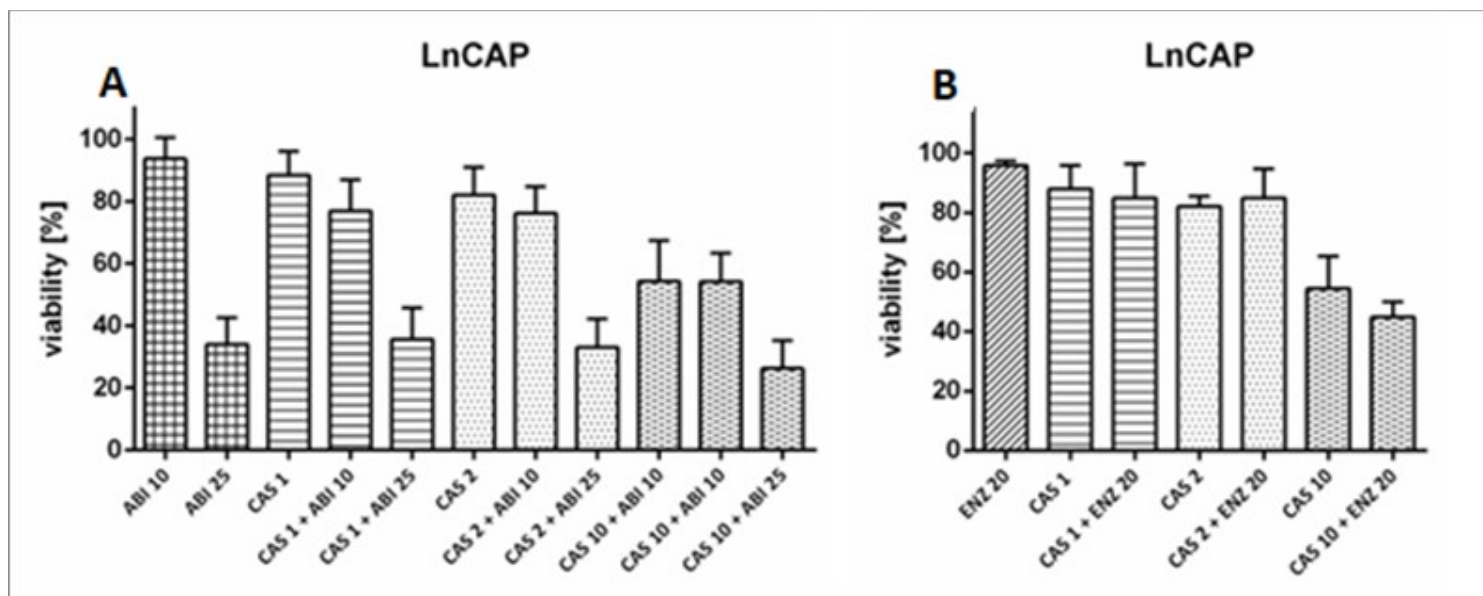


Figure 5

Drug combination assay in LNCaP cells. Cells were preincubated with CAS for 3 hours, after which ABI or ENZ was added. After 72-hour incubation SRB assay was performed. Numbers next to drugs' names represent μM -concentrations. No statistically significant ($p < 0.05$) differences between the combined treatment condition and CAS alone and ABI/ ENZ alone were found (t-Student test).

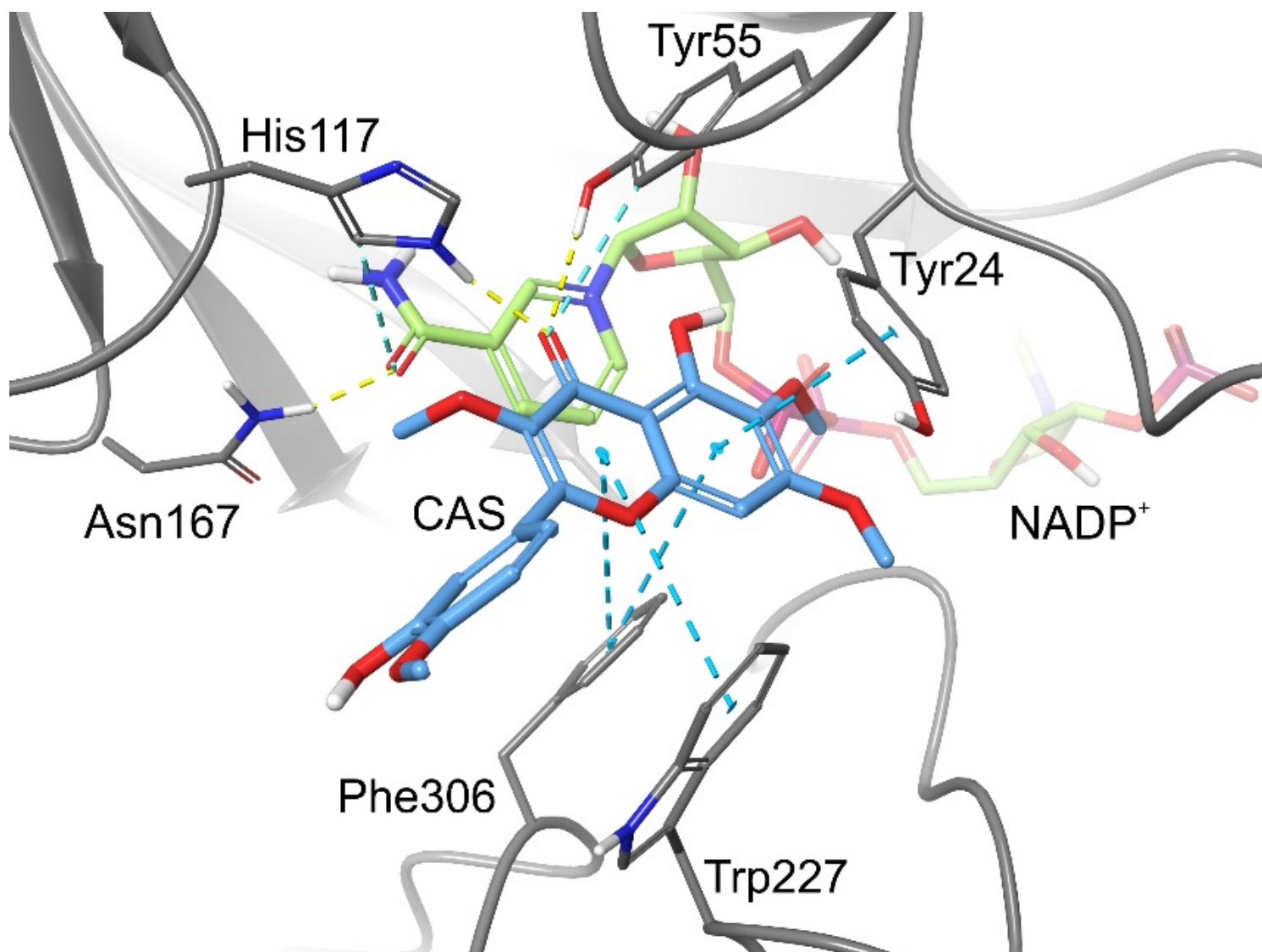


Figure 6

Predicted binding mode of CAS (blue) in the AKR1C3 active site. The ligand forms hydrogen bonds with His117 and Tyr55 (yellow dashed lines) and π -aromatic interactions with Tyr24, Trp227, and Phe306 (cyan dashed lines). The protein model is based on the experimental structure PDB ID: 4DBS, co-crystallised with NADP⁺ (pale green).

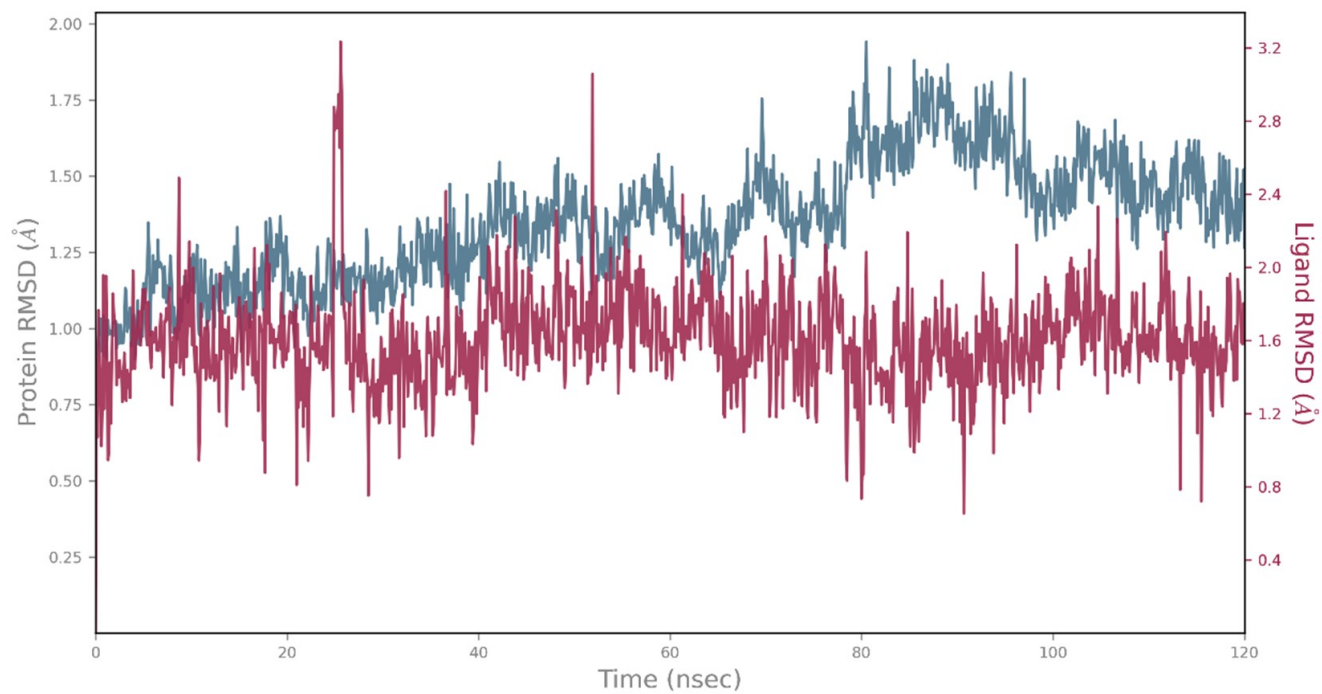


Figure 7

Protein and ligand RMSD values over the 120 ns MD simulation (replicate #1) of the CAS-AKR1C3 complex, indicating minimal conformational changes and sustained ligand residence.

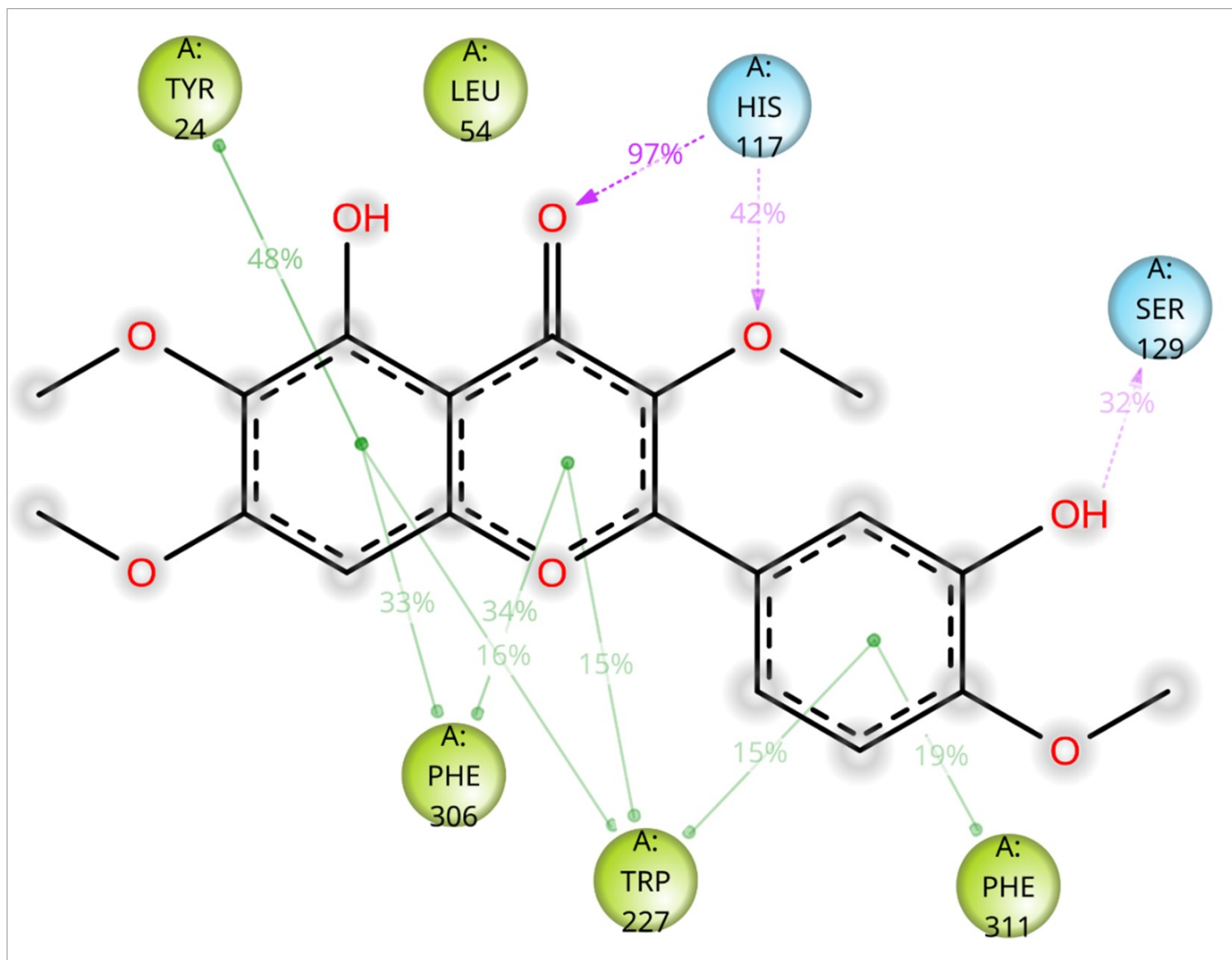


Figure 8

Solid interaction stability throughout the 120 ns MD simulation (replicate #1) of the CAS-AKR1C3 complex, highlighting stable hydrogen bonding and π - π interactions that support the ligand's inhibitory activity.

Supplementary Files

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- [SupplementaryInformation.docx](#)