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Research article

Allosteric Binding of Wild-Type and E17K Mutant AKT By Resveratrol Compared to AKT Inhibitor III (MK-2206): Computational Modeling Studies

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Abstract

AKT is a serine-threonine protein kinase which is hyperactivated in many human cancers, e.g., castration resistant prostate cancer. Studies on chemicals from synthetic and natural sources capable of affecting the activity of AKT have identified the grape-derived resveratrol as an inhibitor of AKT, by mechanisms which have not been completely elucidated. Our recent finding that resveratrol interacts with AKT at its ATP binding site, with a different AKT:ATP binding affinity, had led us to hypothesize that resveratrol also interacts allosterically with AKT at a site distinct from the ATP-binding site. Here we performed computational modeling studies to test this hypothesis. The interaction between the interactive domains of resveratrol (ligand) and AKT (target protein) is computationally analyzed by molecular docking to determine effects of binding of resveratrol and its structural mimetics with AKT. Global docking simulations show that resveratrol binds AKT at an allosteric site located in the vicinity of amino acid residue Trp80, similar to the interaction occurring between AKT and the pan-AKT allosteric inhibitor VIII (MK-2206). Binding affinity between resveratrol and AKT occurs optimally in a pleckstrin homology (PH)-in conformation comparable to binding between AKT and inhibitor VIII. This interaction results in formation of a ligand:protein complex that effectively restricts the access/exposure of AKT to its molecular activators, respectively, ATP and phospholipids. Modeling studies also reveal that resveratrol and several of its biotransformed metabolites but not inhibitor VIII bind to E17K mutant AKT. These results may help to develop agents with efficacy in cancers harboring the mutant E17K AKT.

Keywords: : Resveratrol, AKT, E17K mutated AKT, AKT inhibitor VIII (MK-2206)

Introduction

AKT is a serine-threonine protein kinase that plays an important role in the control of cell proliferation and survival in various human cancers [1-8]. Dysregulation of AKT is frequently accompanied by its hyperactivation, in correlation with oncogenic transformation and tumorigenesis in human cancers [9-12]. The observed molecular connectivity between hyperactivation of AKT and malignancy in humans has underpinned the focus on AKT as an attractive anticancer drug target and the impetus to search for chemicals capable of influencing the activation and downstream signaling events transduced by AKT [13-18].

Several generations of AKT inhibitors with various potency, specificity, mechanisms have surfaced in the last two decades [19]. Combining a radioactive assay with a nonradioactive ELISA and using isoquinoline-5-sulfonamide derivative H-89 as a reference, Reuveni and coworkers identified NL-71-101 as a first generation AKT inhibitor displaying ATP-competitive activity [20, 21]. Since such inhibitors are only modestly selective and tend to overlap with closely related kinases sharing similarity to the catalytic domain of AKT, later efforts to uncover more specific AKT inhibitors utilize approaches focused on allosterism and modulation of changes in AKT conformation during its activation and/or catalysis [22, 23]. This strategy has led to the discovery of novel AKT inhibitors [24, 25], including inhibitor VIII (MK-2206) [26-28].

An interest of our laboratory has been the identification of anti-AKT inhibitors from natural sources. In recent studies we showed that AKT is a cellular target protein of resveratrol which interacts avidly with the high affinity resveratrol target protein, NQO2 [29]. Because resveratrol inhibits phosphoinositide 3-kinase (PI3K) by competing with binding of ATP to the

catalytic site [30] - a mechanism that could well apply to the reported AKT inhibitory effects of resveratrol [31-35], we tested whether resveratrol binds AKT using resveratrol affinity column chromatography. We found that AKT is retained on the resveratrol column and cannot be eluted using buffers containing mM concentrations of ATP [29], suggesting that binding of resveratrol to AKT occurs at an allosteric site distinct from the ATP-binding catalytic site. We also found that AKT activity is significantly increased in NQO2-knockdown CWR22Rv1 prostate cancer cells [36]. Conceivably, resveratrol may act dualistically by targeting both the catalytic and allosteric sites of AKT to fine-tune its binding and accessibility to ATP and other AKT activating and modulatory chemicals. Herein we test this hypothesis by employing computational modeling studies to determine binding positions, orientations, and energies using the UCSF DOCK suite of programs for small molecule-receptor binding [37] and UCSF Chimera for molecular modeling [38]. The E17K mutant AKT is reportedly oncogenic and occurs with a low prevalence in breast, colorectal, and ovarian cancers [39-41]. Moreover, the same mutation is also reported in a number of other solid tumors [42-47] and in pediatric T-cell acute lymphoblastic leukemia [48]. Since studies have shown that this mutation renders AKT resistance to its allosteric inhibitor VIII [49] and has been actively evaluated for treating cancers harboring aberrantly activated AKT [50], we compared resveratrol with inhibitor VIII as allosteric modulators for the AKT E17K mutation. Computational modeling analyses revealed that resveratrol and several of its biotransformed metabolites but not inhibitor VIII bind to AKT expressing the E17K mutation. The demonstration that resveratrol and its metabolites can bind to an allosteric site in both wild type and E17K mutant AKT is a newly discovered effect of resveratrol having the potential for adjunctively treating normal and diseased states harnessing altered AKT activity and function.

Materials and Methods

Docking Analysis

The UCSF DOCK6 v6.3 suite of programs was used to perform Docking [51, 52]. Ligands used for the docking experiments were obtained from the ZINC database. The known X-ray structure of AKT in complex with inhibitor VIII (PDB code: 3O96) was used as a reference receptor structure. The total binding energies between ligand and receptor were calculated. Ligands resulting in highly negative binding energies were grouped as representing those with a high affinity towards the receptor. After the completion of docking, results were analyzed using the UCSF program Chimera and viewed utilizing Chimera's ViewDock. Chimera's FindHBond was used to determine hydrogen-bonds occurring between a specific ligand and the receptor. Docking was performed in a shell program in a Linux based machine [51, 52].

Preparation of receptors for docking with small molecules was achieved using UCSF Chimera v1.8. Receptor structures were obtained from the Protein Data Bank (www.pdb.org) in PDB format. Chimera's DockPrep [38] was used to add hydrogens to the model; charges were added using the AMBER ff12SB library for standard residues while charges for other residues and small molecules were added using ANTECHAMBER and the AM1-BCC method [37, 53]. The prepared structure was saved and a receptor surface file was generated using Chimera's "write DMS". Active site coordinates were also saved using Chimera, either by selecting a co-crystalized ligand, or by selecting one or a set of amino acids close to the desired binding site.

First spheres were generated using the prepared receptor file and the DOCK6 program sphgen was used to fill any pockets in the receptor. Then spheres within a certain distance of the active site (usually 10 angstroms, lower for more constrained docking) were selected using the DOCK6 program sphere selector. For global docking, all spheres were used so that docking could be simulated and checked for all locations of the receptor. Next a box was generated allowing an additional 5 angstroms around the box using DOCK6's showbox program. This box served as the location constraints for docking calculations. The sphere and box files were then used, along with other receptor files, to generate an energy grid to be used for docking calculations with the use of the DOCK6 program. Then docking simulations were carried out utilizing the DOCK6 grid scoring method. The grid scoring method allows for flexibility in the ligand, but not the receptor. For local docking, 5000 rigid orientations were used and the ligand was allowed to be minimized by 1,000 cycles. For global docking, 10,000 orientations were used. The top scoring orientations of each ligand were outputted for analysis. Finally, results were analyzed using UCSF Chimera's ViewDock. Hydrogen bonds were computed between the receptor and ligand using Chimera's Find HBond, allowing for some relaxation in distance constraints and calculating all possible H-bonds up to 3.5 angstroms in length.

In order to assess further binding characteristics in more complex models (like an allosteric inhibitor binding to AKT1) the AMBER scoring method [38, 53] was utilized. Although the AMBER scoring method can only be used to refine prior docking runs, it has the advantage of allowing for flexibility in both receptor and ligand and thus the capability for assessing the "induced-fit" model and the evaluation of possible changes to the receptor upon ligand binding. Receptors were first prepared using the PDB2PQR server [54, 55]. This server converted the receptor file into one that can be read by the AMBER suite. Specifically, in place of PDB files, PQR files are used which have the proviso for replacing the occupancy and B-factor columns with per-atom charge and radius; in addition, states of protonation are assigned for residues using pH 7.0. Next the receptor and ligand file (consisting of results from a prior docking run) was prepared using the UCSF script prepare_amber.pl. This script was used to generate files to be concurrently analyzed by the AMBER docking. Finally DOCK6 was utilized to conduct the docking simulations. Flexibility in the receptor was confined to within 3 angstroms of the active site for AKT (PDB code: 3O96), as an increase in allowed flexibility in the receptor could lead to complications and unrealistic results. This encompassed assessing a total of 79 residues near inhibitor VIII and resveratrol's binding location. Altogether 1,000 minimization cycles and 5,000 ligand orientations were evaluated with the results visualized in Chimera using the method mentioned above.

Results

Computer modeling studies show that resveratrol binds AKT as an allosteric modulator

In our recent studies, we employed resveratrol-bioaffinity column chromatography to test the binding of AKT to resveratrol. We reasoned that affinity of the polyphenol for the ATP site of AKT might be revealed by its displacement from the column with high concentration of ATP. Surprisingly, proteins eluted from the resveratrol-appended column using mM ATP did not include AKT suggesting that AKT likely interacts with the column-bound resveratrol in a way that renders AKT inaccessible to ATP [29]. We then reasoned that resveratrol might function as a heterotrophic AKT allosteric modulator binding the kinase at a location distinct from the ATP site and effectively inhibiting the enzyme. To test this hypothesis, resveratrol was docked to AKT bound to its allosteric inhibitor VIII (PDB code 3O96). In figure 1A resveratrol is shown to interact with the PH domain of AKT binding at a cavity located in the proximity of Trp80, forming a strong low-barrier hydrogen bond (defined as distance between acceptor and donor of - 2.4 Angstroms) with Ser205 in the kinase domain (Figure 1A). Of note, the interaction with both Ser205 and Trp80 is also observed in binding of AKT with its known allosteric effector, inhibitor VIII (Figure 1A). Global docking simulations performed using the grid based scoring method and allowing for 10,000 orientations on the entire receptor confirmed that the most probable binding location of resveratrol occurs in the vicinity of Trp80, similar to inhibitor VIII (Figure 1A). Docking was refined in this locality of AKT by analyzing an additional 5,000 orientations and by including the AMBER-based scoring method in the analysis, to allow for flexibility in both the ligand and receptor to within 3 angstroms of the selected area. This more focused analysis added 79 amino acids in both the PH and kinase domains of AKT, all in the vicinity of the binding site of resveratrol. The expanded analyses showed that binding to resveratrol induces significant conformational changes in AKT: small shifts were found in Trp80, Asn51 and Gln79 of the PH domain, changes were also observed in Ser205, Leu210, Val270, Tyr272 and Asp292 in the kinase domain, while the largest shift occurred in the loop extending from Glu294 to Gly298 (Fig. 1B). These changes provide support that resveratrol binds AKT as an allosteric modulator.

Binding to resveratrol affects AKT activation by altering the interaction and affinity of ATP/phospholipid for AKT

The AKT PH-in conformation (PDB: 3O96) was used in the analysis, with focus being directed to affinity for the PIP3 head group (IP4) and binding to ATP. Figure 2A shows the PH and kinase domains in yellow and red; residues within 5 angstroms of IP4 are presented in green and residues within 5 angstroms of ATP binding are illustrated in blue. Effects of binding to resveratrol on changes in AKT using phospholipids as the activator were assessed by molecular docking and the results are shown in figure 2B. Compared to the AKT site normally involved in binding IP4 (shown in purple), interaction with resveratrol causes a change in AKT that results in a ~4 angstrom change in IP4 orientation towards the exterior (shown in blue) (Figure 2B). Based on these results, it may be suggested that complex formation occurring between resveratrol and AKT induces an alteration in AKT conformation that possibly restricts its binding in the native state to IP4, impinging on the functional activation of AKT. Quantitation of affinity of resveratrol for the allosteric site of AKT and the changes in AKT binding to IP4 and ATP as induced by resveratrol are presented in figure 2C showing that there was an 18 to 25% decrease in the affinity for IP4 and ATP, respectively.

Resveratrol has been shown to act as a phosphoinositide 3-kinase (PI3K) inhibitor by an ATP competitive mechanism [30]. Although the studies above suggest that resveratrol functions as an allosteric modulator to preferentially bind AKT in its inactive, PH-in state, it remains possible that resveratrol also plays a role as an ATP-competitive inhibitor. Hence, we next tested the binding affinity of resveratrol to the ATP site of AKT in its active, PH-out state. Docking of resveratrol to an AKT, PH-out conformation (PDB: 3CQU) shows that resveratrol produces a slightly lowered grid score compared to ATP (~-49kJ/mol vs ~-54kJ/mol) (Figure 3). This result is consistent with the interpretation that resveratrol may also bind to AKT as a low affinity ATP-competitive inhibitor; the net result being that binding to ATP may be blocked while ATP bound to AKT may not be readily displaced. Taken together, the in silico modeling studies raise the possibility that resveratrol has a dual role in controlling the activation and function of AKT, both as an allosteric modulator and ATP competitive inhibitor, and the relative impact of these effects may well depend on the stage of AKT activation when it comes in contact with resveratrol.

Unlike inhibitor VIII, resveratrol also binds E17K mutant AKT

Intramolecular domain interactions maintaining AKT in a PH-in conformation prevents activation of the enzyme while somatic mutations disrupting the interplay between PH and kinase domains lead to oncogenic activation of AKT [56]. A model of interaction and the relevant amino acids and the specific hydrogen bonds (shown in cyan) involved between the PH (yellow) and kinase (red) domains of wild type AKT (PDB: 3O96) is depicted in Figure 4A. We performed computer simulations to examine how somatic mutations might contribute to conformational changes capable of disrupting the interaction between these two domains. Structures for three common AKT mutants (E17K, D323H, L52R) were generated and compared with wild type AKT; hydrogen bond changes induced by the mutations are circled in dashed lines. The hydrogen bonds formed between the PH and kinase domains for both wild-type and the AKT mutants are portrayed in Figure 4B: bonds not affected by the mutations (green columns); bonds affected by the mutations (orange columns); the hydrogen bonds formed between the PIP3 head group (IP4) and the PH domain, and as affected by D323H and L52R mutations (red columns). Parikh et al. reported that activity of AKT allosteric inhibitor factor VIII required an intact PH–Kinase interface and that the inhibition was effectively counteracted by mutations in AKT [56]. Since our studies above show that resveratrol acts as an AKT allosteric inhibitor, we examined whether the effects of resveratrol can be similarly attenuated by AKT mutations. Molecular simulations showed that E17K but not L52R or D323H muta-

tions of AKT negatively affected binding of inhibitor VIII; in contrast, resveratrol showed essentially the same binding energy and binding orientation for the mutant E17K and wild-type AKT (Figure 4C). These results may have therapeutic implications for patients with the E17K mutant AKT status.

Resveratrol metabolites also show potential as allosteric inhibitors of AKT

As a rapidly metabolized compound [57], resveratrol has limited bioavailability [58-60] making it of interest to determine whether its metabolites might potentially function as AKT allosteric inhibitors. Docking analyses showed that piceatannol had a similar binding affinity for the allosteric pocket of AKT (energy score -45.41 kJ/mol), (Table 1) as resveratrol (energy score -45 kJ/mol (Figure 2C). A higher binding affinity for AKT allosteric pocket was found for resveratrol-3-sulfate (-52.6 kJ/mol), piceid (-65.27 kJ/mol) and resveratrol-3-glucuronide (-67.46 kJ/mol) (Table 1), suggesting these metabolites to have a better allosteric inhibitory potential against AKT as compared to resveratrol. We also examined the binding affinity of resveratrol metabolites in E17K mutant AKT and found that they were equally effective in the E17K mutants harboring intramolecular changes between PH and kinase domains (Table 1).

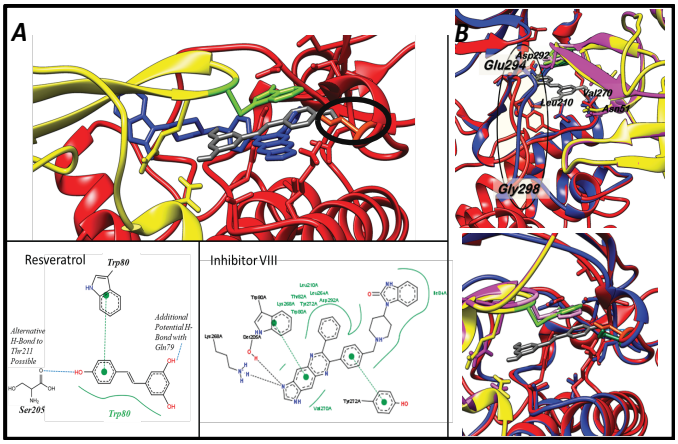
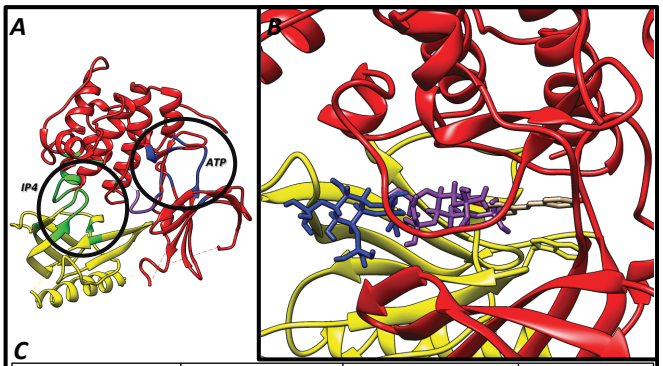
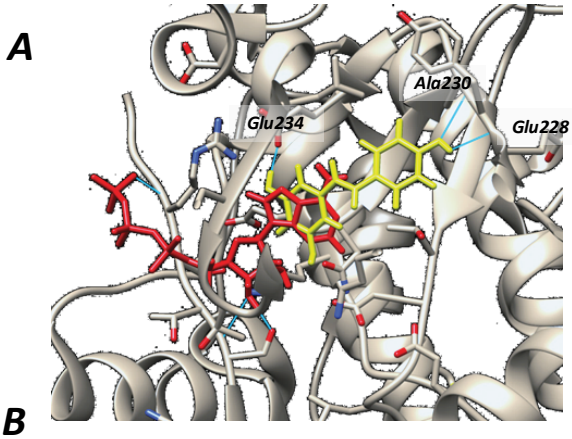


Figure 1. Resveratrol vs inhibitor VIII as an allosteric inhibitor of AKT. (A) Top: Resveratrol (grey) and inhibitor VIII (blue) bound to the allosteric inhibitor binding pocket of AKT. The PH domain of AKT is shown in yellow and the kinase domain is shown in red. The Trp80 residue of the PH domain is shown in green and the Ser205 residue of the kinase domain is shown in orange and circled. It is evident that resveratrol and inhibitor VIII take up similar binding positions beneath the Trp80 amino acid. Bottom: PoseView generated structures showing interactions between resveratrol or inhibitor VIII with the PH and kinase domains of AKT. Shown are the similarities in the ring and hydrophobic interactions occurring between resveratrol or inhibitor VIII and the Trp80 residue of the PH domain, as well as the hydrogen bond formed between Ser205 with either inhibitor. It should be noted that these interactions have been found to be essential in inhibition of AKT by its allosteric inhibitors. (B) Changes induced on receptor (AKT) upon binding to resveratrol as calculated using the AMBER docking method. The PH and kinase domains are shown in yellow and red, respectively and the resveratrol bound conformation is shown in magenta (PH) and blue (kinase.) Residues with the largest conformational changes are labeled, particularly the large shift in the loop from residue 294-298. Comparative changes in Ser205 are shown in orange (initially, without resveratrol) and green (resveratrol bound) and changes in the Trp80 residue are shown in green (starting, without resveratrol) and pink (resveratrol bound.)



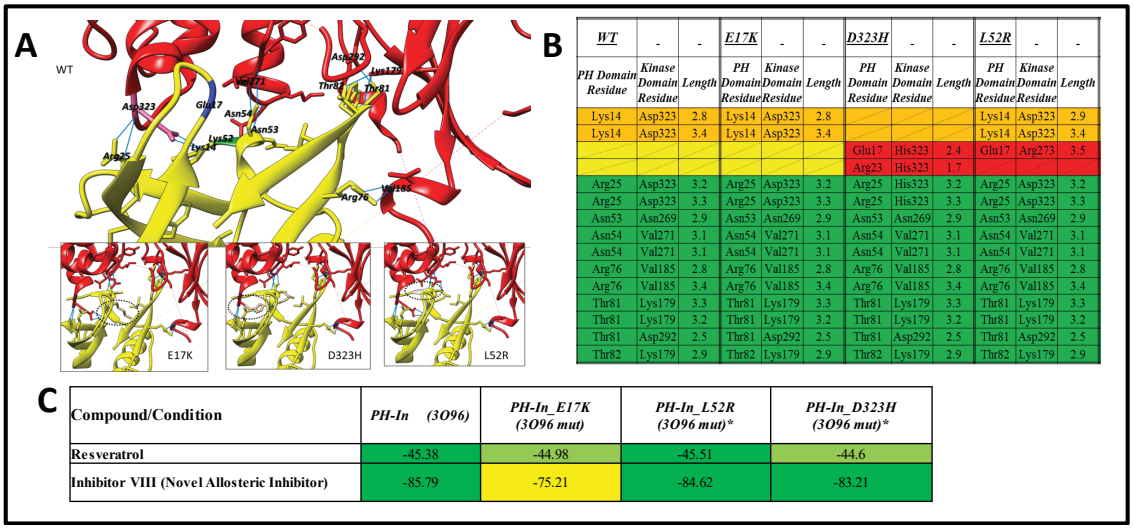
Receptor Conditions/Ligand	Inhibitor Binding Affinity	PIP3 Head Group Binding Affinity	ATP Binding Affinity
3O96 Alone (Purple)	N/A	-90 kJ/mol	-84kJ/mol
Resveratrol & 3O96 (Blue)	-45 kJ/mol	-74 kJ/mol	-62kJ/mol

Figure 2. Changes in AKT binding to PIP3 Head Group (IP4) induced by interaction with resveratrol. (A) Complete structure of PH-in conformation of AKT, showing relative PIP3 head group (IP4) and ATP binding locations. The PH domain is shown in yellow and the kinase domain in red. Residues within 5 angstroms of PIP3 head group (IP4) binding are shown in green while residues within 5 angstroms of ATP binding are shown in blue. (B) The figure shows AKT PH and kinase domains in yellow and red, respectively, with resveratrol presented in grey and the PIP3 head groups (IP4) in purple and blue. The purple represents IP4's native binding location and the blue represents IP4's binding location after resveratrol binding. It is evident that resveratrol prevents IP4 from binding in its native conformation, a change that can be expected to inhibit the initiation of activation of AKT. (C) The table shows both resveratrol's binding affinity to the allosteric pocket of AKT and the changes in PIP3 head group binding affinity as induced by binding to resveratrol.



Ligand	VDW Energy	ES Energy	Total Grid Score
Resveratrol	-44.30 kJ/mol	-4.79 kJ/mol	-49.09 kJ/mol
ATP	-54.31 kJ/mol	-.13 kJ/mol	-54.44 kJ/mol

Figure 3. Resveratrol as a Dual Action Inhibitor of AKT: Binding of Resveratrol to ATP Binding Site in the PH-out conformation. (A) shows relative orientations and positions of both resveratrol (yellow) and ATP (red) bound to the ATP site of the AKT kinase domain (in PH-out conformation) (PDB: 3CQU). Resveratrol (grey) binding to the allosteric inhibitor pocket of AKT and showing the 2.4 angstrom bond formed between resveratrol and the Ser205 residue of the kinase domain of AKT. Also shown is resveratrol's position beneath the Trp80 amino acid (green) of the PH domain of AKT. The PH domain is shown in yellow and the kinase domain is shown in red. (A) also shows resveratrol bound as a potential ATP-competitive inhibitor of AKT. The kinase domain of AKT here is shown in grey, resveratrol is shown in yellow and ATP is shown in red. Hydrogen bonds formed between resveratrol and the receptor are shown in blue and the residues are labeled. (B) Table of receptor (AKT) affinity for both resveratrol and ATP as calculated by the Grid based scoring method. This scoring method is appropriate as no significant change to the receptor is expected upon competitive inhibitor binding. The total grid score is the summation of both the Van Der Waals and electrostatic contributions to binding energies.



WT	-	-	*E17K*	-	-	*D323H*	-	-	*L52R*	-	-
PH Domain Residue	*Kinase Domain Residue*	*Length*	*PH Domain Residue*	*Kinase Domain Residue*	*Length*	*PH Domain Residue*	*Kinase Domain Residue*	*Length*	*PH Domain Residue*	*Kinase Domain Residue*	*Length*
Lys14	Asp323	2.8	Lys14	Asp323	2.8				Lys14	Asp323	2.9
Lys14	Asp323	3.4	Lys14	Asp323	3.4				Lys14	Asp323	3.4
						Glu17	His323	2.4	Glu17	Arg273	3.5
						Arg23	His323	1.7			
Arg25	Asp323	3.2	Arg25	Asp323	3.2	Arg25	His323	3.2	Arg25	Asp323	3.2
Arg25	Asp323	3.3	Arg25	Asp323	3.3	Arg25	His323	3.3	Arg25	Asp323	3.3
Asn53	Asn269	2.9	Asn53	Asn269	2.9	Asn53	Asn269	2.9	Asn53	Asn269	2.9
Asn54	Val271	3.1	Asn54	Val271	3.1	Asn54	Val271	3.1	Asn54	Val271	3.1
Asn54	Val271	3.1	Asn54	Val271	3.1	Asn54	Val271	3.1	Asn54	Val271	3.1
Arg76	Val185	2.8	Arg76	Val185	2.8	Arg76	Val185	2.8	Arg76	Val185	2.8
Arg76	Val185	3.4	Arg76	Val185	3.4	Arg76	Val185	3.4	Arg76	Val185	3.4
Thr81	Lys179	3.3	Thr81	Lys179	3.3	Thr81	Lys179	3.3	Thr81	Lys179	3.3
Thr81	Lys179	3.2	Thr81	Lys179	3.2	Thr81	Lys179	3.2	Thr81	Lys179	3.2
Thr81	Asp292	2.5	Thr81	Asp292	2.5	Thr81	Asp292	2.5	Thr81	Asp292	2.5
Thr82	Lys179	2.9	Thr82	Lys179	2.9	Thr82	Lys179	2.9	Thr82	Lys179	2.9

Compound/Condition	*PH-In (3O96)*	*PH-In_E17K (3O96 mut)*	*PH-In_L52R (3O96 mut)**	*PH-In_D323H (3O96 mut)**
Resveratrol	-45.38	-44.98	-45.51	-44.6
Inhibitor VIII (Novel Allosteric Inhibitor)	-85.79	-75.21	-84.62	-83.21

Figure 4. Oncogenic Mutations in AKT affect interactions of PH and Kinase Domains with binding to inhibitor VIII and phospholipid activator, but not binding to resveratrol. (A) Hydrogen bonding interactions between the PH (yellow) and Kinase (red) domains of AKT. Hydrogen bonds are shown in cyan and amino acids that underlie three key mutations are labeled and colored pink, blue and green. The images show changes in hydrogen binding induced by the mutations to the receptor (AKT). (B) Table showing the hydrogen bonds formed between the PH and kinase domains in both wild-type (WT) and three common AKT mutations. Also shown are the hydrogen bonds formed between the PIP3 head group (IP4) and the PH domain of WT AKT. Bonds shown in green are not affected by the mutations whereas bonds in orange are affected by the mutations. Bonds shown in red are those formed between IP4 and the PH domain of WT AKT, affected by the mutations. (C) Table showing how the mutations affect binding of ATP and PIP3 (IP4) activator by allosteric and ATP competitive inhibitors. Color ranks efficacy per compound, from green (highest affinity) to red (lowest affinity). Unlike the AKT allosteric inhibitor, inhibitor VIII, binding of ATP and PIP3 by resveratrol is not affected by the AKT E17K mutation.

Table 1. Resveratrol metabolites as allosteric inhibitors for wild type and E17K mutant AKT. Shown are resveratrol metabolites and their binding affinities to the allosteric pocket of AKT. The first column shows the AMBER scoring method results; in AMBER scoring, flexibility in both the receptor and ligand is allowed. The second column shows the GRID scoring method; this method does not allow flexibility in either the ligand of receptor. The third column shows the GRID scoring method which allows for flexibility in the ligand, but not the receptor. Compounds which scored lower in the second or third column but higher in the first indicate that changes to the receptor occur by induced-fit. Colors represent ranking from green (best) to red (worst?).

Compound	GRID Score (With Ligand Minimization / Flexibility; No receptor Flexibility)	E17K GRID	Diff
piceatannol	-45.41	-45.98	-0.57
resveratrol-3-O-sulfate	-52.60	-59.28	-6.68
piceid	-65.27	-67.70	-2.43
resveratrol-3-O-glucuronide	-67.46	-69.16	-1.7

Discussion

Aberrant AKT activation is a molecular feature found in many cancers making the discovery of AKT inhibitors of clinical importance. Resveratrol has been reported to inhibit AKT activity [5-8, 11, 12, 31-35]; however, molecular details of the underlying mechanisms have not been fully elucidated. In our previous studies we have proposed resveratrol to exert its multitude of biological effects by binding to its target protein NQO2 [29, 61] and that a link exists between NQO2 and kinase control by resveratrol. That such link occurs intracellularly is supported by results of published studies. For example, designer kinase inhibitors for BCR-ABL or CKII, not only bind and inhibit their respective intended targeted kinases, but also bind and inhibit NQO2 enzymatic activities [62-64]. Control of cell survival kinases including JNK, AKT, p38, and p44/p42 MAPK by NQO2 has been reported using cultured keratinocytes [65]. We recently showed that knockdown of NQO2 in CWR22Rv1 prostate cancer cells is accompanied by a parallel increase in AKT activity [29, 36]; moreover, binding of AKT to resveratrol alters its interaction with NQO2 [29]. Further, in profiling proteins eluted from the resveratrol affinity column using a variety of elution conditions, AKT was eluted by resveratrol and not ATP [29] suggesting that binding of resveratrol to AKT is not likely to involve the ATP binding site. We interpret these results as showing that resveratrol, despite its ability to bind AKT, is a poor ATP competitive inhibitor and instead could additionally affect AKT activity by binding to a location distinct from the ATP binding site. Accordingly, we further investigated the role NQO2 plays in resveratrol mediated inhibition of AKT in the present study, by employing in silico approaches to predict and gain information on the location, binding and interaction between resveratrol and AKT. Several findings from the present study are of clinical and therapeutic relevance.

To determine whether resveratrol could act as a non-competitive allosteric modulator of AKT binding to a site distinct from the ATP pocket [29], we compared effects of resveratrol with inhibitor VIII. We found that resveratrol utilizes two molecular features considered essential for allosteric inhibition: a planar interaction with Trp80 located in the PH domain of AKT, and also via hydrogen bonding with Ser205 in the kinase domain [50, 66]. Global docking analysis further demonstrated that resveratrol preferentially binds the allosteric site of AKT; the residues involved in binding include Asn51, Gln79, Trp80 of the PH domain, and Ser205 Leu210, Val270, Tyr272 and Asp292 in the kinase domain (Figure 1). Our studies also revealed that resveratrol binds with an orientation and interaction similar to inhibitor VIII favoring a forced PH-in conformation and inactive state of AKT; these results support the thesis that resveratrol exerts AKT inhibitory activity by acting as an allosteric modulator.

Disruption of the interaction between PH and kinase domains secondary to somatic mutations in AKT, e.g., E17K mutation, is correlated with oncogenic activation in human cancers [50, 56]. As support that the PH-in conformation is an essential molecular feature for allosteric inhibitors to exert their efficacy in inhibiting AKT, AKT E17K mutation is incapable of responding to pan-AKT inhibitor VIII [50]. In the case of resveratrol even though it appears to act as an allosteric modulator of AKT, molecular docking simulation shows that it is equally effective in wild type and E17K mutated AKT, both with respect to binding energies and orientation (Figure 4C). These results raise the possibility that resveratrol may be developed as adjunct therapy for clinical intervention in patients showing E17K mutated AKT status.

Studies of the CK2 kinase have suggested six categories of kinase inhibitors [67]: the classical ATP-competitive inhibitors (type I); inhibitors that partially bind to an allosteric pocket (type II); inhibitors that bind specifically to an allosteric pocket next to the ATP binding site (type III); substrate competitive inhibitors (type IV); allosteric inhibitors that bind to a pocket completely unrelated to substrate or ATP binding sites (type V); and other noncompetitive inhibitors with unclear mechanisms of action (type V). Based on the binding characteristics and molecular actions revealed in the present study, vis-à-vis, impairment of binding of phospholipids and ATP, disruption of interaction between the PH and kinase domains, and stabilization of interaction occurring between the PH and kinase domains, it is likely that resveratrol falls into a mosaic, multi-action category whose mixed-inhibitory attributes may well depend on the stage of activation of AKT. In this regard, resveratrol may be relevant by allosterically preventing both the

initial pre-activation and membrane translocation of AKT; equally plausible is its engagement in hindering the binding of AKT by membrane bound co-activators. Future studies may reveal that resveratrol has other effects targeting hitherto unexplored states of AKT.

As a final note, we also performed modeling studies using a number of bioactive metabolites sharing in common with resveratrol a 1,2-diphenylethylene nucleus. All chemicals tested bind to E17K mutant AKT with varying degree of increased affinity than wild type AKT, with the most pronounced binding attributed to resveratrol-3-O-glucuronide. It may be suggested that chemicals containing a stilbene-derived scaffold fit an allosteric site present both in wild type and E17K mutant AKT. If verified experimentally this may represent a novel biological activity of resveratrol that has the potential of being developed as adjunctive therapeutic platform for preventing and treating human diseases expressing E17K mutant AKT.

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Author Contributions

Conceived and designed the experiments: TCH. Analyzed the data: TCH, DJB. Wrote and edited the paper: TCH, DJB, BBD, EW, JMW.

Competing Interests

The authors have declared that no competing interests exist.

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