Received: 23 February 2018 | Accepted: 10 July 2018

DOI: 10.1002/jcb.27401

RESEARCH ARTICLE

WILEY Journal of Cellular Biochemistry

Structural insights of resveratrol with its binding partners in the toll-like receptor 4 pathway

Uzma Saqib[1] | **Syed M. Faisal**[2] | **Rohit Saluja**[3] | **Mirza S. Baig**[4]

[1]Discipline of Chemistry, School of Basic Sciences, Indian Institute of Technology (IIT), Indore, India

[2]National Institute of Animal Biotechnology (NIAB), Hyderabad, India

[3]Department of Biochemistry, All India Institute of Medical Sciences (AIIMS), Bhopal, India

[4]Centre for Biosciences and Biomedical Engineering (BSBE), Indian Institute of Technology (IIT), Indore, India

Correspondence
Mirza S. Baig, Centre for Biosciences and Biomedical Engineering, Indian Institute of Technology Indore (IITI), Simrol, Indore 453552, India.
Email: msb@iiti.ac.in

Funding information
Science and Engineering Research Board, Grant/Award Number: YSS/2015/001279; Department of Biotechnology, Ministry of Science and Technology, Grant/Award Number: BT/ HRD/35/02/2006

Abstract

The benefits associated with resveratrol (Resv; 3,4′,5-trihydroxy-trans-stilbene) are known for a long time. The therapeutic properties of Resv are observed in diseases like cancer, neurological disorders, atherosclerosis, aging, inflammation, etc. Multiple studies suggest that the beneficial properties of Resv are due to its binding to targets in multiple pathways. The same has been reflected in inflammation, where Resv has been shown to inhibit nuclear factor κ light-chain enhancer of activated B cells in the toll-like receptor 4 (TLR4) pathway. There are multiple cellular targets which bind to Resv, however the mode and the key interactions involved remain elusive for many of them. In the current work, we have investigated the structural insights of Resv with three of its binding partners involved in the inflammatory TLR4 signaling pathway. Through a structure-based modelling and molecular dynamics study, we have unraveled the molecular and atomic interactions involved in the Resv-binary complexes of inhibitor of κB kinase, cyclooxygeanse-2, and tank-binding kinase I, all three of which are key players in TLR4 inflammatory signaling. This study is the latest addition to the investigations of the structural partners of Resv and its molecular interactions.

KEYWORDS

inflammation, phytochemicals, resveratrol, TLR4

1 | INTRODUCTION

Many phytochemicals like curcumin, vanillic acid, ferulic acid, quercetin, etc, have been shown to have potent activities in various diseases.[1] Although many of these act in varied disease models, but the bandwagon of resveratrol (Resv; 3,4′,5-trihydroxy-trans-stilbene) seems to be never ending with newer targets discovered every year.[2] Multiple studies have shown the potential of Resv as a potent cardioprotective,[3] neuroprotective,[4] anticancer and anti-inflammatory agent.[5] For many of the Resv targets, detailed structural interaction information is available. The protein data bank (PDB) (http://www.rcsb.org/) also witnesses many Resv-protein complexes implicated in various signaling events inside the cell.[6,7] For few others, only the effects or the mechanism of action of Resv is known.[8] The three-dimensional (3D) ligand-receptor binding information is important because it guides us to understand the pharmacology of the compound which thereby helps in enhancing its efficacy. Studies have shown that Resv modifications performed better in disease models further emphasizing the importance of studying interactions at the molecular level.[9] To fully utilize the therapeutic potential of Resv, the understanding of its molecular interactions with its targets is important. Resv regulates the function of key players in the toll-like receptor 4 (TLR4) pathway.[10] Although, substantial cellular data is available indicating

the direct binding of Resv with many TLR4 targets, there is no information on the mode of binding at the molecular level. In continuation with this, we investigated the mode of interaction of Resv with three of the major players of the TLR4 inflammatory pathway: inhibitor of κB kinase (IKK), cyclooxygeanse-2 (COX-2), and tank-binding kinase I (TBK1). All three of these proteins are directly involved in the TLR4 inflammatory pathway (Figure 1A).

1.1 | Resv as a promiscuous drug

Resv, is a natural phytoalexin found in grapes and wine.[11] It has a planer ring system composed of *m*-hydroquinone (ring A) and 4-hydroxystyryl moieties (ring B), Figure 1B. From the past many years its anti-inflammatory, anticancer, and antidiabetic properties have been published by investigators across the world.[5] Multiple reviews have discussed its potential as a potent therapeutic agent.[2,3,12]

1.2 | Resv as an IKK inhibitor

Extracellular stimuli can activate nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) through signal transduction pathways.[13] This is turn activates IKK complex that phosphorylates NF-κB inhibitor, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α on serines leading to its ubiquitination and degradation by the proteasome.[14] This further enables NF-κB to translocate to the nucleus where it activates the expression of proinflammatory genes. Both, Holmes-McNary and Baldwin,[15] and Ren et al[16] have shown that Res could modulate NF-κB activity and downstream gene expression by inhibiting IKK activity. Detailed reports of Resv action on IKK could be referred from these studies.[15,16]

1.3 | Resv as a COX-2 inhibitor

COX-2 catalyzes the conversion of arachidonic acid to proinflammatory substances such as prostaglandins (PGs). Prostaglandin E2 (PGE_2) plays an important role in induced cell proliferation and wound repair.[17] The anti-inflammatory activity of Resv seems to be mainly associated with the suppression of COX-2.[18] Subbaramaiah et al[19] clearly showed that Resv suppressed the synthesis of PGE_2 by inhibiting COX-2 enzyme activity in human mammary epithelial cells. Similarly, Zykova et al[20] have shown that Resv directly targets COX-2 in human colon adenocarcinoma HT-29 cells. These and other studies document the potential of Resv inhibitory activities against COX-2.

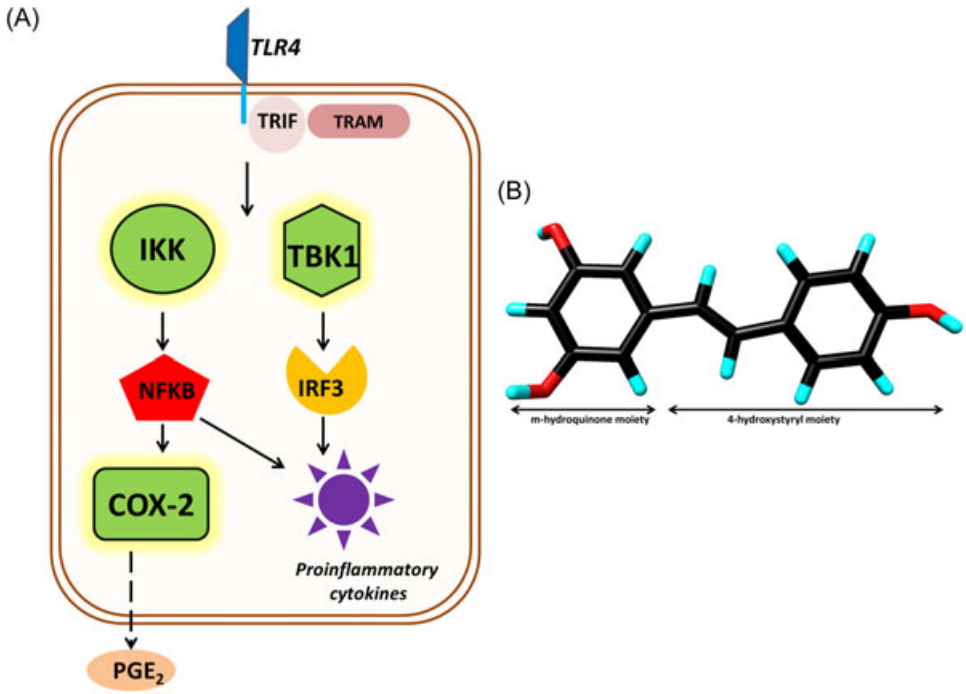

FIGURE 1 A, TLR4 pathway depicting critical roles played by IKK, COX-2, and TBK1 in inflammatory signaling. IKK and TBK1 activate the release of proinflammatory cytokines via NF-κB and IRF3, respectively. On the other hand, COX-2 releases PGE2 which in turn modulates the inflammatory response. B, Three-dimensional chemical structure of Resv. Atoms are colored as carbon (black), oxygen (red), and hydrogen (cyan). The two chemical moieties are shown as marked. Image created by DS Visualizer 2.5. COX, cyclooxygeanse; IKK, inhibitor of κB kinase; NF-κB, nuclear factor κB; PGE, prostaglandin E; TBK, tank-binding kinase; TLR, toll-like receptors

1.4 | Resv as a TBK1 inhibitor

TBK1 is activated by pattern-recognition receptors, such as TLRs, and other intracellular receptors, whereby it mediates downstream signaling pathways via interferon regulatory factor 3, which in turn triggers the expression of proinflammatory cytokines in the nucleus.[21] Resv has been shown to inhibit the kinase activity of TBK1 in a dose-dependent manner.[22] In their study, Youn et al[22] showed that TBK1 is the molecular target of Resv in inhibiting TLR3 and TLR4 downstream signaling pathways and proinflammatory gene expression. For a detailed understanding of Resv inhibition of TBK1, readers are advised to refer recent paper by Liu et al.[23]

2 | MATERIALS AND METHODS

2.1 | Molecular docking

Docking of the respective cocrystallized inhibitors was performed on the IKK, COX-2, and TBK1 structures. The 3D crystal structures of IKK (3RZF),[24] COX-2 (PDB:5IKR),[25] and TBK1 (PDB:4IWQ)[26] were used for the docking analysis. The LibDock program in Discovery Studio 2.5 (http://www.accelrys.com/) was used to redock the bound ligands to their respective receptors. For this, the cocrystallized inhibitors were first extracted from the binding site of protein and redocked back to the parent receptor utilizing the default parameters of the LibDock program. Further, Resv was docked into the respective proteins by specifying the binding site as used for the bound inhibitors.

2.2 | Scoring

To score the docked Resv poses, a consensus scoring method was applied with LigScore[27] (comprising LigScore1_Dreiding, LigScore2_Dreiding), piecewise linear potential 1&2 (PLP1 and PLP2),[28,29] Jain,[30] potential of mean force (PMF)[31] and PMF04 (an updated version of the original PMF score)[32] as the scoring functions. LigScore1 computed in units of pK_i ($-\log K_i$), is calculated based on three descriptors including vdW, C+ pol, and TotPol^2, whose individual contributions provide the overall LigScore1 value. The vdW is a Lennard-Jones potential on a grid. The c+ pol is a count of the buried polar surface area between a ligand and a protein involving attractive ligand-protein interaction. The TotPol^2 is a count of the buried polar surface area between a ligand and a protein involving both attractive and repulsive ligand-protein interactions. Similar to LigScore1, LigScore2 is also based on the descriptors vdW and C+ pol, however instead of TotPol^2, BuryPol^2 is used as a measure for buried polar surface area of the receptor and the ligand molecule. We used Dreiding force-field[33] parameters in LigScore for grid based and exact pairwise calculations of vdW. In PLP1, each nonhydrogen ligand or nonhydrogen receptor atom is assigned a PLP atom type including (a) hydrogen-bond (H-bond) donor, (b) H-bond acceptor, (c) both H-bond donor and acceptor, and (d) nonpolar. On the other hand, in PLP2 function, PLP atom typing remains the same as in PLP1, an atomic radius is assigned for (a) small: a value of 1.4 for F and metal ions (including Zn, Mn, Mg, and Fe); (b) medium: a value of 1.8 for C, O, and N; and (c) large: a value of 2.2 for S, P, Cl, and Br. Jain is an empirical scoring function describing (a) lipophilic interactions, (b) polar attractive interactions, (c) polar repulsive interactions, (d) solvation of the protein and ligand, and (e) an entropy term for the ligand. PMF and PMF04 scores are calculated by summing pairwise interaction terms overall interatomic pairs of the receptor-ligand complex. The active site grid has been selected based on the "PDB site records" for each of the LigandFit calculations. Resulting poses were automatically saved as SD files and analyzed in Discovery studio 2.5.

2.3 | Molecular dynamics

The high scoring ligand-receptor complexes of Resv with the three receptors were further used to perform the molecular dynamics simulations. The PDB deposited crystal structures were already refined hence atom and bond corrections were not needed. Further, the complexes were first energy minimized with 1000 steps of steepest descent and a RMS gradient of 1.0 and then by 2000 steps of adopted basis NR with a RMS gradient of 0.1. After substantial minimization, the complex was subjected to heating with a simulation time of 4 picoseconds with a time step of 2 femtoseconds, at an initial temperature of 50 K and target temperature of 300 K, and a velocity frequency of 50. The equilibrium and production simulation times were kept at 100 and 1000 picoseconds, respectively, with time step and target temperature same as that mentioned for the Heating phase above. Shake constraints were applied to the overall complex.

3 | RESULTS

3.1 | Docking and scoring

The docking protocol was applied to redock the cocrystallized inhibitors to the respective proteins.

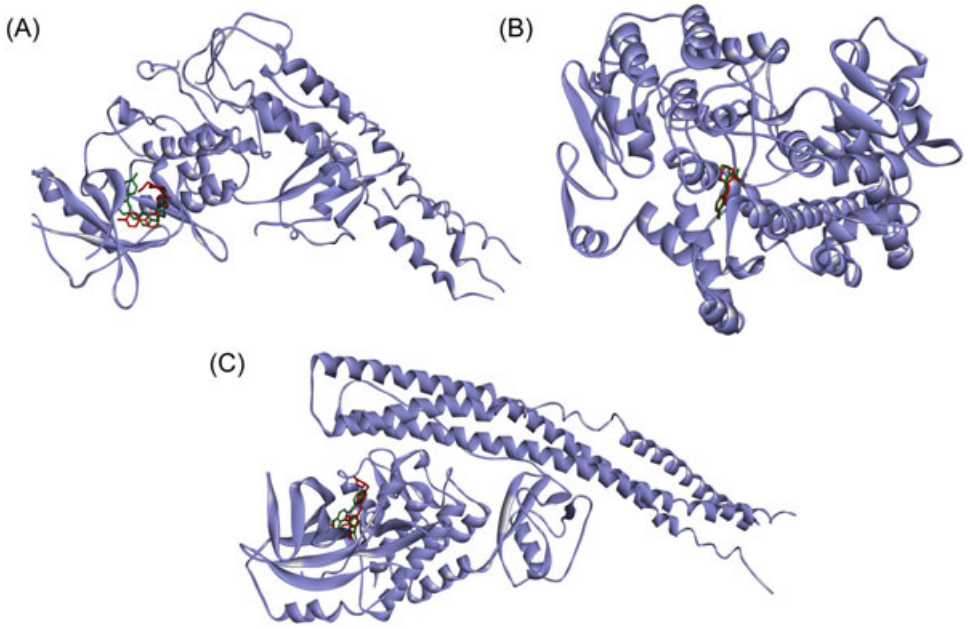

FIGURE 2 Redocked conformations of cocrystallized inhibitors in the receptor binding sites. The crystal-bound inhibitors were redocked in (A) IKK, (B) COX-2, and (C) TBK1 binding sites. Receptors are colored in blue ribbons, cocrystallized (red), and docked inhibitors (green) are depicted in sticks representation. COX, cyclooxygeanse; IKK, inhibitor of κB kinase; TBK, tank-binding kinase

Hence, XNM was redocked into IKK, mefenamic acid into COX-2 and MRT67307 into TBK1. The redockings were done to ensure that the docking program is competent enough to reproduce the closest crystallized conformations. Figure 2 shows that the Libdock program repositions the extracted and redocked ligands in a more or less similar 3D space as that of their cocrystalized conformations in the receptor binding site. Further, Libdock was used to carry the docking simulations of Resv with each of the three targets of study. The binding site for Resv has been identified similar to that for the bound inhibitors described above. The docked conformations of Resv were scored for each of the complex utilizing an intensive scoring analysis using the Score Ligand Poses functionality. Various empirical, force-field, and knowledge-based scoring functions (LigScore1_Dreiding; LigScore2_Dreiding[32]; PLP1; PLP2[30,33]; Jain,[30] PMF, and Dock_Score[27]) part of the LigandFit scoring program, implemented in Discovery Studio 2.5 were used to evaluate the best docked poses. Table 1 tabulates the comparative docking scores of each of three Resv complexes. Figure 3 shows the superimposed conformations of the cocrystallized inhibitors with docked Resv clearly indicating that both bind in the same binding space of the receptor molecule in each of the three complexes.

3.2 | Molecular dynamics

The molecular dynamics study has been done to study the interactions of Resv with the receptor in a time-dependent manner. After scoring and selecting the best Resv poses in the receptors, we performed molecular dynamics of the three complexes respectively. This would provide insights on whether the docked Resv conformations are flexible or static, besides providing a detailed picture of the bound complexes. The Resv-complex dynamics has been monitored over time and evaluated based on their energy during the course of dynamics. It has been observed that all the three complexes showed more or less similar pattern in the stability of Resv-binary complexes. All the three Resv docked complexes did not deviate much from their docked conformations and were more or less

TABLE 1 Top scoring Resv conformations screened by various score functions available in the score ligand poses functionality of Discovery Studio Program

Structure	LigScore1_Dreiding	LigScore2_Dreiding	PLP1	PLP2	Jain	PMF	PMP04	Absolute Energy	LibDock Score
3RZF	1.39	4.22	55.1	43.4	−2.03	49.4	16.99	28.162	82.042
4IWQ	1.44	4.43	59.7	53.2	−1.76	17.2	−4.68	28.223	81.757
5IKR	0.39	3.4	69.9	70.5	2.09	59.2	24.27	28.223	95.875

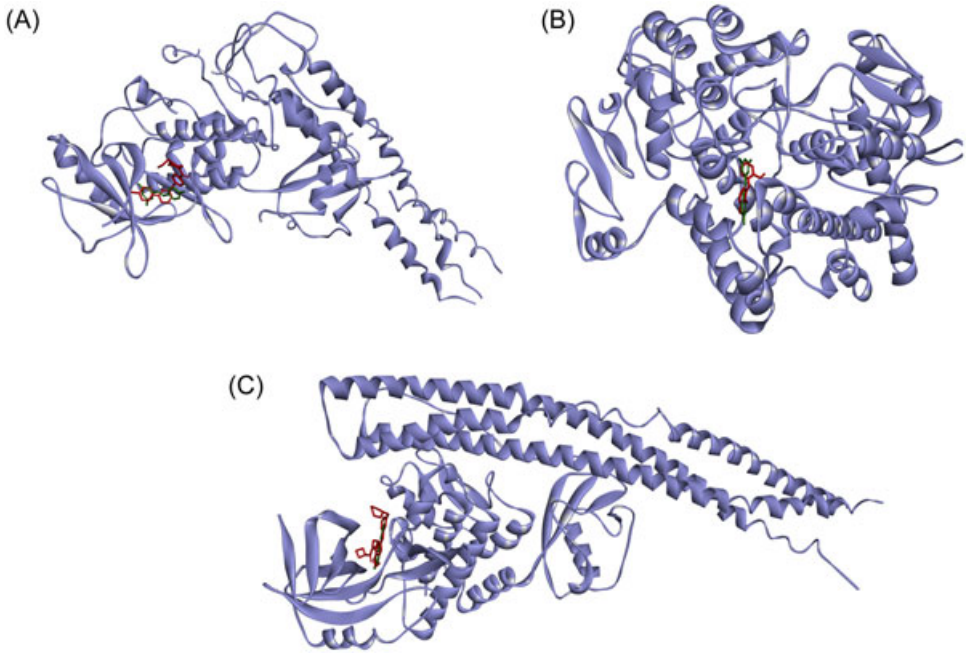

FIGURE 3 Superimposed conformations of cocrystallized inhibitors with Resv. The docked Resv in (A) IKK, (B) COX-2, and (C) TBK1. Receptors are colored in blue ribbons, cocrystallized inhibitors (red), and docked Resv (green) are depicted in sticks representation. COX, cyclooxygeanse; IKK, inhibitor of κB kinase; Resv, resveratrol; TBK, tank-binding kinase

localized at the original point of docking. The total energy vs time graph for each of the complex is shown in the Supporting Information Figure. Also the total energy was more or less stable after 800 picoseconds post-production phase for all of the three complexes. As discussed above, the molecular dynamics simulations concluded with not much change in the initial and final conformations of the bound Resv (Figure 4).

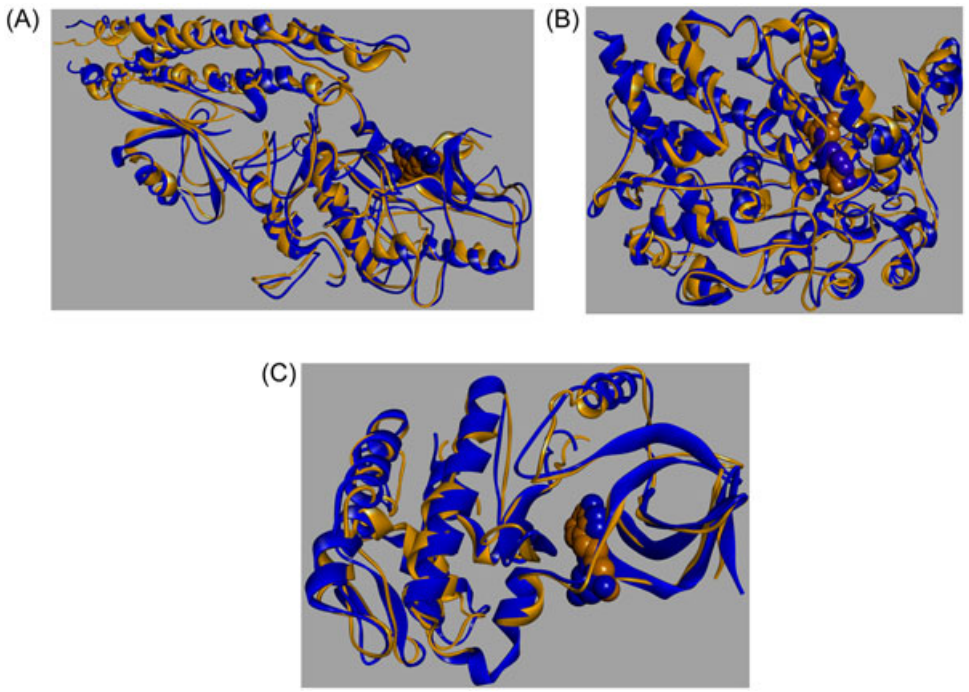

FIGURE 4 Resv docked conformations of receptors. The conformations of Resv (CPK styled) docked into (A) IKK, (B) COX-2, and (C) TBK1 before (blue) and after (golden). Molecular dynamics simulations reveal negligible difference in conformations of Resv and of the overall complex. COX, cyclooxygeanse; IKK, inhibitor of κB kinase; Resv, resveratrol; TBK, tank-binding kinase

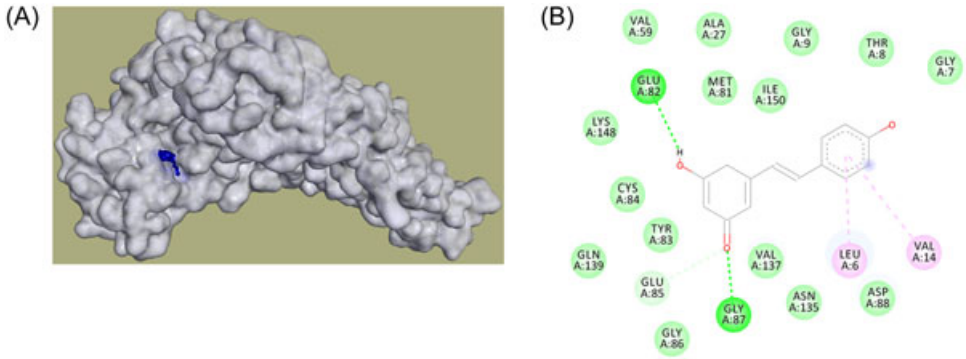

FIGURE 5 Surface representation and two-dimensional binary representation of Resv bound IKK complex. A, Resv (blue CPK styles) bound to the surface cavity in IKK structure (gray). B, Resv (atom color lines) is shown to form many polar and nonpolar interactions with the IKK binding site. Residues are color coded according to the type of interaction involved; dark green depicting the conventional hydrogen bonds, light green as the van der Waals, and light pink as pi-alkyl bonds. IKK, inhibitor of κB kinase; Resv, resveratrol

3.3 | Resv-IKK complex

It could be observed (Supporting Information Figure) the total energy of the IKK-Resv complex is consistent for most of the simulation time. It varied from −25 643 to −26 348 kcal/mol over a period of 1 nanoseconds. Resv binds to IKK in a narrow surface groove (Figure 5A). It interacts with the kinase domain (KD) of IKK at the ATP binding site making it incompatible as an active kinase. In the 3D space, Resv binds the region by making several key interactions through its A and B rings. These include hydrogen-bond interactions with Glu82 and Gly87, and hydrophobic interactions with Tyr83, Cys84, Met8, Ile150, etc via its ring A. Few pi-alkyl interactions with Leu6 and Val14 along with the van der Waals interactions with Gly7, Thr8, Gly9, Asp88, etc via its ring B were also present (Figure 5B).

3.4 | Resv-COX-2 complex

Resv docks into a very deep and narrow cavity inside the COX-2 binding site (Figure 6A). The total energy of the Resv-COX-2 complex remains more or less consistent ranging from −25 875 to −26 657 kcal/mol (Supporting Information Figure). As there was no much movement of Resv during the simulation, the interactions of the binding site residues in the vicinity remain conserved. The ring A of Resv makes two important hydrogen bonds with Val523 and Arg120 via its –OH rings. It also makes multiple hydrophobic Pi-alkyl interactions with various COX-2 binding site residues including Ala527, Val349, Leu352, Val523, etc. On the other hand the ring B of Resv makes a close hydrogen bond with Phe518 in the vicinity, along with a pi-alkyl bond with the nearby Val523 residue. Ring B is also occluded by several key residues involved in hydrophobic interactions (Figure 6B).

3.5 | Resv-TBK1 complex

The binding pocket of TBK1 for Resv is deep buried as in COX-2 (Figure 7A). As for COX-2 and IKK, the total energy of the TBK1-Resv complex does not deviate much and ranges from −13 350 to −13 832 kcal/mol

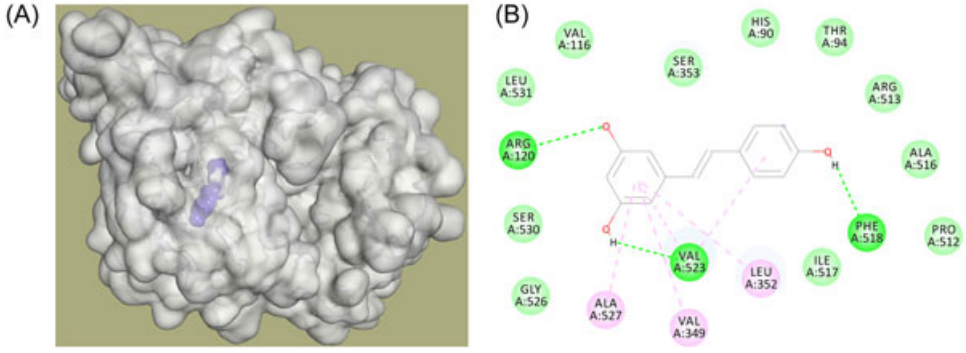

FIGURE 6 Surface representation and two-dimensional binary representation of Resv bound COX-2 complex. A, Resv (blue CPK styles) bound to a deep buried cavity (gray) in COX-2 structure. B, Resv (atom color lines) is shown to form many polar and nonpolar interactions with the COX-2 binding site. Residues are color coded according to the type of interaction involved with dark green depicting the conventional hydrogen bonds, light green as the van der Waals and light pink as pi-alkyl bonds. COX, cyclooxygeanse; Resv, resveratrol

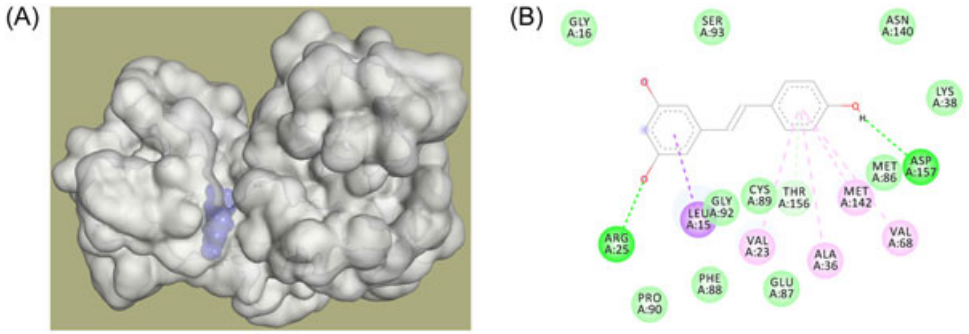

FIGURE 7 Surface representation and two-dimensional binary representation of Resv bound TBK1 complex. A, Resv (blue CPK styles) bound to a deep buried cavity (gray) in TBK1 structure. B, Resv (atom color lines) is shown to form many polar and nonpolar interactions with the TBK1 binding site. Residues are color coded according to the type of interaction involved with dark green depicting the conventional hydrogen bonds, light green as the van der Waals, purple as pi-sigma, and light pink as pi-alkyl bonds. TBK, tank-binding kinase

(Supporting Information Figure). This further confirms that Resv occupies its space quite strongly and with little movement in the TBK1 binding site. As in IKK, TBK1 inhibitors also bind to its KD where they position themselves in the cleft reserved for ATP binding. Resv also binds to TBK1 at the same site designated for ATP or inhibitors. Hence a competitive binding between Resv and ATP would result in inhibition of the kinase activity of TBK1 as well as of IKK as described above. The binary interactions of Resv ring A includes a hydrogen bond with Arg25 via the hydroxyl group of ring A, and a pi-sigma interaction with Leu15. On the other hand, ring B makes several noncovalent interactions with residues of the binding site including a hydrogen bond between its –OH group and Asp 157 and other hydrophobic interactions with Val23, Thr156, Ala36, Val68, Met142 via its phenyl ring (Figure 7B).

4 | DISCUSSION

To truly understand the mechanism of the biological effects, the direct interactions between Resv with its target biomolecules must be identified. Although an increasing number of biochemical tools to measure and quantify direct physical interactions between biomolecules are available, a prior insilico analysis of the binary interactions would be beneficial in dissecting the molecular interactions involved. Resv is a promiscuous molecule with binding partners encompassing hydrolases, oxidoreductase, metal binding proteins, transcription regulators, motor proteins, ligases contractile proteins, transferases, transport proteins, etc.[6-8] Our soon to be published review on Resv, details the structural aspects of Resv binding with its bandwagon of targets. So far, the structural interaction profile of Resv suggests a nonconserved mode of binding which could not be assigned any particular signature motif or pattern. Hence, it could be suggested that only certain local structural complementarity triggers Resv-target interactions and hence its promiscuity for the large array of targets it binds. This could also explain why Resv appears to have so many health benefits.[2-5]

The reports of the biological activities of Resv against TLR4 targets IKK, COX-2, and TBK1 have been published previously.[15-23] Resv has shown to have a clear cut role in inhibiting these targets by direct interactions. Hence, we further wanted to pursue this important phytochemical for its ability to bind these targets at the molecular level. We first extracted the information of the three key TLR4 targets from the PDB. This was followed by redocking of the bound cocrystalized inhibitors back to the receptors. This enabled us to understand whether the docking program could well reproduce the cocrystallized conformations. Further, we docked Resv into the three receptors and compared the overall conformations of Resv and the bound compounds in the receptor binding site. This led us to understand that Resv occupies similar binding space as those occupied by the cocrystallized inhibitors in the three receptors. The docked conformations of Resv in respective receptors were further scored to screen the best scoring conformations, which were further analyzed by molecular dynamics analysis. Molecular dynamics simulations allow us to study protein-ligand interactions in a large conformational space. Each molecular dynamics run encompassing the binary complex minimization, heating, equilibrium, and production phase was analyzed to assess whether Resv moves or changes its conformations inside the binding sites of the receptors in a time-dependent manner. It was observed that Resv snugly and stably fits in the binding pockets. It occupied a surface groove in the IKK binding site, while it was present in a deep cavity in both of the COX-2 and TBK1 binding pockets. As could be

deduced from the unchanged conformations, the energies of the binary complexes were also consistent in the production phase of the molecular dynamics simulations, further confirming that Resv binding is quite stable and strong. We further elucidated the 2D binary interactions of Resv with binding site residues of the three receptors. As described above, Resv more or less binds at the same conformational space as that of the bound inhibitors and hence makes similar set of polar and nonpolar interactions with the binding site residues.

5 | CONCLUSION

The beneficial effects of Resv in various diseases are much attributed to its efficacy to bind multiple receptors in major signaling pathways. Moreover, the unique structural interaction pattern it makes with all these diverse receptors makes it a very interesting phytochemical to study. The multi-targeting role of Resv is inflammation involving IKK, COX-2, and TBK1 led us to investigate its structural fingerprints with its respective receptors. Our study is a novel idea incorporating repurposing, reverse docking and multi-targeting strategies to optimize drug discovery against TLR4 targets. The polypharmacology of Resv could be utilized to probe more targets which would shed light on its immense therapeutic potential. This would eventually facilitate future drug discovery against TLR4 inhibition along with other targets.

ACKNOWLEDGMENTS

This study was supported by the Department of Biotechnology sponsored Ramalingaswami Fellowship (BT/HRD/35/02/2006) to MSB and the Science and Engineering Research Board (SERB) Young Scientists Start-Up Research Grant under Grant Number YSS/2015/001279 to US. The authors also gratefully acknowledge the Indian Institute of Technology Indore for providing facilities and other support.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

ORCID

Mirza S. Baig http://orcid.org/0000-0001-6422-1353

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Saqib U, Faisal SM, Saluja R, Baig MS. Structural insights of resveratrol with its binding partners in the toll-like receptor 4 pathway. *J Cell Biochem*. 2018;1-9. https://doi.org/10.1002/jcb.27401