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An insight into paracetamol and its metabolites using molecular docking and molecular dynamics simulation

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Abstract

Paracetamol is a relatively safe analgesia/antipyretic drug without the risks of addiction, dependence, tolerance, and withdrawal when used alone. However, when administrated in an opioid/paracetamol combination product, which often contains a large quantity of paracetamol, it can be potentially dangerous due to the risk of hepatotoxicity. Paracetamol is known to be metabolized into N-(4-hydroxyphenyl)-arachidonamide (AM404) via fatty acid amide hydrolase (FAAH) and into N-acetyl-p-benzoquinone imine (NAPQI) via cytochrome P450 (CYP) enzymes. However, the underlying mechanism of paracetamol is still unclear. In addition, paracetamol has the potential to interact with other drugs that are also involved with CYP family enzymes (inducer/inhibitor/substrate), an example being illicit drugs. In our present work, we looked into the relationship between paracetamol and its metabolites (AM404 and NAPQI) using molecular docking and molecular dynamics (MD) simulations. We first carried out a series of molecular docking studies between paracetamol/AM404/NAQPI and their reported targets, including CYP 2E1, FAAH, TRPA1, CB1, and TRPV1. Subsequently, we performed MD simulations and energy

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decomposition for CB1-AM404, TRPV1-AM404, and TRPV1-NAPQI for further investigation of the dynamics interactions. Finally, we summarized and discussed the reported drug–drug interactions between paracetamol and central nervous system drugs, especially illicit drugs. Overall, we are able to provide new insights into the structural and functional roles of paracetamol and its metabolites that can inform the potential prevention and treatment of paracetamol overdose.

Keywords

Acetaminophen; Drug abuse; Overdose; CB1; TRPA1; TRPV1

Introduction

Paracetamol, also known as acetaminophen or APAP, is a commonly used analgesia/antipyretic drug and a major active constituent in numerous prescription analgesics [1, 2]. It has been used for more than a century since it was first synthesized in 1878, while the underlying mechanism of its pain and fever reduction effect still remains elusive [2]. Unlike ibuprofen, a non-steroidal anti-inflammatory drug (NSAID), it is uncertain that the analgesic effect of paracetamol can be attributed to the inhibition of cyclooxygenases (COXs) [3]. Paracetamol also does not have side effects on the gastrointestinal tract, blood coagulation, kidney function, etc. [4] Unlike opioid analgesics [5], paracetamol will not generate euphoria or cause any alteration of mood. Therefore, paracetamol can be considered a relatively safe analgesia drug without the risks of addiction, dependence, tolerance, and withdrawal when administered alone. However, when used in opioid/paracetamol combination, which often contains a large amount of paracetamol, it can be potentially dangerous due to paracetamol's well-known hepatotoxicity [6–9]. Data have shown that the proportion of acute liver failure (ALF) cases resulting from deliberate or accidental overdoses of paracetamol, mainly in opioid/paracetamol combination products, increased from 28% in 1998 to 51% in 2003. Thus, the elimination of prescription paracetamol combination products was recommended by the FDA advisory committee in June 2009 [6, 10].

Although the exact mechanism of paracetamol is still unclear, the literature supports the view that paracetamol exerts its therapeutic effects via its active metabolites rather than itself. In the human body, paracetamol can be metabolized into two key metabolites called N-(4-hydroxyphenyl)-arachidonamide (AM404) and N-acetyl-p-benzoquinone imine (NAPQI) [11, 12]. Animal experiments have confirmed that paracetamol is first metabolized into p-aminophenol by the deacetylation in the liver and then into AM404 by fatty acid amide hydrolase (FAAH) in the brain [13]. Literature shows that the transient receptor potential vanilloid 1 (TRPV1) [14, 15] is one of the main receptors of AM404 in dorsal root ganglia (DRG) neurons, where AM404 can bind to the vanilloid binding site of TRPV1. Moreover, it is reported that, under high concentration, AM404 is a direct activator of TRPV1 in the brain, which mediates paracetamol-evoked hypothermia and analgesia [14]. The activation of TRPV1 can consequently lead to inhibition of its downstream T-type calcium channels, especially those of the Cav3.2 subtype, which produces an analgesic response [2, 11]. Furthermore, paracetamol also exerts a possible effect on the endocannabinoid system [15–17]. Anandamide (AEA) is an endocannabinoid

neurotransmitter that activates cannabinoid type 1 receptor (CB1) [18–21], and is degraded by FAAH, the same enzyme that metabolizes paracetamol into AM404. Therefore, degradation and reuptake of AEA can be inhibited competitively by AM404. Hence, AM404 can indirectly activate CB1 in the rostral ventromedial medulla (RVM) [12]. Recently, a study has found that, upon administration of AM404 (10 mg/kg, i.v.) to wild-type mice, respiratory rate and atrial oxygen saturation were reduced significantly, while the respiratory parameters of CB1 knock-out mice showed no notable difference [22]. The insensitivity of CB1 knockout mice and rats pretreated with the CB1 antagonist (AM251) to paracetamol further supports the involvement of CB1 in the mechanism of paracetamol [2, 23–25]. Recently, AEA has been reported to activate both CB1 and TRPV1 receptors on cultured DRG neurons [26, 27].

NAPQI, the other metabolite of APAP, is highly reactive and toxic. It sensitizes and activates transient receptor potential ankyrin 1 (TRPA1) slowly but directly by interacting with distinct intracellular cysteine residues [14]. Recently, Eberhardt et al. [28] reported that both parabenzoquinone (pBQ) and NAPQI can activate TRPV1. Furthermore, TRPA1 can co-express with TRPV1 on most of the sensory neurons in the liver, spinal cord, and kidney [15, 29]. In addition, the analgesia effect mediated by the activation of TRPA1 has been reported to be similar to that of TRPV1 [30, 31].

Regarding the relationship between paracetamol and 5-HT family receptors, even though paracetamol increases in brain areas such as cortex, hypothalamus, striatum, hippocampus, and brainstem correspond to the concentration of 5-HT1A, 5-HT2A, 5-HT2C, and 5-HT7 receptor subtypes (except 5-HT3) in a concentration-dependent manner [2, 32], it remains uncertain whether 5-HT family receptors are involved in the action of paracetamol.

It is also worth mentioning that paracetamol is metabolized mostly into NAPQI by CYP 3A4, CYP 2E1, CYP 2C9, CYP 2D6, CYP 2A6, and CYP 1A2. Among them, CYP 2E1 and CYP 3A4 are believed to be the major contributors to the metabolism. Therefore, drugs that can act as substrates/inhibitors/inducers of cytochrome P-450 CYP 2E1/3A4 may have the potential to interact with paracetamol [33].

The detailed information regarding the molecular level of the interaction between paracetamol/AM404/NAPQI and the targets is currently unavailable. In our present work, we performed computational systematic analyses of paracetamol and its metabolites (AM404 and NAPQI) to look into the underlying mechanism of paracetamol (a flow chart of our study can be found in Fig. S1 in the Supplementary Material).

Materials and methods

Homology modeling and validation of TRPV1 and structural preparation of CB1 structure

In this study, we used our previous homology model of human TRPV1 (hTRPV1) for further studies. Briefly, this model was constructed based on the cryo-EM-derived structure of *Rattus norvegicus* TRPV1 (rTRPV1)-capsaicin (PDB entry:3J5R, EM resolution: 4.2 Å) [34]. The three-dimensional (3D) TRPV1 structural model has been previously validated by

our MD simulation and bioassay data [35, 36]. The binding site is a groove pocket, including residues Tyr511, Leu518, Phe543, Leu547, Thr550, Arg557, Glu570, Leu663, and Leu670.

Moreover, we used the crystal structure of CB1 (PDB: 5XR8) [37] in our studies. Modeller 9.18 [38] was used to repair the residues. In addition, we used Modeller to ‘fill in’ the missing residues/loops by treating the original structure (without the missing residues) as a template. The important residues involved in the binding pocket included Phe102 (N-terminus), Met103 (N-terminus), Ile105 (N-terminus), Phe170, Phe174, Val196, Thr197, Phe268, Leu276, Trp279, Trp356, Val364, Phe379, and Ser383.

Docking study of ligand-receptors

We adopted the MOLCAD module implemented in SYBYL-X 1.3 to explore the potential binding pocket for receptors. The docking program Surflex-Dock GeomX (SFXC) in SYBYL-X 1.3 was applied to construct receptor–ligand complexes in which the docking scores were expressed in $-\log_{10}(K_d)$ [39]. The main protocols or parameters of docking were addressed in our previous publications [35, 40–43]. Briefly, the docking parameters used were as follows: (1) the number of starting conformations per ligand was set to 10, and the number of max conformations per fragment was set to 20; (2) the maximum number of rotatable bonds per molecule was set to 100; (3) flags were turned on at “pre-dock minimization”, “post-dock minimization”, “molecule fragmentation”, and “soft grid treatment”; (4) activate spin alignment method with density of search was set to 9.0; and (5) the number of spins per alignment was set to 12.

Molecular dynamics simulations

Three systems were set up using the web-based tool CHARMM-GUI [44, 45]. For the TRPV1 receptor complexed with AM404 or NAPQI, the system included 328 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) lipids, 75,639 water molecules, 67 Na⁺ ions, and 95 Cl[−] ions. The initial configurations of protein receptors and ligands were taken from docking studies. The sizes of the initial simulation boxes were $\sim 121 \text{ \AA} \times 121 \text{ \AA} \times 106 \text{ \AA}$. The system of CB1 with AM404 included 127 POPE lipids, 8237 water molecules, 21 Na⁺ ions, and 31 Cl[−] ions. The box size of the initial simulation was $\sim 71 \text{ \AA} \times 71 \text{ \AA} \times 100 \text{ \AA}$.

The AMBER ff14SB force field [46] was applied to proteins and the AMBER Lipid14 force field [47] was applied to lipids. Water molecules were treated with the TIP3P water model [48]. The partial atomic charges of ligands were derived by the semi-empirical with bond charge correction (AM1-BCC) method [49, 50]. The other force field parameters came from GAFF in AMBER16 [50]. The residue topologies for ligands were prepared using the ANTECHAMBER module [51].

Molecular dynamics (MD) simulations were carried out using the PMEMD.mpi and PMEMD.cuda modules in the AMBER16 [52–54] package. First, several minimization steps were carried out on the systems to avoid possible steric crashes. Then, each system was gradually heated from 0 K to 300 K during the heating stage and kept at 300 K during the following equilibrium and production stages. A time step of 2 fs was used for the heating stage, the equilibrium stage, and the entire production stage. A periodic boundary condition was employed to maintain constant temperature and the pressure (NPT) ensembles. The

pressure was set at 1 atm and controlled by the anisotropic (x-, y-, z-) pressure scaling protocol with a pressure relaxation time of 1 ps. The temperature was regulated using Langevin dynamics with a collision frequency of 2 ps^{-1} [55, 56]. The particle mesh Ewald (PME) method [57, 58] was adopted to handle long-range electrostatics and a $10 \text{ \AA}$ cutoff was set to treat real-space interactions. All covalent bonds involving hydrogen atoms were constrained with the SHAKE algorithm [59]. Each system was subjected to 100 ns MD simulation and the trajectories of simulated systems were saved every 100 ps.

Molecular mechanics/generalized born surface area calculations

For the saved trajectories of MD simulations, the molecular mechanics/generalized Born surface area (MM/GBSA) [60–63] method was used to calculate the binding energies of receptors under the treatment of different ligands, respectively. Every 200 ps from 90 to 100 ns, 50 snapshots were extracted from each trajectory in order to calculate the mean binding energy. The formula was used as follows:

$$\Delta E_{\text{bind}} = \Delta E_{\text{MM}} + \Delta E_{\text{SOL}} = \Delta E_{\text{MM}} + \Delta E_{\text{GB}} + \Delta E_{\text{SA}}$$

where E_{bind} is the binding energy, and E_{MM} denotes the sum of molecular mechanical energies in vacuo and can be further divided into the contributions from electrostatic, van der Waals, and internal energies. This term could be computed through molecular mechanics methods. E_{SOL} is the solvation energy, which includes the polar solvation energy (E_{GB}) calculated with the generalized Born (GB) approximation model [64, 65], and the non-polar part (E_{SA}) obtained by fitting solvent accessible surface area (SASA) [66] with the linear combinations of pairwise overlaps (LCPO) model [67, 68]. Additionally, the energies of each residue were decomposed into the backbone and side-chain atoms. Through energy decomposition, we can analyze the contributions of key residues to binding [69].

Energy decomposition analysis (EDA) with ab initio methods, such as absolutely localized molecular orbitals (ALMO) and symmetry-adapted perturbation theory (SAPT), can in principle provide more accurate results [70, 71]; however, due to the high computational demand, their applications are currently limited to the small model systems of hydrogen bonding, π - π interaction, halogen interactions, etc., and served as a tool to develop next-generation MM force fields [70, 71]. MM-based EDA methods have been widely and successfully applied to systems of macromolecules [60–69].

Results and discussion

Interactions between paracetamol and CYP 2E1

As mentioned previously, CYP 2E1 and CYP 3A4 are believed to be the major contributors to the metabolism of paracetamol. Here, we docked paracetamol into both CYP 2E1 (Figs. 1, S2) and CYP 3A4 (Results and Fig. S3 in the Supplementary Material) to explore their interactions.

First, we docked the reported compound pilocarpine back to the crystal structure of CYP 2E1 (PDB: 3T3Z) [72] as a validation of our docking protocol, as shown in Fig. S3a in the

Supplementary Material. We can see that several key residues are involved in the binding pocket of CYP 2E1, including Phe106, Phe115, Phe116, Phe207, Phe298, Thr303, Val364, Leu368, and Phe478 [72]. Particularly, the docked pilocarpine (magenta) overlapped well with the crystallized structure (orange) with a root mean square deviation (RMSD) of 1.5 Å, indicating that our docking protocol is reliable.

Then, we docked paracetamol (blue) into CYP 2E1 with the same protocol, as shown in Fig. 1. For comparison, the alignment between paracetamol (blue) and crystallized pilocarpine (magenta) can be found in Fig. S3b in Supplementary Material. The docking results showed that crystallized pilocarpine (magenta) and paracetamol (blue) share a similar binding pose, hydrophobic interactions, and coordination bond. For example, a coordination bond can be found between the heme iron and the hydroxyl group of paracetamol with a distance of ~2.1 Å (Fig. 1), while the distance between pilocarpine and the heme iron in CYP 2E1 in the co-crystal structure is ~2.2 Å. Moreover, Ile115, Phe116, and Phe298 in CYP 2E1 interact with paracetamol/pilocarpine through hydrophobic interactions, which can future stabilize coordination bonds between paracetamol and the heme iron. We also observed some stronger hydrophobic interactions between Phe207/Phe478 and pilocarpine with a distance of ~3.5 Å, which helps explain the strong binding affinity of pilocarpine ($K_I = 360 \mu\text{M}$ ~765 μM) [72] compared with that of paracetamol ($K_I = 830 \mu\text{M}$) [73].

The docking results and discussion of paracetamol in CYP 3A4 can be found in the Supplementary Material.

Interactions between paracetamol and FAAH

As described above, paracetamol can be metabolized by FAAH to form AM404 in rat and mouse brains [13]. Here, we docked paracetamol into FAAH using the same docking protocol.

Carprofen was first docked back to the crystal structure of FAAH (PDB: 4DO3) [74], as shown in Fig. S4a in the Supplementary Material. The key residues involved in the binding pocket of FAAH included Ser193, Phe194, Phe381, Leu401, Gly402, Asp403, Leu404, Ile407, Met436, Phe432, Gly485, Arg486, Ala487, Thr488, Ile491, and Trp531. As shown in Fig. S4a, the docked carprofen (orange) and the crystallized carprofen (magenta) overlapped very well with each other, with RMSD of about 0.8 Å, indicating the accuracy of our docking protocol.

Sequentially, we docked paracetamol (blue) into FAAH, as shown in Fig. 2. For comparison, the alignment of crystallized carprofen (magenta) and paracetamol (blue) is shown in Fig. S4b in the Supplementary Material, which showed that carprofen and paracetamol shared very similar binding poses and hydrogen bonds (H-bonds) in the binding pocket. From Fig. 2, we found that oxygen in the carboxylate group of crystallized carprofen (magenta) and oxygen on the benzene ring of paracetamol (blue) formed strong H-bonds with multiple residues, including Asp403 (~4.5 Å vs ~2.9 Å), Arg486 (~4.7 Å vs ~2.9 Å), and Gly402 (~4.0 Å vs ~3.9 Å). Residues including Asp403, Arg486, and Gly402 in Fig. 2 are reported to play key roles in the recognition for ligand binding of FAAH [74]. In addition, the oxygen

in the carbetamide group of paracetamol forms an H-bond with the oxygen on Thr488 with a distance of 2.8 Å, which further stabilizes paracetamol in the binding pocket.

Interactions between NAPQI and TRPA1

TRPA1, a member of transient receptor potential channel family, is a potential target for pain and neurogenic inflammation. Increase in $[Ca^{2+}]$ is an indication of the activity of the TRPA1 receptor. A study [75] reported that upon administration of NAPQI (500 nM) to dorsal root ganglion neurons, 39% of neurons cultured from TRPA1^{+/+} mice elicited an increase in $[Ca^{2+}]$, in contrast to 0.4% in neurons cultured from TRPA1^{-/-} mice. This finding affirmed NAPQI as a ligand for the receptor TRPA1 [75]. In the present work, we applied the same docking protocol to dock NAPQI into TRPA1.

NAPQI (blue) was docked into the structure of TRPA1 (PDB:3J9P) [76] to explore their detailed interaction, as shown in Fig. 3. The binding pocket was defined according to the literature [76]: “antagonist binding site: the cryo-EM map for the double-antagonist-treated sample contains a unique density corresponding to A-967079 and located within a pocket formed by S5, S6 and the first pore helix.” Residues Thr874, Ile878, Leu881, Ile905, Gln906, Phe909, Val942, and Ile946 play important roles in the ligand recognition of TRPA1. We observed three important interactions between NAPQI and TRPA1, which we believe to be the main contributors to its recognition. A strong H-bond can be found between the oxygen on the benzene ring of NAPQI and the oxygen on Thr874, with a distance of around 2.9 Å. In addition to the H-bond, two hydrophobic interactions were observed according to its binding pose. They are the interaction between the benzene ring on NAPQI and the benzene ring on Phe909 (~4.7 Å), and between the same benzene ring on NAPQI and a carbon on Ile878 (~3.7 Å). All these interactions and key residues were supported by the crystal structure information of TRPA1 (PDB:3J9P) [76].

Interactions between AM404 and CB1

The CB1 receptor is a G protein-coupled receptor that mediates adenylate cyclase, neurotransmitters release, and calcium channel activity when activated [18–21]. Recently, Sinning et al. [77] reported that AM404 acts as a weak agonist at CB1 with K_I value of 1.5 μM and at CB2 with K_I value of 1.3 μM. Therefore, we docked AM404 into CB1 and conducted the MD simulation to explore these interactions.

First, we docked the reported compounds back to the CB1 crystal structure complexed with agonists (PDB: 5XR8) [37] as a validation of our docking process. From Fig. S5 in Supplementary Material, we can find that the reported AM841 (agonist, orange) was able to overlap with the crystallized AM841 (magenta), with an RMSD of about 0.2 Å, indicating that our docking process is reliable.

As shown in Fig. 4, we docked AM404 into the crystal structure of CB1. The binding pocket of CB1 was formed mainly by helices I, II, III, V, VI, VII, ECL2 (extracellular loop 2) and the N-terminus. The important residues involved in the binding pocket included Phe177, Phe189, Leu193, Val196, Phe200, Phe268, Leu359, Met363, and Ser383. All the residues described here are consistent with the information acquired from the CB1 crystal structures [37, 78, 79]. From our docking results, we found that Phe189 and Ser383 interacted with

AM404 via H-bonds (3.6 Å and 2.9 Å), while Phe177, Leu196, Phe200, Leu359, and Met363 contributed to the hydrophobic interactions with distances of 3.5 Å, 3.5 Å, 3.4 Å, 3.5 Å, and 3.5 Å, respectively. All these results were supported by the CB1 crystal structure.

MD simulation between AM404 and CB1

In order to further validate the interactions described previously, we carried out 100 ns MD simulation for the system of CB1 and AM404. The RMSD of CB1 and AM404 fluctuated around 1.5 Å and 3.5 Å, respectively (black and red lines in Fig. 4c). Meanwhile, the average structure of the last 20 ns simulations was generated and used for analyses. As a CB1 agonist, AM404 was found to be relatively stable during the MD simulation with some conformational changes. For example, Phe189, Val196, Phe268, Leu359 formed strong hydrophobic interactions with AM404 during the MD simulation. However, the hydroxyphenyl group on AM404 moved away from Phe177 and Ser383, making hydrophobic interactions and breaking the H-bond (Fig. 4b). Interestingly, Ser383 still got close to, and interacted with, AM404 through hydrophobic interactions. In order to explore the contribution of key residues, we conducted the free energy decomposition (Table 1) using the MM/PBSA method. Figure 4d shows that Phe189 ($-1.674/-1.287$ kcal mol $^{-1}$), Leu193 ($-2.224/-1.844$ kcal mol $^{-1}$), Val196 ($-1.662/-1.635$ kcal mol $^{-1}$), Phe268 ($-2.688/-2.164$ kcal mol $^{-1}$), and Ser383 ($-1.618/-1.379$ kcal mol $^{-1}$) can stabilize the interaction with alkyl chains owing to the contribution of van der Waals interactions/non-polar solvation. Meanwhile, electrostatic contributions are not important, except at Ser383 (-0.485 kcal mol $^{-1}$); however, the polar solvation contribution can deteriorate the interaction. The contribution of the aromatic ring on Phe268 (total energy: -4.564 kcal mol $^{-1}$), and methyl on Val196 (total energy: -3.564 kcal mol $^{-1}$) were the most important factor for its binding with CB1. All these MD results were consistent with the docking results, providing detailed information on the interactions between AM404 and CB1.

Interactions between AM404 and TRPV1

TRPV1, a nonselective cationic channel that promotes depolarization and synaptic activity, functions as a detector and regulator of body temperature and can provide a sensation of pain (nociception) [14, 15]. Recently, Stueber et al. [11] reported that AM404 activated hTRPV1 at concentrations higher than 1 μ M and in a concentration-dependent manner. Moreover, Eberhardt et al. [28] reported that NAPQI, another key metabolite of paracetamol, can activate TRPV1. Thus, we docked AM404/NAPQI to TRPV1 to study their interactions.

The binding mode of AM404 with TRPV1 is shown in Fig. 5a. We found that the atom oxygen and nitrogen of AM404 can form a strong H-bond with the hydroxyl group on Thr550 at a distance of about 3.0 Å, and the hydroxyl or carbonyl on aromatic ring can bind with the nitrogen atom on Arg557 through a weak H-bond at a distance of about 4.4 Å. Importantly, AM404 has a long fat chain adjacent to acetyl, which can form a hydrophobic interaction with benzene on Phe543, and the methyl of Leu518 and Leu547, at distances of 3.4 Å, 3.2 Å and 3.5 Å, respectively.

MD simulation between AM404 and TRPV1

In order to verify the proposed interaction and investigate the dynamic interactions, we carried out 100 ns MD simulations for the system of TRPV1 complexed with AM404 (see movie in the Supplemental Material).

The RMSD of the protein increased slowly from ~ 2.4 Å at $t = 0$ ns to ~ 2.6 Å at $t = 40$ ns, and then kept stable for the rest of simulation time (black line in Fig. 6a). Moreover, the RMSD of AM404 dropped from ~ 3.4 Å to ~ 2.5 Å at $t = 28$ ns and then fluctuated around this value. The average structure of the last 20 ns simulations was taken and is shown in Fig. 5c. Comparing with Fig. 5a, the binding positions and bind pose of AM404 were almost the same, except that the aromatic head went deeper into the binding cavity and got close to Arg557 and Glu570 (Fig. 5c). The H-bond between the hydroxyl O in the aromatic head and the N atom in Arg557 got much stronger (from 4.4 Å to 4.2 Å). Most of the interactions revealed by the docking study were preserved in MD (Fig. 5c): (1) the H-bond between the hydroxyl group in Thr550 and the carbonyl O atom in AM404 remained stable (Fig. 5c, 2.6 Å); (2) the benzene ring of Phe543 got close to the linear alkyl chain of AM404 and formed a similar hydrophobic interaction; and, moreover, (3) Leu663 and Leu547 formed a similar hydrophobic interaction with the alkyl chain of AM404 during the MD simulation. Additionally, the hydrophobic side chain of Leu518 contributed van der Waals energy for their interaction (distance < 5.0 Å, not shown in Fig. 5c).

In order to discover the contribution of key residues to the binding between TRPV1 and AM404, we performed free energy decomposition with MM-GBSA and the results are shown in Fig. 6c and Table 2. We found that the contribution of Thr550 (total energy: -7.624 kcal mol $^{-1}$) was larger than that of the other residues owing to the electrostatic energy (-7.121 kcal mol $^{-1}$) from two H-bonds. Similarly, the strong hydrophobic interaction between Phe543 (van der Waals: -2.108 kcal mol $^{-1}$), Leu 547 (van der Waals : -2.155 kcal mol $^{-1}$), Leu663 (van der Waals: -1.403 kcal mol $^{-1}$), and AM404 can promote their binding.

Interactions between NAPQI and TRPV1

The binding modes of NAPQI with TRPV1 are shown in Fig. 5b. We also found that the atom oxygen and nitrogen of NAPQI can form a strong H-bond with the hydroxyl group on Thr550 at a distance of about 3.0 Å, and the hydroxyl or carbonyl on aromatic ring can bind with nitrogen atom on Arg557 through a weak H-bond at a distance of about 4.5 Å. Moreover, the methyl of NAPQI can form a hydrophobic interaction (3.8 Å) with the side chain of Leu670. The details of interactions between NAPQI and TRPV1 can be found in our docking data.

MD simulation between NAPQI and TRPV1

For the TRPV1-NAPQI system, the RMSD of TRPV1 remained stable at around 3.1 Å after 35 ns (black line in Fig. 6b). Moreover, the RMSD of NAPQI fluctuated slightly between 1.2 Å and 2.0 Å (red line in Fig. 6b). The average structure of the last 20 ns simulations of the TRPV1-NAPQI system was shown in Fig. 5d. Comparing with the docking structure (Fig. 5b), the binding pose of NAPQI predicted by MD simulation was similar but underwent some conformational changes. The H-bond between the hydroxyl O of NAPQI and the N

atom in Arg557 became much stronger (from 4.5 Å to 3.0 Å), just like that of AM404. Both of the bifurcated H-bonds between the hydroxyl group in Thr550 and the two H-bond receptors (N and carbonyl O atoms) in the ligand (Fig. 5b) were totally broken (Fig. 5d). However, the carbonyl group of NAPQI formed a new H-bond with the hydroxyl group in Tyr511 (Fig. 5d). The hydrophobic bond between methyl on NAQPI and Leu670 was retained (3.8 Å). Unlike the TRPV1-AM404 system, the position of the benzene ring of Phe543 did not change too much.

In order to discover the contribution of key residues to the binding between TRPV1 and NAPQI, we performed free energy decomposition with MM-GBSA and the results are shown in Fig. 6d and Table 3. Our results showed that the electrostatic energy from Tyr511 ($-3.485 \text{ kcal mol}^{-1}$) and Arg557 ($-5.191 \text{ kcal mol}^{-1}$) was larger than that of the other residues owing to their strong H-bonds. Additionally, the hydrophobic interaction between Thr550 ($-0.808 \text{ kcal mol}^{-1}$)/Gln570 ($-0.881 \text{ kcal mol}^{-1}$) and NAPQI was favorable for binding. However, Tyr511 ($0.392 \text{ kcal mol}^{-1}$) and Arg557 ($1.738 \text{ kcal mol}^{-1}$) provided positive polar solvation energy for bonding, which meant the hydrophobic interaction energy here was favorable.

Conclusions

Paracetamol has been used worldwide for many years but when administrated in an opioid/paracetamol combination product that contains a large amount of paracetamol, the risk of hepatotoxicity increases tremendously. Paracetamol can be metabolized into NAPQI and AM404, and several novel targets are identified for these two metabolites recently, including TRPV1, CB1, and TRPA1.

In present work, we were able to provide new insights into the interaction between paracetamol/its active metabolites and their targets at a molecular level, which will help inform the potential prevention and treatment of paracetamol overdose. The binding result between paracetamol and CYP 2E1 showed a stable coordination bond between the heme iron and the hydroxyl group on paracetamol, which made possible the metabolism of paracetamol in CYP 2E1. The binding result between paracetamol and FAAH showed four strong hydrophobic interactions, which secured paracetamol inside the binding pocket of FAAH. The detailed binding results of these metabolic processes have the potential to aid in future drug design of paracetamol derivatives that is selective for one pathway instead of being process by both pathways and producing both metabolites. In addition to metabolic processes, the binding and MD result of the interaction between the metabolites and their targets could also provide new insights into the further development of paracetamol derivatives. Binding results between NAPQI and TRPA1 showed a total of three interactions between NAPQI and the target, including one strong hydrogen bond and two hydrophobic interactions; the binding result of AM404 and CB1 showed that, inside the binding pocket of CB1, AM404 is secured by one hydrogen bond and multiple hydrophobic interactions, while the MD result showed that AM404 is relatively stable inside the binding pocket; binding results between AM404/NAPQI and TRPV1 compared the binding affinity and interaction between two metabolites, while MD results compared their stability. The binding results between the metabolites and their targets provided us with a closer look at these interactions,

which could be used to assist with the design of future drugs using these metabolites as the active ingredient.

Apart from the reported mechanism that TRPV1 is the common direct target of the two metabolites of paracetamol (AM404 and NAPQI), we suggest two other underlying mechanisms for the therapeutic effect of paracetamol based on our data: (1) NAPQI directly targets TRPA1, which is co-expressed with TRPV1, and both TRPA1 and TRPV1 may be correlated with paracetamol-dependent hypothermia. (2) The direct activation of the cannabinoid system (maybe CB1) by AM404 might be involved in the paracetamol-modulated nociception-related signal transduction.

Last but not least, we summarized the reported drug–drug interactions of paracetamol. More results and discussion can be found in the Supplementary Material.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Interaction between paracetamol and CYP 2E1. Key residues in CYP 2E1 include Phe106, Phe115, Phe116, Phe207, Phe298, Thr303, Val364, Leu368, and Phe478

Fig. 2.

Interaction between paracetamol and fatty acid amide hydrolase (FAAH). Ser193, Phe194, Phe381, Leu401, Gly402, Asp403, Leu404, Ile407, Met436, Phe432, Gly485, Arg486, Ala487, Thr488, Ile491, and Trp531 contribute to the recognition of ligands

Fig. 3.
Interaction between NAPQI and TRPA1. Thr874, Ile878, Leu881, Ile905, Gln906, Phe909, Val942, and Ile946 contribute to the recognition of ligands

Fig. 4 a–c.

Interaction and molecular dynamic (MD) simulation between AM404 and CB1. **a**

Interaction between AM404 and CB1. **b** Interaction between AM404 and CB1 after 100 ns

MD simulation. **c** Energy decomposition analysis (EDA) of complex CB1-AM404

Fig. 5 a–d.

Interaction and MD simulation between AM404/NAPQI and TRPV1. **a** Interaction between AM404 and TRPV1. **b** Interaction between NAPQI and TRPV1. **c** Interaction between AM404 and TRPV1 after 100 ns MD simulation. **d** Interaction between NAPQI and TRPV1 after 100 ns MD simulation. Tyr511, Leu518, Phe543, Leu547, Thr550, Arg557, Glu570, Leu663, and Leu670 in TRPV1 contribute to the recognition of ligands

Fig. 6 a–d.

Root mean square deviation (RMSD) plots and EDA for MD of TRPV1 complexed with AM404 and NAPQI. **a** RMSD of complex TRPV1-AM404. **b** RMSD of complex TRPV1-NAPQI. **c** EDA of complex TRPV1-AM404. **d** EDA of complex TRPV1-NAPQI

Table 1

Energy decomposition of the CB1–AM404 interaction

Residues	Energy (kcal mol ⁻¹)				Total
	van der Waals	Electrostatic	Polar solvation	Non-polar solvation	
Phe177	-0.019	-0.004	0.010	0.000	-0.013
Phe189	-1.674	-0.313	0.273	-1.287	-3.000
Leu193	-2.224	0.036	0.093	-1.844	-3.938
Val196	-1.662	-0.115	-0.153	-1.635	-3.564
Phe200	-0.352	0.027	-0.001	-0.179	-0.504
Phe268	-2.688	-0.034	0.321	-2.164	-4.564
Leu359	-0.019	-0.008	-0.018	0.000	-0.046
Met363	-0.723	0.011	-0.027	-0.657	-1.397
Ser383	-1.618	-0.485	0.393	-1.379	-3.089

Energy decomposition of TRPV1 and AM404 interaction

Residue	Energy (kcal mol ⁻¹)				
	van der Waals	Electrostatic	Polar solvation	Non-polar solvation	Total
Tyr511	-0.345	0.037	-0.087	-0.116	-0.512
Leu518	-0.048	-0.019	-0.022	0.000	-0.089
Phe543	-2.108	0.159	-0.001	-1.855	-3.804
Leu547	-2.155	0.436	-0.255	-1.666	-3.640
Thr550	-0.699	-7.121	1.438	-1.243	-7.624
Arg557	-0.961	-0.700	-0.971	-0.656	-3.288
Glu570	-1.200	0.157	1.529	-0.700	-0.214
Leu663	-1.403	-0.009	0.007	-1.098	-2.503
Leu670	-0.975	-0.158	0.130	-0.941	-1.944

Table 3

Energy decomposition of TRPV1 and NAPQI interaction

Residue	Energy (kcal mol ⁻¹)			
	van der Waals	Electrostatic	Polar solvation	Non-polar solvation
Tyr511	-0.409	-3.485	1.185	-0.696
Leu518	-0.011	0.025	-0.017	0.000
Phe543	-0.004	-0.076	0.075	0.000
Leu547	-0.116	-0.312	0.264	-0.051
Thr550	-0.808	-0.535	0.392	-0.590
Arg557	-0.286	-5.191	1.738	-0.495
Glu570	-0.881	1.143	-1.049	-0.564
Leu663	-0.003	-0.018	0.017	0.000
Leu670	-0.415	0.017	-0.004	-0.455
				-0.857