Curcumin analogs as the inhibitors of TLR4 pathway in inflammation and their drug like potentialities: a computer-based study

Md. Asad Ullah¹, Fatema Tuz Johoraa¹, Bishajit Sarkara¹, Yusha Ararf and MD. Hasanur Rahmanc

¹Department of Biotechnology and Genetic Engineering, Faculty of Biological Sciences, Jahangirnagar University, Dhaka, Bangladesh; cDepartment of Biotechnology and Genetic Engineering, Faculty of Life Sciences, Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj, Bangladesh

ABSTRACT
Toll-like receptor 4 (TLR4) pathway is one of the major pathways that mediate the inflammation in human body. There are different anti-inflammatory drugs available in the market which specifically act on different signaling proteins of TLR4 pathway but they do have few side effects and other limitations for intended use in human body. In this study, Curcumin and its different analogs have been analyzed as the inhibitors of signaling proteins, i.e. Cyclooxygenase-2 (COX-2), inhibitor of kappa kinase (IKK) and TANK binding kinase-1 (TBK-1) of TLR4 pathway using different computational tools. Initially, three compounds were selected for respective target based on free binding energy among which different compounds were reported to have better binding affinity than commercially available drug (control). Upon continuous computational exploration with induced fit docking (IFD), 6-Gingerol, Yakuchinone A and Yakuchinone B were identified as the best inhibitors of COX-2, IKK, and TBK-1 respectively. Then their drug-like potentialities were analyzed in different experiments where they were also predicted to perform well. Hopefully, this study will uphold the efforts of researchers to identify anti-inflammatory drugs from natural sources.

1. Introduction
Inflammation is delineated as normal biological as well as immune response against harmful stimuli including pathogens (bacteria, virus), toxins, stress, radiation, damaged cells, etc. It is one of the protective mechanisms of an organism to vanish invading stimulation, wound healing and for the restoration of body’s normal physiology [1,2]. Inflammation is the result of several biological processes. Tissue injury or infection triggers inflammation as well as subsequent inflammatory cascade. Typical inflammatory cascade consists of four components (Figure 1).

(i). Inducers: Exogenous immune inducers (pathogens, virus, bacteria, allergens, etc.) or endogenous inducers (damaged cells and stress) result in infection. (ii). Sensors: Specific receptors, i.e. toll-like receptors (TLRs, sensors) recognize these inducers. These receptors are localized on the cell membrane of immune cells (mast cells, dendritic cells, and macrophages). (iii). Mediators: Immune cells release different types of mediators i.e. tumor necrosis factor (TNF), interleukin-1 (IL-1), interferon-β (IFN-β), interleukin-6 (IL-6) etc. which vary depending on the type of inducers. (iv). Inflammation in target tissue: Immune mediators elicit their inflammatory effects i.e. dilation of blood vessels, increased vascular permeability, movement of leukocytes from blood vessels to inured area, etc. on target tissues [3,4].

There are two forms of inflammation, i.e. acute inflammation and chronic inflammation. Acute inflammation is pictured as immediate, short-term response and innate immunity to injury. Augmented movement of leukocytes from blood to the injured area results in acute inflammation within minutes or hours and lasts for short time to irradiate harmful stimuli. When the inflammatory responses last for long periods, it leads to chronic inflammation. Chronic inflammation leads to more complicated physiological conditions and several diseases [5], i.e. neuroinflammation (brain), metabolic disorders (liver/pancreas) [6], osteoporosis (bone) [7,8], cardiovascular disease (heart) [9], cancer [10], rheumatoid arthritis [11], obesity, asthma, and Alzheimer’s disease [12], etc.

1.1. Role of toll-like receptor 4 (TLR4) pathway in inflammation
In response to inflammatory inducers (signals, i.e. bacterial lipopolysaccharides) pattern recognition receptors as well as transmembrane receptors play a pivotal role in the initiation of immune response, among these, TLRs are most significant [13]. TLR4 senses the harmful stimuli and mediates the coordinate activation of two distinct transcription factors, i.e. nuclear factor kappaB (NF-κB) and interferon regulatory factor 3 (IRF3) (Figure 2) [14-16].

In the resting cells, NF-κB is isolated by inhibitory proteins called, IκappaB/IXB kinase (IKK) in cytoplasm where these proteins act like a mask and inactivate NF-κB. In response to signals, TLR4 gets activated which in turn
activates both canonical IκB kinases (i.e. IκBα/β-IKKα/β) and non-canonical IκB kinase, i.e. TANK binding kinase-1 (TBK1) IkappaB kinases (IKKβ) and these kinases activate the NF-κB and IRF3 transcription factors respectively. These transcription factors then translocate into the nucleus and facilitate the transcription of proinflammatory genes.

1.2. Current treatments of inflammation and limitation

Non-steroidal anti-inflammatory drugs (NSAIDs) are most commonly prescribed drugs for inflammation. These drugs reduce pain and immune response by inhibiting COX enzymes, TBK-1 (Amlexanox [22]) and IKK kinases (Quilonoxaline) that take part in the synthesis of PGs [23,24]. These drugs are classified into two groups, i.e. non-selective NSAIDs (Aspirin, Ibuprofen, Naproxen, etc.) and selective NSAIDs (Celecoxib and Rofecoxib etc.) [25–29]. Treatment of inflammation with selective NSAIDs is lucrative because selective NSAIDs selectively inhibit target enzymes. As a result, body’s homeostatic functions are carried out perfectly. However, recent studies suggest that selective NSAIDs can cause cardiovascular complications and disturb normal renal function as COX-2 is involved in renal development [30,31]. As the use of these existing drugs are very challenging, plant-based medicines are more desirable as they found to have less side effects [32].

1.3. Curcumin analogs as anti-inflammatory agents

Plants are the sources of wide varieties of phytochemicals which provide ranges of therapeutic benefits in human body [33,34]. Curcumin is a dietary yellow pigment which is naturally found in turmeric (Curcuma longa). This is a potent anti-inflammatory agent as it inhibits the PG synthesis which is crucial for the initiation of inflammation. There are various types of analogs of Curcumin found in other plants of the mother nature [35]. Curcumin has been proven to have anti-inflammatory activities and down regulate the NF-κB activation in inflammation in different laboratory experiments [36–38]. In a laboratory experiment, Curcumin has been shown to inhibit COX-2 as well as COX-1 activity by 50% in a concentration of 15 μM with slight selectivity [39]. In yet other studies, Curcumin has been shown to inhibit IKK activity and downregulate NF-κB activation [40]. Curcumin analogs...
Yakuchinone A and Yakuchinone B were also reported to inhibit COX-2 activity in laboratory experiment [41]. Moreover, 6-Shogaol has similar structure as of Curcumin and a structural analog of 6-Gingerol is an inhibitor of TBK-1 [42]. Since TLR4 pathway is one of the major pathways that are mainly involved in mediating the inflammatory responses, blocking one or multiple intracellular signaling proteins with specific inhibitor(s) of this pathway is an effective strategy for the development of anti-inflammatory drug. Different anti-inflammatory drugs based on the signaling proteins, i.e. COX-2, IKK, and TBK-1 of TLR4 pathway are already available in the market but they come up with few limitations as mentioned earlier.

In this experiment, different Curcumin analogs have been analyzed to understand their inhibitory effects on multiple signaling proteins (targets), i.e. COX-2, IKK, and TBK-1 involved in TLR4 pathway based on the hypothesis that, since they have similar structures and few analogs have target specific anti-inflammatory activities so, one or more analog(s) could have even better activity in a search for anti-inflammatory drugs from natural sources since natural compounds are often nontoxic, cheap and have less side effects.

2. Materials and methods

A total of 14 compounds including Curcumin, its derivatives and analogs were selected from literature review (Tables 1 and 2). Then they were subjected to drug-likeness property analysis, molecular docking, and other in silico drug potential assessing experiments to identify best inhibitor(s) of the respective targets (Figure 3).

2.1. Drug-likeness property analysis

The selected ligand molecules were analyzed to determine whether they obey Lipinski’s rule of five or not which states that a drug is considered to have poor bioavailability and low permeation if it violates the standard rule [55,56]. Canonical smile of each intended ligand molecule was retrieved from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and the canonical smile was then analyzed using the

| Compound name          | Source                  | PubChem CID | References          |
|------------------------|-------------------------|-------------|---------------------|
| Bisdemethoxycurcumin   | Turmeric (Curcuma longa) | 5315472     | Park et al. [43]    |
| Curcumin               | Turmeric (Curcuma longa)| 969516      | Anand et al. [44]   |
| Cyclocurcumin          | Turmeric (Curcuma longa)| 69879809    | Kuch et al. [45]    |
| Demethoxycurcumin      | Turmeric (Curcuma longa)| 5469424     | Park et al. [43]    |

| Compound name          | Source                  | PubChem CID | Reference           |
|------------------------|-------------------------|-------------|---------------------|
| 6-Gingerol             | Ginger (Zingiber officinale Roscoe) | 442793     | Kim et al. [46]     |
| 6-Paradol              | Ginger (Zingiber officinale Roscoe) | 94378      | Huang et al. [47], Keum et al. [48] |
| 6-Shogaol              | Ginger (Zingiber officinale) | 5281794    | Ling et al. [49]    |
| Cassumunin A           | Ginger (Zingiber cassumunar) | 10460395   | Masuda et al. [50]  |
| Cassumunin B           | Ginger (Zingiber cassumunar) | 10054109   | Masuda et al. [50]  |
| Dehydrozingerone       | Ginger (Zingiber officinale Roscoe) | 5354238   | Yogosawa et al. [51] |
| Dibenzoylmethane       | Licorice (Glycyrrhiza echinata) | 8433      | Lin et al. [52]     |
| Isoeugenol             | Cloves (Eugenia carophyllus) | 853433     | Fujisawa et al. [53] |
| Yakuchinone A          | Galanga (Alpinia officinarum) | 133145    | Flynn et al. [54]   |
| Yakuchinone B          | Galanga (Alpinia officinarum) | 6440365   | Flynn et al. [54]   |

Figure 3. Strategies employed in the overall study. Molinspiration Cheminformatics server (https://www.molinspiration.com/cgi-bin/properties) for different drug-likeness parameters (Table 3) [57,58]. Compounds violating the rule were then opted out from consideration of further evaluation.

2.2. Molecular docking experiment

2.2.1. Protein preparation

Three dimensional crystallographic structures of Human COX-2 (PDB ID: 5F1A), Inhibitor of kappaB kinase beta (catalytic subunit of IKK) (PDB ID: 3RZF) and TBK-1 (PDB ID: 5W5V) were downloaded in PDB format from Protein Data Bank
was again eradicated during the minimization step. Extraordinary water molecule under 3H-bonds to non-water particle root-mean-square-deviation (RMSD) to 30 Å and any minimization was performed setting the greatest substantial potentials for Liquid Simulations (OPLS_2005) force field. Features were refined and then minimized utilizing Optimized Potentials for Liquid Simulations (OPLS_2005) force field. Minimization was performed setting the greatest substantial particle root-mean-square-deviation (RMSD) to 30 Å and any extraordinary water molecule under 3H-bonds to non-water was again eradicated during the minimization step.

2.2.2. Ligand preparation
A total of 12 ligand structures except those that violated Lipinski’s rule of five were downloaded in SDF format from PubChem database (https://pubchem.ncbi.nlm.nih.gov/) [63]. These structures were then processed and prepared using the LigPrep wizard of Maestro Schrödinger suite [64]. Minimized 3D structures of ligands were generated using Epik2.2 within pH 7.0 ± 2.0 in the suite. At last, the structures were refined and then minimized utilizing Optimized Potentials for Liquid Simulations (OPLS_2005) force field. Minimization was performed setting the greatest substantial particle root-mean-square-deviation (RMSD) to 30 Å and any extraordinary water molecule under 3H-bonds to non-water was again eradicated during the minimization step.

2.2.3. Receptor grid generation
Grid usually confines the active site to specific area of the receptor protein for the ligand to dock specifically within that selected area. Receptor grid was generated using default Van der Waals radius scaling factor 1.0 and charge cutoff 0.25 which was then subjected to OPLS_2005 force field for the minimized structure in Glide [65]. A cubic box was then generated around the cyclooxygenase and peroxidase active sites of COX-2 and serine/threonine kinase catalytic site of both I KK and T BK-1 by clicking the co-crystallized reference ligand. The grid box dimension was then adjusted to 14 Å × 14 Å × 14 Å for docking to be carried out.

2.2.4. Glide standard precision (SP) and extra precision (XP) ligand docking
Extra precision (XP) ligand docking works more accurately for small number of ligand molecules than standard precision (SP) ligand docking which is recommended for large compound libraries [66]. But both of the docking methods were applied for the selected ligand molecules and intended targets to make comparison among different docking parameters. The Van der Waals radius scaling factor and charge cutoff were set to 0.80 and 0.15, respectively, for all the ligands and molecules under study. Final score was assigned according to the pose of docked ligand within the binding cleft of the receptor molecules. Best possible poses and types of ligand-receptor interactions were then analyzed utilizing Discovery Studio Visualizer version 4.5 (Dassault Systèmes, San Diego, CA) (Figure 4) [67].

2.2.5. Prime molecular mechanics – generalized born and surface area (MM-GBSA) rescoring
After SP and XP ligand docking the docked complexes were then again subjected to molecular mechanics – generalized born and surface area (MM-GBSA) rescoring with the help of Prime module of Maestro Schrödinger suite for further evaluation. This technique utilizes an implicit solvent which uses more accurate scoring function that then improves the overall free binding affinity score upon the reprocessing of the docked complex [66,68]. It combines OPLS molecular mechanics energies ($E_{MM}$), surface generalized born solvation model for polar solvation ($G_{SGB}$), and a nonpolar salvation term ($G_{NP}$) for total free energy ($\Delta G_{bind}$) calculation. The total free energy of binding was calculated by the following equation:

$$\Delta G_{bind} = G_{complex} - (G_{protein} - G_{ligand})$$,

where,

$$G = E_{MM} + G_{SGB} + G_{NP}$$

The result of SP docking, XP docking and MM-GBSA rescoring is summarized in Table 4.

2.2.6. Induced fit docking (IFD)
Three compounds were selected based on the lowest MM-GBSA score for each of the receptor molecules which were

| SL. No. | Compound Name | MW (g/mol) | mlLogP | HBA | HBD | nROTB | TPSA (Å²) | Lipinski Violation |
|--------|--------------|------------|--------|-----|-----|-------|-----------|------------------|
| 01.    | 6-Gingerol   | 294.39     | 3.22   | 4   | 2   | 10    | 66.76     | 0                |
| 02.    | 6-Paradol    | 278.39     | 4.60   | 3   | 1   | 10    | 46.53     | 0                |
| 03.    | 6-Shogaol    | 276.38     | 4.35   | 3   | 1   | 9     | 46.53     | 0                |
| 04.    | Cassumunin A | 558.63     | 4.96   | 8   | 2   | 13    | 111.53    | 1                |
| 05.    | Cassumunin B | 588.65     | 4.77   | 9   | 2   | 14    | 120.77    | 1                |
| 06.    | Curcumin     | 368.38     | 2.30   | 6   | 2   | 8     | 93.07     | 0                |
| 07.    | Cyclocurcumin| 368.38     | 3.03   | 6   | 2   | 5     | 85.23     | 0                |
| 08.    | Dehydrozingerone | 192.21 | 1.55   | 1   | 3   | 3     | 46.53     | 0                |
| 09.    | Demethoxycurcumin | 338.36 | 2.48   | 5   | 2   | 7     | 83.83     | 0                |
| 10.    | Dibenzoylethane | 224.26 | 2.88   | 2   | 0   | 4     | 34.14     | 0                |
| 11.    | Isoeugenol   | 164.20     | 2.38   | 2   | 1   | 2     | 29.46     | 0                |
| 12.    | 6-Shogaol    | 276.38     | 4.35   | 3   | 1   | 9     | 46.53     | 0                |
| 13.    | Yakuchinone A| 312.41     | 4.24   | 3   | 1   | 9     | 46.53     | 0                |

HBA: hydrogen bond acceptors; HBD: hydrogen bond donors; nROTB: number of rotatable bonds; TPSA: topological polar surface area

(www.rcsb.org) [59–61]. The structures were then prepared and processed using the Protein Preparation Wizard in Maestro Schrödinger Suite version 11.4 (Schrödinger Inc., New York, NY). Bond orders were assigned to the structures, hydrogens were added to heavy atoms. All of the water molecules were erased from the atoms, missing side chains were added to the protein backbone using Prime and het states were erased from the atoms, missing side chains were added to the protein backbone using Prime and het states were erased from the atoms.
then used for further evaluation since it is more robust scoring method. At this stage, different scores of three best-docked compounds were compared with one approved known inhibitor (control), i.e. Celecoxib, Sulfasalazine, and Fostamatinib for COX-2, IKK, and TBK-1 (Table 5) receptor respectively [69]. After that, the best three ligands for each receptor were subjected to induced fit docking (IFD) which is even more accurate docking method to generate the native poses of the ligands [70]. Again, OPLS_2005 force field was applied after generating grid around the co-crystallized ligand of the receptor and this time the best three ligands were docked rigidly. Receptor and Ligand Van Der Waals

Figure 4. Three dimensional representation (left) of best possible poses of ligand molecules (stick) inside the binding pocket of intended target (ribbon). Two dimensional representation (right) of ligand-receptor interaction. Interacting amino acids are represented in three letter code and their respective number within specific chain inside circular disk. Dotted lines represent interactions.
screening was set at 0.70 and 0.50, respectively, residues within 2 Å were refined to generate two best possible poses with XP. Best performing ligand was selected based on the lowest IFD score for one receptor molecule (Table 6). Then all three selected ligands for three receptors were used in the next phases of this study.

### 2.3. Ligand-based ADME/T prediction

**In silico** prediction of ADME/T profile of candidate drug molecule helps to increase the success rate of drug discovery expenditure [71,72]. Canonical smiles of the best three ligands were used to predict drug-like potential and tentative pharmacokinetic and pharmacodynamic parameters. ADME/T profile of each ligand was predicted using admetSAR 2.0 (http://lmmd.ecust.edu.cn/admetsar2/) and pkCSM server (http://biosig.unimelb.edu.au/pkcs/) [73,74] (Table 7).

#### 2.4. Pharmacological and biological activity prediction

Pharmacological and biological activities of the best ligand molecules were predicted using Prediction of Activity Spectra of Substances (PASS) Online (http://www.pharmaexpert.ru/passonline/) and Molinspiration Cheminformatics servers, respectively (Tables 8 and 9) [58,75]. These tools predict the probable activities of compounds based on structure activity relationship (SAR) in correlation with known compounds existing in the database.

#### 2.5. P450 site of metabolism prediction

**In silico** analysis of potential sites of metabolism of candidate drug metabolism provides insights into the metabolic vulnerability of the molecule inside the human body which then drives the *in vitro* assay to understand the accurate drug metabolism parameters [76]. Best metabolism sites of best three ligands for three isoforms, i.e. CYP3A4, CYP2D6, and
CYP2C9 of Cytochrome P450 family of enzymes were predicted utilizing online-based RS-WebPredictor server (http://reccr.chem.rpi.edu/Software/RS-WebPredictor/) (Figure 5) [77].

2.6. Density functional theory (DFT) calculation

Minimized ligand structures obtained from LigPrep were used for DFT calculation with the aid of the Jaguar panel of Maestro Schrödinger Suite using Becke’s three-parameter exchange potential and Lee-Yang-Parr correlation functional (B3LYP) theory with 6–31G* basis set in the suite [78–81]. Quantum chemical properties, i.e. surface properties (MO, density, potential) and multipole moments were calculated along with highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) energy. Global frontier orbital was analyzed and hardness (\( \eta \)) and softness (\( S \)) of selected molecules were also calculated using the following equation as per Parr and Pearson interpretation and Koopmans theorem [82,83]. The result of DFT calculation is summarized in Table 10. HOMO and LUMO occupations of the ligands are illustrated in Figure 6.

\[
\eta = \left( \frac{\text{HOMO}_e - \text{LUMO}_e}{2} \right), \quad S = \frac{1}{\eta}
\]

3. Results

3.1. Drug likeness property

A total of 14 selected ligand molecules were analyzed to understand whether they comply with Lipinski’s rule of five or not. Cassumunin A and Cassumunin B violated the standard rule and hence were removed from consideration of further evaluation (Table 3). All other ligand molecules were reported to obey Lipinski’s rule of five. These 12 ligand molecules were then utilized in the next phases of the experiment.

Alongside the standard rule, the ligands were also analyzed for their topological polar surface area (TPSA). Isoeugenol was reported to have lowest TPSA and Curcumin was reported to have highest TPSA. Other ligands have TPSA within the moderate range between the highest and lowest values.

3.2. Molecular docking experiment

A total of 12 selected ligand molecules from the drug likeness property analysis were then utilized in the docking

| Compound name | IUPAC name | Chemical formula | 2D structure |
|---------------|------------|------------------|--------------|
| 6-Gingerol    | (S)-5-hydroxy-1-(4-hydroxy-3-methoxyphenyl)decan-3-one | C_{17}H_{26}O_{4} | ![Image](image1.png) |
| Yakuchinone A | 1-(4-hydroxy-3-methoxyphenyl)-7-phenylheptan-3-one | C_{20}H_{24}O_{3} | ![Image](image2.png) |
| Yakuchinone B | (E)-1-(4-hydroxy-3-methoxyphenyl)-7-phenylhept-1-en-3-one | C_{20}H_{22}O_{3} | ![Image](image3.png) |

| Properties | 6-Gingerol | Yakuchinone A | Yakuchinone B |
|------------|------------|---------------|---------------|
| Absorption | High       | High          | High          |
| Human intestinal absorption | Low       | Low           | Low           |
| Human oral bioavailability     | High       | High          | Low           |
| Caco-2 permeability           | No         | Yes           | Yes           |
| Distribution | No         | Yes           | Yes           |
| P-glycoprotein substrate | Yes       | No            | No            |
| P-glycoprotein inhibitor       | No         | Yes           | No            |
| Blood-brain barrier penetration | Yes       | Yes           | Yes           |
| Metabolism | Yes         | Yes           | Yes           |
| CYP3A4 substrate | Yes       | Yes           | Yes           |
| CYP3A4 inhibition | No         | No            | No            |
| CYP2D6 substrate | No         | Yes           | Yes           |
| CYP2D6 inhibition | No         | No            | No            |
| Excretion | Total clearance | 1.339 | 0.346 | 0.231 |
| OCT2 substrate | No         | No            | No            |
| Toxicity | AMES toxicity | No        | No            | No            |
| hERG inhibition | Yes       | Yes           | Yes           |
| Eye irritation | Yes       | No            | No            |
| Acute oral toxicity | Type III | Type III | Type III |

OCT2: organic cation transporter 2; hERG: human ether-a-go-go related gene; CYP: cytochrome P450.
Table 8. Result of pharmacological activity prediction of selected ligand molecules.

| Pharmacological activity          | 6-Gingerol | Yakuchinone A | Yakuchinone B |
|-----------------------------------|------------|---------------|---------------|
| Anti-inflammatory                  | 0.532      | 0.532         | 0.497         |
| Anti-inflammatory, intestinal      | 0.566      | 0.004         | 0.437         |
| Anti-inflammatory, ophthalmic      | 0.343      | 0.343         | 0.330         |
| MAP kinase stimulant              | 0.482      | 0.049         | 0.667         |
| TNF expression inhibitor          | 0.633      | 0.010         | 0.595         |
| JAK2 expression inhibitor         | 0.679      | 0.020         | 0.863         |
| NF kappa A inhibitor              | 0.228      | 0.115         | 0.278         |
| NF kappa B inhibitor              | 0.264      | 0.015         | 0.240         |
| Macrophage colony stimulating factor agonist | 0.762     | 0.007         | 0.687         |
| Cyclooxygenase substrate          | 0.326      | 0.011         | 0.230         |

Table 9. Result of biological activity prediction of best ligands. GPCR: G protein-coupled receptors

| Bioactivity                      | 6-Gingerol | Yakuchinone A | Yakuchinone B |
|----------------------------------|------------|---------------|---------------|
| GPCR ligand                      | 0.16       | 0.07          | -0.00         |
| Ion channel modulator            | 0.04       | -0.02         | -0.17         |
| Kinase inhibitor                 | -0.33      | -0.31         | -0.36         |
| Nuclear receptor ligand          | 0.20       | 0.12          | 0.17          |
| Protease inhibitor               | 0.15       | 0.01          | -0.09         |
| Enzyme inhibitor                 | 0.38       | 0.16          | 0.13          |

Table 8.

3.2.1. Binding mode of 6-Gingerol with COX-2

6-Gingerol docked with COX-2 with an IFD score of $-2474.460 \text{ Kcal/mol}$ and XP Gscore of $-9.264 \text{ Kcal/mol}$ (Table 5). It formed five conventional hydrogen bonds with Gln454, His214, Tyr385, Thr212, and Asn382 amino acid residues inside the binding pocket of COX-2 at 2.01Å, 2.29Å, 1.93Å, 3.60Å, and 2.52Å, distance apart respectively. It also formed one non-conventional hydrogen bond with His388 amino acid and few other hydrophobic interactions, i.e. Pi-Pi Stacked, Pi-Pi T Shaped, and Pi-Alkyl interactions with few other interacting amino acids inside the binding cleft of COX-2. It interacted with 11 amino acids in total inside the binding site of COX-2 (Figure 4).

3.2.2. Binding mode of Yakuchinone A with IKK

Yakuchinone A docked with IKK with an IFD score of $-1081.370 \text{ Kcal/mol}$ and XP Gscore of $-9.111 \text{ Kcal/mol}$ (Table 5). It formed two conventional hydrogen bonds with Lys106 amino acid residue inside the binding pocket of IKK at 5.46Å and 2.00Å distance apart, respectively. Again, it formed two additional conventional hydrogen bonds with Glu100 and Cys99 amino acids at 2.72Å and 1.96Å distance apart, respectively. Yakuchinone A formed one nonconventional hydrogen bond with Glu100 amino acid residue and few other Pi-Alkyl interactions with other interacting amino acids inside the binding cleft of IKK. It interacted with 7 amino acids in total inside the binding site of IKK (Figure 4).

3.2.3. Binding mode of Yakuchinone B with TBK-1

Yakuchinone B docked with TBK-1 with an IFD score of $-1309.340 \text{ Kcal/mol}$ and XP Gscore of $-7.971 \text{ Kcal/mol}$ (Table 5). It formed two conventional hydrogen bonds with Ser93 and Cys89 amino acid residues inside the binding pocket of TBK-1 at 2.84Å and 2.09Å distance apart, respectively. Again, it formed three additional non-conventional hydrogen bonds with Gly139 and Phe88 amino acids. Yakuchinone B also formed few other hydrophobic interactions, i.e. Pi-Sigma, Pi-Alkyl, and Amide-Pi Stacked with other different interacting amino acids inside the binding cleft of TBK-1. It interacted with 7 amino acids in total inside the binding site of TBK-1 (Figure 4).
3.3. ADME/T prediction

Best three selected ligand molecules (Table 6) were analyzed for their potential ADME/T profiles (Table 7). All of the ligand molecules were predicted to be highly absorbed in intestine but have low oral bioavailability. Only Yakuchinone B showed sign of lower Caco-2 permeability. All of them were predicted to be non-substrates of membrane P-glycoproteins and capable of penetrating blood–brain barrier. Only 6-Gingerol was reported to be inhibitor of P-glycoproteins. All of them were reported to be the substrate of CYP3A4 and additionally, 6-Gingerol and Yakuchinone A to be the substrates of CYP2D6. None of them showed sign of inhibition toward CYP3A4, CYP2D6, and CYP2C9 enzymes.

None of the ligands was reported to be organic cation transporter 2 (OCT2) substrate and show sign of AMES toxicity and Hepatotoxicity. All of the ligands were reported to be the inhibitors of Human ether-a-go-go related gene (hERG) channel. Only 6-Gingerol showed sign of eye irritation. All ligands showed Type III acute oral toxicity.

### Table 10. Result of DFT calculation.

| Compound name     | HOMO   | LUMO   | Gap    | Hardness ($\eta$) | Softness (S) | Dipole moment |
|-------------------|--------|--------|--------|-------------------|--------------|---------------|
| 6-Gingerol        | -0.19924 | -0.01477 | 0.18447 | 0.09223           | 10.84167     | 3.3525        |
| Yakuchinone A     | -0.19952 | -0.01594 | 0.18358 | 0.09179           | 10.89443     | 2.1614        |
| Yakuchinone B     | -0.21682 | -0.05847 | 0.15835 | 0.07917           | 12.63104     | 4.5276        |

The unit of HOMO, LUMO, gap, hardness and softness are in Hartree and the unit of dipole moment is in Debye.

Figure 5. Results of P450 site of metabolism prediction. Best three vulnerable atoms are marked in encircled number.
Overall, all the ligands showed promising result in ADME/T prediction experiment.

3.4. Pharmacological and biological activity prediction

Best three ligand molecules were analyzed for their probable pharmacological activities (Table 8). They were analyzed to understand their association with anti-inflammatory and other activities with enzymes, signaling proteins, transcription factors, and cytokines involved in different inflammatory cascades (Table 8).

Probability scores of particular pharmacological activities of investigated ligands varied with variety of extent and Yakuchinone B performed slightly better in the pharmacological activity prediction experiment followed by Yakuchinone B and 6-Gingerol. Yakuchinone B showed activities as TNF expression inhibitor and JAK2 expression inhibitor with probability score greater than 0.7. 6-Gingerol showed Macrophage colony-stimulating factor agonist activity with probability score greater than 0.7. Other scores of different activities by selected ligand molecules ranged from moderate to low.

Thereafter, the selected ligands were investigated for their biological activities against GPCR (G protein-coupled receptor) ligand, ion channels, enzyme, etc. (Table 9). 6-Gingerol showed better positive scores as enzyme inhibitor, nuclear receptor ligand, and GPCR ligand modulator with higher positive probability scores. However, Yakuchinone A and Yakuchinone B also showed almost similar activities against few parameters.

3.5. P450 site of metabolism prediction

Best selected ligand molecules were examined for their potential sites of metabolism against three major isoforms of Cytochrome P450 family of enzymes, i.e. CYP3A4; CYP2D6 and CYP2C9 (Figure 5). All of the selected ligand molecules were reported to have multiple atoms which are vulnerable to a specific enzyme of CYP450 family. 6-Gingerol showed almost similar sites of metabolism for all three isoforms but Yakuchinone A and Yakuchinone B showed different potential sites of metabolism for different enzymes.

3.6. Analysis of frontier’s orbitals

Detailed HOMO energy, LUMO energy, energy gap (HOMO-LUMO gap), hardness, and softness of the selected three compounds are summarized in Table 10 and the HOMO and LUMO occupation of the ligands is illustrated in Figure 6. Highest gap was observed for 6-Gingerol and lowest gap was observed for Yakuchinone B. 6-Gingerol showed highest hardness and Yakuchinone B showed lowest hardness.

Figure 6. The HOMO and LUMO occupation for the selected compounds; (A) 6-Gingerol, (B) Yakuchinone A and (C) Yakuchinone B. Blue and red are positive and negative respectively in its wave function.
whereas Yakuchinone A was reported to have medium hardness as compared with other two molecules.

According to the energy gap, the order of the compounds is: 6-Gingerol > Yakuchinone A > Yakuchinone B. Along with the HOMO and LUMO energy, dipole moment of the selected ligand molecules were also calculated and according to dipole moment the order of compounds: Yakuchinone A > 6-Gingerol > Yakuchinone B.

4. Discussions

In silico analysis of drug-likeness helps to filter out compounds with poor drug-like potentials usually those that have poor physicochemical properties. Violation of Lipinski’s rule by a compound indicates that the compound is more likely to fail in the later stages of a drug discovery approach [84,85]. In this experiment, the ligand molecules were analyzed in accordance with Lipinski’s rule of five. Cassumunin A and Cassumunin B violated the standard rule and then they were removed from the consideration of further experiment (Table 3). Thereafter, 12 ligand molecules except these two were analyzed in the molecular docking experiment. Molecular docking is one of the most commonly used methods in computer-aided drug designing. This method works on specific algorithm which assigns binding energy to the docked complex after docking which in turn reflects binding affinity of a ligand to a molecular target [56,86,87]. Lowest binding energy of ligand-receptor complex reflects higher affinity meaning they remain more time in contact [88]. In this experiment, SP and XP ligand docking were carried out to make comparison among docking parameters of different ligands with different targets. However, best three ligands for one receptor were selected based on lowest MM-GBSA scores because it is more rigorous scoring method than SP and XP docking (Table 4) [89,90]. Few ligands among the three selected best ligands showed betterbinding free energies than approved inhibitors (controls). Then the selected ligand molecules were subjected to IFD which is even powerful docking method to generate poses and assigning scores [91]. Upon continuous exploration with different methods of molecular docking, 6-Gingerol, Yakuchinone A, and Yakuchinone B were selected as the best inhibitors of COX-2, IKK, and TBK-1, respectively (Tables 5 and 6). Hydrogen bonding and hydrophobic interactions play significant role in strengthening the ligand-receptor interactions [92]. All selected ligands were reported to form multiple hydrogen bonds and other forms of hydrophobic interactions inside the binding cleft of respective receptors (Figure 4). 6-Gingerol formed hydrophobic and hydrogen interactions with multiple amino acids of both peroxidase and cyclooxygenase active sites of COX-2 [93]. Yakuchinone A and B were also found to form multiple interactions within the serine/threonine kinase catalytic site containing kinase domains of both IKK (amino acids 16–307) and TBK-1 (amino acids 9–301) and thus the selected best compounds are expected to interfere with the normal function of the target proteins (Figure 4) [94,60].

In silico analysis of absorption, distribution, metabolism, excretion, and toxicity is crucial to determine whether a drug is likely to survive or not in the later stages of drug development process and these data again help to reduce the time and cost of drug discovery approach by assisting in vitro assays [71,95]. Blood–brain barrier permeability is a major concern for the drugs targeting primarily the cells of the central nervous system (CNS). Oral delivery system is the most commonly used route of drug delivery and the delivered drug migrates through the digestive tract into the intestine and so the drug under investigation is expected to be highly absorbed in human intestinal tissue. P-glycoproteins are the cell membrane glycoproteins that are responsible to facilitate the transport of many drugs through the cell membrane and hence their inhibition by candidate drugs may affect the normal drug transport inside human body. Caco2 permeability to drug reflects the human intestinal tissue permeability since this cell line is commonly used for in vitro permeability assay [96–98]. Cytochrome P450 family of enzymes is center to control the drug interaction, and metabolism inside human body, and drug excretion outside of the body. Inhibition of these enzymes may lead to acute drug toxicity, slow clearance, and eventually malfunction of the drug compound inside human body [99–101]. AMES toxicity is used to examine the toxicity endpoint of chemicals in question [102,103]. hERG channels are the voltage-gated potassium ion channels that play key roles for potassium ion transport through the cell membrane. Different structurally and functionally unrelated drugs have been reported to block the hERG potassium channel which has raised the concern of off-target drug interaction and so, screening compounds for activity on hERG channels early in the lead optimization process is crucial [104]. Renal organic cation transporter 2 (OCT2) is important for drug and xenobiotic excretion through kidney. The substrates of this transporter protein are considered to be easily excreted through urine [105]. All of the selected ligands exhibited almost similar properties in ADME/T test (Table 7).

Pharmacological activity (PASS prediction) is predicted in the context of probability of activity (Pa) and probability of inactivity (Pi) of a compound and the result of the prediction varies between 0.000 and 1.000. The activity is considered possible when Pa > Pi [106]. When Pa > 0.7, the compound is very likely to exhibit the activity but possibility of the compound being analog to a known pharmaceutical is also high. Compound with 0.5 < Pa < 0.7 score is likely to exhibit the activity but the probability is less along with the chance of being a known pharmaceutical agent is also lower. When Pa < 0.5, then the compound is less likely to exhibit the activity [107]. Pharmacological activity was predicted for the compounds to understand their relation with anti-inflammatory activities and other activities with proteins, transcription factors, enzymes, and cytokines involved in different inflammatory cascades. Yakuchinone B performed slightly better for few activities whereas all other ligands were predicted to have almost similar scores for other intended activities (Table 8). Then the ligands were analyzed for their potential biological activities against G protein-coupled receptors (GPCRs),
ion channels, enzymes, nuclear receptors, etc., which are the most potent drug targets in human body. Among these, only GPCRs are the targets of 50% of currently available drugs in the market [108–110]. 6-Gingerol showed better significant connections (probability scores) followed by Yakuchinone A and B against all targets which might be useful for drug discovery expenditure but at the same time could also raise the concern of unexpected drug-target interaction as useless (Table 9).

Then the ligands were analyzed for their potential metabolism sites for three major enzymes, i.e. CYP3A4, CYP2D6, and CYP2C9 of Cytochrome P450 family and multiple sites for each molecule were reported (Figure 5). The best ligands were also analyzed for their HOMO and LUMO energy and occupation. HOMO is usually a constraint portion in a molecule that is capable of donating electrons whereas LUMO is responsible for accepting electrons (Figure 6). HOMO–LUMO gap is used to define the stability of a compound, and a compound having highest gap is likely to undergo a chemical reaction more easily [111,112]. Yakuchinone A and 6-Gingerol showed almost similar energy gap whereas Yakuchinone B showed slightly lower gap (Table 10).

Finally, 6-Gingerol, Yakuchinone A and Yakuchinone B were the best findings of this study, however, other three selected ligands showing comparable binding free energies should also have promising inhibitory effects on respective targets (Table 3). Anti-inflammatory activities of best three compounds have already been proven in laboratory experiments [41,113,114]. These compounds also performed quite similar in different post-screening experiments after docking which could be useful for further investigation on anti-inflammatory drug discovery approach from natural sources. However, these findings might be required to be supported by further in vivo or in vitro study.

Overall, this study recommends 6-Gingerol, Yakuchinone A, and Yakuchinone B as the best inhibitors of COX-2, IKK, and TBK-1, respectively, among the selected Curcumin analogs. However, other compounds could also be investigated since they also performed well in docking experiment.

5. Conclusion

Inflammation is mediated by different inflammatory pathways inside human body. TLR4 pathway is one of the major pathways that regulate inflammatory responses. Many signaling proteins of this pathway are potential targets of commercially available anti-inflammatory drugs. In this study, 14 Curcumin analogs were utilized to explore their anti-inflammatory activities against three signaling proteins of the TLR4 pathway in a search for anti-inflammatory drug from natural sources. Upon continuous computational exploration, 6-Gingerol, Yakuchinone A, and Yakuchinone B were identified as the best inhibitors of COX-2, IKK, and TBK-1, respectively. Thereafter, their different drug-like potentials were also analyzed and they were found to have different promising and similar drug-like parameters. Therefore, these compounds could be potential anti-inflammatory agents from natural source. However, authors suggest further in vivo and in vitro experiments with these compounds to confirm their anti-inflammatory activities and strengthen these findings.

Acknowledgments

Authors acknowledge the members of Swift Integrity Computational Lab, Dhaka, Bangladesh, a virtual platform of young researchers for their support during the preparation of the manuscript.

Disclosure statement

Authors declare no conflict of interest regarding the publication of the manuscript.

ORCID

Md. Asad Ullah http://orcid.org/0000-0002-6615-6433
Fatema Tuz Johora http://orcid.org/0000-0003-0591-2183
Bishajit Sarkar http://orcid.org/0000-0001-8439-6994
Yusha Arif http://orcid.org/0000-0002-0144-5875
MD. Hasanur Rahman http://orcid.org/0000-0001-9238-3149

Data availability

Authors made all the data generated during experiment and analysis available within the manuscript.

References

[1] Ricciotti E, FitzGerald GA. Prostaglandins and inflammation. Arterioscler Thromb Vasc Biol. 2011;31(5):986–1000.
[2] Liu CH, Abrams ND, Carrick DM, et al. Imaging inflammation and its resolution in health and disease: current status, clinical needs, challenges, and opportunities. FASEB J. 2019;33(12):13085–13097.
[3] Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428–435.
[4] Lon HK, Liu D, Jusko WJ. Pharmacokinetic/pharmacodynamic modeling in inflammation. Crit Rev Biomed Eng. 2012;40(4):295–312.
[5] Horadagoda NU, Knox NM, Gibbs HA, et al. Acute phase proteins in cattle: discrimination between acute and chronic inflammation. Vet Rec. 1999;144(16):437–441.
[6] Lee JW, Lee YK, Yik DY, et al. Neuro-inflammation induced by lipopolysaccharide causes cognitive impairment through enhancement of beta-amyloid generation. J Neuroinflammation. 2008;5(1):37.
[7] Feghali CA, Wright TM. Cytokines in acute and chronic inflammation. Front Biosci. 1997;21(1):412–26.
[8] Cox SS, Speaker KJ, Beninson LA, Craig WC, et al. Adrenergic and glucocorticoid modulation of the sterile inflammatory response. Brain Behav Immun. 2014;36:183–192.
[9] Libby P. Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr. 2006;83(2):456S–4560. S.
[10] Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860–867.
[11] Zvaifler NJ. The immunopathology of joint inflammation in rheumatoid arthritis. Adv Immunol. 1973;16:265–336.
[12] Bauer ME, Teixeira AL. Inflammation in psychiatric disorders: what comes first? Ann NY Acad Sci. 2019;1437(1):57–67.
[13] Kaczorowski DJ, Nakao A, Vallabhaneni R, et al. Mechanisms of toll-like receptor 4 (TLR4)-mediated inflammation after cold ischemia/reperfusion in the heart. Transplantation. 2009;87(10):1455–1463.
Blackwell TS, Christman JW. The role of nuclear factor-κ B in cytokine gene regulation. Am J Respir Cell Mol Biol. 1997;17(1):3–9.

Zhang B, Ramesh G, Uematsu S, et al. TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity. J Am Soc Nephrol. 2008;19(9):923–932.

Poligone B, Baldwin AS. Positive and negative regulation of NF-κB by COX-2 ROLES OF DIFFERENT PROSTAGLANDINS. J Biol Chem. 2001;276(42):38658–38664.

Fitzgerald KA, McWhirter SM, Faia KL, et al. IKKα and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol. 2003;4(5):491–496.

Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest. 2001;107(1):7–11.

Zhao P, In Wong K, Sun X, et al. TBK1 at the crossroads of inflammation and energy homeostasis in adipose tissue. Cell. 2018;172(4):731–743.

Kishore N, Huynh QK, Mathialagan S, et al. IKK-α and TBK-1 are enzymatically distinct from the homologous enzyme IKK-2 COMPARATIVE ANALYSIS OF RECOMBINANT HUMAN IKK-α, IKK-1, AND IKK-2. J Biol Chem. 1997;272(34):21181–21186.

Hasan M, Yan N. Therapeutic potential of targeting TBK1 in autoimmune diseases and interferopathies. Pharmacol Res. 2016;111:336–342.

Burke JR, Pattoli MA, Gregor KR, et al. BMS-345541 is a highly selective inhibitor of IκB kinase that binds at an allosteric site of the enzyme and blocks NF-κB-dependent transcription in mice. J Biol Chem. 2003;278(3):1450–1456.

Voille N, de Weille J, Mamet J, et al. Nonsteroid anti-inflammatory drugs inhibit both the activity and the inflammation-induced expression of acid-sensing ion channels in nociceptors. J Neurosci. 2001;21(20):8026–8033.

Bjarnason I, Hayllar J, Dre A, Macpherson NJ, et al. Side effects of nonsteroidal anti-inflammatory drugs on the small and large intestine in humans. Gastroenterology. 1993;104(6):1832–1847.

Mendes RT, Stanczyk CP, Sordi R, et al. Selective inhibition of cyclooxygenase-2: risks and benefits. Rev Bras Reumatol. 2012;52(5):767–782.

Rao P, Knaus EE. Evolution of nonsteroidal anti-inflammatory drugs (NSAIDs): cyclooxygenase (COX) inhibition and beyond. J Pharm Pharmac. 2008;60(12):81–110.

Martelet-Pelletier J, Jaleunesse D, Reboul P, et al. Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. Ann Rheum Dis. 2003; 62(6):501–509.

Payne R. Limitations of NSAIDs for pain management: toxicity or lack of efficacy? The Journal of Pain. 2000;1(3):14–18.

Lisowska B, Kosson D, Domaracka K. Positives and negatives of the enzyme and blocks NF-κB dependent transcription in mice. J Biol Chem. 2003;278(3):1450–1456.

Kiuchi F, Goto Y, Sugimoto N, et al. Nematocidal activity of turmeric. J Pharm. 2001;276(42):38658–38664.

Masuda T, Matsumura H, Oyama Y, et al. Synthesis of (±)-cassuvine. J Org Chem. 2001;66(9):2913–2916.

Ling H, Yang H, Tan SH, et al. 6-Shogaol, an active constituent of ginger. Biochem Pharmacol. 2003;65(8):1643–1649.

Anand P, Thomas SG, Kunnumakkara AB, et al. Activities of curcumin, on 7, 12-dimethylbenz [a] anthracene-induced mammary tumorigenesis. Proc Natl Acad Sci. 2001;98(5):569–573.

Kohli K, Ali J, Ansari MJ, et al. Curcumin: a natural anti-inflammatory agent. Indian J Pharmacol. 2005;37(3):141.

Fujisawa S, Atsumi T, Ishihara M, et al. Cytotoxicity, ROS-generating activity and radical-scavenging activity of curcumin and related compounds. J Environ Pathol Toxicol Oncol. 2002;21(2):9.

Simerska P, Moyle PM, Toth I. Modem lipid-, carbohydrate-, and peptide-based delivery systems for peptide, vaccine, and gene products. Med Res Rev. 2011;31(4):520–547.

Park SJ, Lee MY, Son BS, et al. TBK1-targeted suppression of TRIF-dependent signaling pathway of Toll-like receptors by 6-shogaol, an active component of ginger. Biosci Biotechnol Biochem. 2009;73(7):1474–1478.

Kumar V, Bhardwaj M, Pahwa R, et al. Effect of curcumin on experimental ischemia/reperfusion injury in rat liver. Arch Biochem Biophys. 2003;410(2):250–257.

Chun KS, Kang JY, Kim OH, et al. Effects of yakuchinone A and yakuchinone B on the Phorbol ester-induced expression of COX-2 and TNF-α and activation of NF-κB in mouse skin. J Environ Pathol Toxicol Oncol. 2002;21(2):9.

Kim SO, Kundu JK, Shin YK, et al. [6]-Gingerol inhibits COX-2 expression by blocking the activation of p38 MAP kinase and NF-κB in phorbol ester-stimulated mouse skin. Oncogene. 2005;24(15):2558–2567.

Huang WY, Cai YZ, Zhang Y. Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. Nutr Cancer. 2009;62(1):1–20.

Masuda T, Matsumura H, Oyama Y, et al. Synthesis of (±)-curcumin and its analogues (Congeners) made by man and Mother Nature. Biochem Pharmacol. 2008;76(11):1590–1611.

Koike K, Yamaoka Y, Yasuda S, et al. Cytotoxicity and radical-scavenging activity of curcumin, on 7, 12-dimethylbenz [a] anthracene-induced mammary tumorogenesis. Proc Natl Acad Sci U S A. 1999;96(5):2693–2697.

Maione F, Russo R, Khan H, et al. Medicinal plants with anti-inflammatory activities. Nat Prod Res. 2010;24(12):1343–1352.

Fujisawa S, Atsumi T, Ishihara M, et al. Cytotoxicity, ROS-generating activity and radical-scavenging activity of curcumin and related compounds. Anticancer Res. 2004;24(28):563–570.

Flynn DL, Rafferty MF, Doctor AM. Inhibition of 5-hydroxy-eicosatetraenoic acid (5-HETE) formation in intact human neutrophils by naturally-occurring diarylheptanoids: inhibitory activities of...
curcuminoids and yaku chinones. Prostaglandins Leukot Med. 1986;22(3):357–360.

[55] Lipinski CA, Lombardo F, Dominy BW, et al. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. Adv Drug Deliv Rev. 1997;22(1–3):3–25.

[56] Ullah A, Prottoy NI, Araf Y, et al. Molecular docking and pharmacological property analysis of phytochemicals from clitoria ternatea as potent inhibitors of cell cycle checkpoint proteins in the cyclin/CDK pathway in cancer cells. Comput Mol Biochem. 2019;09(03):81–94.

[57] Bolton EE, Wang Y, Thiessen PA, et al. PubChem: integrated platform of small molecules and biological activities. Ann Rep Comput Chem. 2008;4:217–241.

[58] Cheminformatics M. Nova ulica, SK-900 26 Slovensky Grob, Slovak Republic [Internet]. Bratislava University; 1986.

[59] Lucido MJ, Orlando BJ, Vecchio AJ, et al. Crystal structure of aspirin-acetylated human cyclooxygenase-2: insight into the formation of products with reversed stereochemistry. Biochemistry. 2016;55(8):1226–1238.

[60] Xu G, Lo YC, Li Q, et al. Crystal structure of inhibitor of j cyclin/CDK pathway in cancer cells. J Med Chem. 2016;55(8):1226–1238.

[61] Deng X, Lo YC, Li Q, et al. Crystal structure of inhibitor of j cyclin/CDK pathway in cancer cells. J Med Chem. 2016;55(8):1226–1238.
Anzenbacher P, Anzenbacherova E. Cytochromes P450 and metabolism of xenobiotics. Cmls, Cell Mol Life Sci. 2001;58(5): 737–747.

Lamb DC, Waterman MR, Kelly SL, et al. Cytochromes P450 and drug discovery. Curr Opin Biotechnol. 2007;18(6):504–512.

De Graaf C, Vermeulen NP, Feenstra KA. Cytochrome P450 in silico: an integrative modeling approach. J Med Chem. 2005;48(8): 2725–2755.

Ames BN, Gurney EG, Miller JA, et al. Carcinogens as frameshift mutagens: metabolites and derivatives of 2-acetylaminofluorene and other aromatic amine carcinogens. Proc Natl Acad Sci. 1972; 69(11):3128–3132.

Xu C, Cheng F, Chen L, et al. In silico prediction of chemical Ames mutagenicity. J Chem Inf Model. 2012;52(11):2840–2847.

Priest B, Bell IM, Garcia M. Role of hERG potassium channel assays in drug development. Channels. 2008;2(2):87–93.

Hacker K, Maas R, Kornhuber J, et al. Substrate-dependent inhibition of the human organic cation transporter OCT2: a comparison of metformin with experimental substrates. PLoS One. 2015; 10(9):e0136451.

Stepanchikova AV, Lagunin AA, Filimonov DA, et al. Prediction of biological activity spectra for substances: evaluation on the diverse sets of drug-like structures. Curr Med Chem. 2003;10(3): 225–233.