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# *In silico* methods reconfirm CDK2 as a potential molecular target of 5-fluorouracil

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The extensive use of 5-fluorouracil (5-FU) in chemotherapy has given rise to drug-resistance. The fact that some compounds have shown additive and/or synergistic effects with 5-fluorouracil has led to the exploration of combination therapy. This has triggered the quest for computer aided design and screening of molecular targets for 5-FU based on Molecular Dynamics simulations. The current study was undertaken with the objective of designing and screening compounds which can serve as putative targets for 5-FU using '*in silico* reverse screening' techniques and evaluation of their ligand-binding properties with 5-FU. The identification of molecular target was achieved employing two pharmacophore screening servers, namely, PharmMapper and PharmaGist. The potential targets were selected after virtual screening, and their efficiency was compared with 5-FU based on docking analyses. This led to the identification of novel targets which were further evaluated based on free energy calculations by molecular dynamics simulations. The molecular dynamics based analysis revealed that cell division protein kinase 2 (CDK2) can act as molecular target for 5-FU. It also showed a binding affinity towards 5-FU which was comparable with human thymidine phosphorylase, a well-studied 5-FU target. Therefore, CDK2 in combination with 5-FU has great potential to be used of in chemotherapy.

**Keywords:** Anticancer, 5-Fluorouracil, Cell division protein kinase 2,Molecular dynamics,Molecular docking, Reverse screening

5-Fluorouracil (5-FU) remains one of the most frequently prescribed chemotherapeutic drug for the treatment of head, neck, breast and gastrointestinal cancer as its metabolites can effect cytotoxicity by getting incorporated into DNA and RNA[1,2]. There are two known pathways for 5-FU metabolism in mammalian cells[3]. In the first pathway, 5-FU is metabolized to fluorourodeoxyuridine by thymidine phosphorylase, and it is further metabolized to triphosphate form by thymidine kinase and other cellular enzymes. A final metabolite of 5-FU, fluorouridine triphosphate (FUTP), inhibits the DNA synthesis and lead to the apoptotic cell death. In the second pathway, 5-FU is metabolized to the triphosphate form by orotatephosphoribosyl transferase (OPRT), and other nucleotide-metabolizing enzymes may be involved as well. The cell death is achieved because of the inhibition of RNA synthesis by 5-FUTP. Among the two metabolic path ways, the first pathway is well studied, and DNA fragmentation of mammalian cells by 5-FU treatment is documented in several studies[4,5]. Moreover, combination therapy of 5-FU has been examined with other chemotherapeutic drugs, and compounds[6]. 5-FU is chemically derived from 5-Flucytosine (5FC) which is non-toxic to human cells. 5-FC is an antimicrobial drug which can be metabolized to 5-FU by bacterial and fungal enzymes whereas; mammalian cell enzymes cannot metabolize it[7]. For these reasons, 5-FC mediated prodrug therapy has attracted several researchers, and in this strategy, 5-FC converting enzymes are introduced

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**Abbreviations**: ADT, AutoDockTools; CDK2, Cell Division Protein Kinase 2; 5-FC, 5-Flucytosine; 5-FU, 5-fluorouracil; 5-FUMP, 5-FluoroUracilMonoPhosphate; GUI, Graphic user interface; MD, molecular dynamics; MM-GBSA, Molecular Mechanics/Generalized Born Surface Area; MM/PBSA, Molecular Mechanics/Poisson Boltzmann Surface Area; OPRT, OrotatePhosphoRibosylTransferase; PBTOT/GBTOT, total binding free energies using the PBSA and GBSA; RMSD, Root Mean Square Deviation; RMSF, Root Mean Square Fluctuation; TP, Thymidine Phosphorylase; VS, Virtual Screening

into cancerous cells and subsequent treatment with 5-FC can lead to the targeting killing of cancer cells and non-cancer cells would be protected[8]. Though many molecular targets have been identified for 5-FU, there still exists a mystery regarding its exact molecular mechanism which leads to the therapeutic effects.

In this study, molecular targets of 5-FU were identified by virtual or *in silico* screening which is used to scan large databases to identify a small number of candidates for biological tests. At present, two virtual screening (VS) methods are available for the drug discovery studies but each one of them has advantages and disadvantages[9-11]. In the first method, the structure of the receptor is used to identify the ligands, and it is called as structure based VS. In the second method, ligand is used to identify the molecular targets, and it is called ligand based VS or reverse screening. In our study, about 15 probable molecular targets were selected after initial virtual screening and the ligand-binding efficiency of these molecular targets with 5-FU were subsequently analyzed by docking analyses and compared. This resulted in the identification of cell division protein kinase 2 (CDK2) as a novel target for 5-FU. The total binding free energy calculations from Molecular dynamics simulations using the Molecular Mechanics/Generalized Born Surface Area (MM-GBSA) and Molecular Mechanics/Poisson Boltzmann Surface Area (MM/PBSA) methods, showed that binding affinity of CDK2 towards 5-FU is almost similar and comparable with human thymidine phosphorylase (TP), which is a much established 5-FU target.

## Materials and methods

### Virtual Screening of Molecular Targets for 5-fluorouracil

The 3D structure of 5-FU was downloaded from PubChem (CID3385) and was submitted to the PharmMapper server[12]. Around 300putative molecular targets for 5-FU molecule were identified using pharmacophore mapping strategy against its own database. Out of the above 300 putative molecular target complexes, 91 ligands were identified and initially shortlisted and screened by similarity with 5-FU. These 91 ligands and 5-FU were submitted to the PharmaGist web server in Mol2 format[13, 14]. Since the server could handle only 32 molecules at a time, the ligands files were divided into four separate groups, but still5-FU was retained as pivot molecule in all. Among all, only 15 ligands, which showed cut off score of above 33.29 were chosen and their corresponding molecular target complexes in PharmMapper results were considered for further analysis.

### Molecular docking analysis

The PDB structures of target protein molecules selected from PharmaGist results were obtained from PDB database. The PDBsum database was used to find the native ligand binding sites for the given receptors[15]. The putative ligands were obtained from PubChem and Zinc database in Mol2 format and then these files were converted into PDB format using Open Babel GUI (Graphic user interface) software[16]. Molecular docking of target proteins with their native ligands and with 5-fluorouracil was carried out using AutoDock software package (ver. 4.2)[17]. Initially, protein and ligand molecules were edited using AutoDockTools (ADT) (ver. 1.5.6.). Water molecules and cofactors were removed while hydrogen atoms were added to proteins. The output files were saved in PDBQT format. While performing docking, the grid box and grid spacing were selected to cover the area considered for docking space and a distance dependent function of dielectric constant was also used for the calculation of the energetic map. Conformation runs were generated with default docking parameters using Lamarckian genetic algorithm (LMA). The output grid parameter file (GPF) and docking parameter file (DPF) were prepared by ADT and executed in command prompt. AutoDockTools displayed 10 most favorable conformations for docking based on binding energy, the conformation with lower binding energy was considered as favorable conformation.

### Molecular Dynamics simulation

We carried out molecular dynamics (MD) simulation on CDK2-5-FU complex and human thymidine phosphorylase-5-FU complex systems. The initial complex structures of CDK2-5-FU and human thymidine phosphorylase- 5-FU complex were obtained from the docking method. These complex systems were then subjected to MD simulations using sander module of AMBER[18] software package with ff99SB Amber force field[19]. We used LEaP and antechamber module in AMBER[18] to obtain the initial coordinate and topology files for the two systems. In case of each system, the resultant initial structure was unambiguously solvated using TIP3P water model in a box having size of 10Å from the solute in the x, y, and z coordinates[20]. The initial structure was then minimized by subjecting it to 1000 steps of

steepest descent minimization of the potential energy, followed by 2000 steps of conjugate gradient minimization. In the minimization step, we fixed the protein molecule (and the solvent water molecules were excluded) using harmonic constraints with a force constant of 30 kcal $mol^{-1}Å^{-2}$, to remove the bad contacts between water molecules and the solute. The structure minimization was then carried out at 40ps of MD using a 2femtoseconds time step for integration. During the MD step, the system was gradually heated from 0-300 K using weak 20 kcal $mol^{-1}Å^{-2}$ harmonic constraints on the solute to its starting structure. This actually allows the structure to undergo slow relaxation. In addition SHAKE constraints[21] using a geometrical tolerance of $5\times10^{-4}$ Å was imposed on all covalent bonds involving hydrogen atoms. Later, MD simulation was performed at NPT condition with temperature regulation achieved with the help of Berendsen weak coupling method (0.5 ps time constant for heat bath coupling and 0.2 ps pressure relaxation time)[21,22]. This was followed by another 5000 steps of conjugate gradient minimization while decreasing the force constant of the harmonic restraints from 20, 30, and 20 kcal $mol^{-1}Å^{-2}$ to zero in steps of 5, 30, 20 kcal $mol^{-1}Å^{-2}$. Finally, for analysis of structures and properties, we carried out 20 ns long NPT MD simulation using a heat bath coupling time constant of 1 ps. The analysis of structural parameters like RMSD, B-factor values were carried out using ptraj module of AMBER package for structure and dynamics analysis of trajectories[18].

#### MM-GBSA/PBSA calculations of CDK-5-FU and TP-5-FU complexes

To determine the relative ligand-binding free energy of CDK2/TP-5-FU complexes, we used the MM-GBSA/PBSA method[23]. For the MM-PBSA calculations, we extracted the snapshots of the complex system (without water and ions) from our molecular dynamics production run. Thereafter, GBSA/PBSA analysis was performed on the three components of each of the complex system: (i) the protein (CDK2/TP), (ii) ligand (5-FU) and (iii) the complex (CDK2/TP-5-FU). For each of these components, the interaction energy and solvation free energy was calculated and the averages of these results were considered to ascertain an estimate of the ligand-binding free energy. The binding free energy of the CDK2/TP in complex with 5-FU was calculated as given below,

$$\Delta G_{bind} = G_{tot}\ (\text{CDK2/TP} - \text{5-FU Complex}) - G_{tot}\ (\text{CDK2/TP}) - G_{tot}(\text{5-FU})$$

In MM-GBSA calculations, we have used the pairwise GB model[24] with parameters described previously by Tsui and Case[25].For all other settings we have kept the default set of values. However, for MM-PBSA[23] calculations, we set the internal and external dielectric constant to be 1.0 and 80.0, respectively.

## Results and discussion

#### Screening of molecular targets for 5-FU

5-FU is a potent anti-cancer drug and its molecular mechanism has been demonstrated in several studies and two molecular targets have been found in human beings[3]. TP and OPRT are two well-studied molecular targets for 5-FU in apoptosis mediated cell death in variety of mammalian cancer cells[4]. In order to identify novel molecular targets, we adopted virtual screening and docking strategies. Nearly 300 potential molecular targets for 5-FU were obtained by PharmMapper server after screening with its own database. This server could find the molecular targets by pharmacophore mapping approach. Around 91 out of 300 molecular targets were shortlisted by removing repeated ligands and targets with same binding sites (Supplementary Table1). The ligands of the 91 molecular target complexes were collected separately and pooled on the basis of similarly with 5-FU by PharmaGist server which gave the similarity scores with the pivot molecule. 5-FU was used as a pivot molecule for all the 91 ligands and selected the 15 ligands with a cut off score of higher than 32.292 (Supplementary Table 2). Further, 15 selected ligands and their corresponding molecular targets were chosen for further analysis. Four out of 15 molecular targets were from plant sources (*Spinaciaoleracea*, *Arabidopsis thaliana* and *Pisumsativum*), six were from bacterial sources (*Escherichia coli*, *Methanothermobacterthermautotrophicus* and *Streptomyces venezuela*e), one each from yeast (*Schizosaccharomycespombe*), mammal (*Mus musculus*) and human (*Homo sapiens*) sources.

#### Docking analysis of selected targets with 5-FU

Fifteen ligands selected form PharmaGist were docked with their corresponding PDB structures obtained from PharmMapper. The putative ligand-binding sites of each target and their native ligands were identified by ligplots which were obtained for PDBsum web server. Supplementary Table3 shows the list of putative binding residues of molecular targets forming the hydrogen and/or hydrophobic bonds with their native ligands. As all the 15 shortlisted ligands had high similarity with 5-FU, we

chose native ligand binding sites for docking the molecular targets with 5-FU. We determined the interactions of all the 15 molecular targets with 5-FU and their molecular targets in the same binding clefts, where their native ligands were also present. Binding energy and inhibition constants for both the native ligand and 5-FU were obtained for each of the 15 targets and are summarized in Table 1. 5-FU showed higher binding affinity with adenylosuccinatesynthetase and deoxyuridine 5'-triphosphate nucleotide hydrolase of *E. coli* with binding energy of −5.59 and −5.21 kcal $mol^{-1}$, respectively. Experimental evidence has shown that *E. coli* uracil phosphoribosyl transferase is a high affinity enzyme for 5-FU metabolism as evident from the
previous report of the prodrug enzyme therapy (PDET)[26]. Similarly, adenylosuccinatesynthetase and deoxyuridine 5'-triphosphate nucleotide hydrolase of *E.coli* would be examined in PDET and these could metabolize the 5-FU. Interestingly, adenylosuccinatesynthetase of *E. coli* has shown high binding affinity than its native ligand hydantocidin 5'phospahte. TDP-glucose-4, 6-dehydratase *of Streptomyces venezuelae* has shown significant binding affinity towards the 5-FU with a binding energy of −4.79 kcal $mol^{-1}$. CDK2 is the only human enzyme which has shown significant affinity to towards the 5-FU with a binding energy of −4.09 kcal $mol^{-1}$.

**Comparative binding analysis of TP and CDK2 with 5-FU**

It is evident from previous reports that 5-FU can be metabolized by TP in mammalian cells[3,4]. This enzyme helps in conversion of 5-FU to 5-FUMP. Down regulation of CDK2 is evident from previous study on 5-FU resistant cancer cell lines[27] but phosphorylation of 5-FU by CDK2 has not been reported yet. But in one of the *in vitro* study[28] observed altered CDK2 activity on 5FU treated cancer cells. It is also evident from preliminary clinical trials that, combination therapy of 5-fluorouracil resulted in effective treatment in variety of malignancies by reducing CDK2 activity[29]. In our study, CDK2 has shown significant affinity toward the anti-cancer drug, 5-FU. From our results we see the significant binding of 5FU to CDK2 and it is coinciding with the results that were obtained in earlier in vitro and in vivo studies[28,29]. As CDK2 is a novel target, we compared the binding energy of this protein with the known 5-FU target, TP by docking analysis. The interaction patterns of the two potential target molecules with 5-FU were compared in Table2. The O-H bonds contribute significantly in binding with 5-FU. CDK2 has shown less binding energy than TP against 5-FU which indicates the high binding affinity of CDK2 towards 5-FU. Binding energies and of both the proteins with 5-FU and the amino acids which had bond length of less than 2.5 Å with 5-FU were analyzed in Fig. (1). The H- atom of amino group of Lys33 of CDK2 formed the strongest bond with O atom of 5-FU with the lowest bond distance of about 1.90 Å Fig.(1A). Additionally, Glu51 and Asp145 also forms O—H bond with 5-FU. As lysine (at 33 position) is one of the key amino acid of CDK2 active site, therefore it might plays an imperative role in the binding interaction of CDK2 with 5-FU. For TP, two O—H bonds were formed with 5-FU. First, the

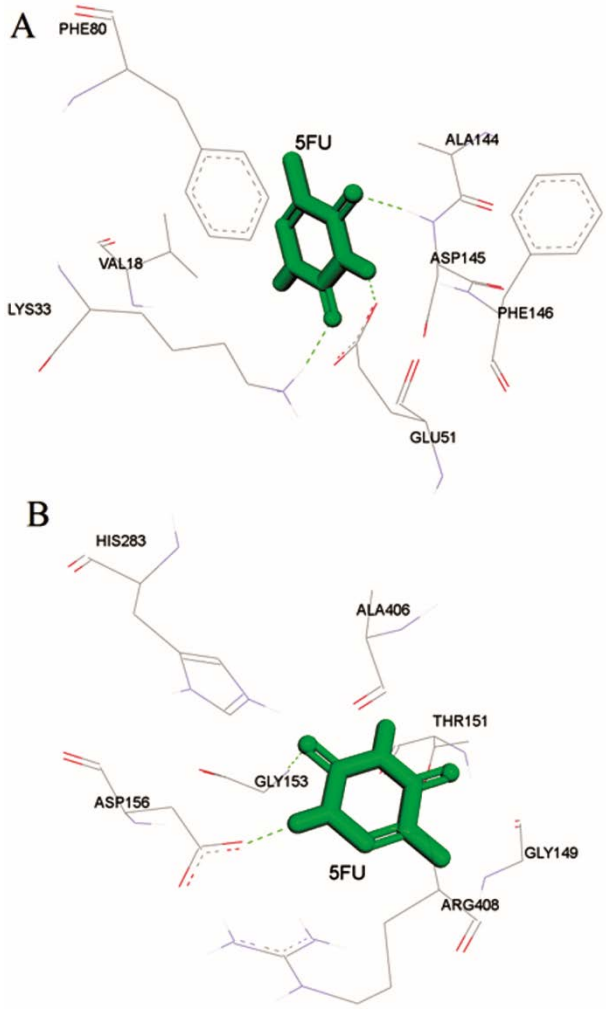


Fig. 1 — Binding interaction modes of 5FU with A) human CDK2 and B) human TPacitve site amino acids. Hydrogen bonds are indicated as dotted line.Discovery Studio (Ver. 3.5), visualization tool, was used to acquire this image.

oxygen atom of aspartic acid (156) forms a bond with hydrogen atom of 5-FU (1.94 Å) and second, H-atom of amino group of glycine (153) forms a bond with oxygen of 5-FU (2.21 Å) as shown in Fig. (1B). It can be inferred that CDK2 develops a stronger interaction with 5 FU than TP because of higher number of O—H bonds and lowest binding energy.

**Molecular Dynamics simulations and total binding free energy calculations**

From the Molecular Dynamics simulation study, we compared the stability of the reference human thymidine phosphorylase-5-FU complex with the CDK2-5-FU complex.

**RMSD Analysis**

The conformational stability of CDK2-5-FU complex (Fig. 2A) and TP-5-FU complex (Fig. 2B) have been studied by the RMSD analysis. For the RMSD analysis, in both the complex systems, we have used the first snapshot in the production step as the reference structure. From the Fig. 2, we can see that the CDK2-5-FU complex was as stable as TP-5-FU complex. The RMSD value for both the above mentioned complex structures resolved to well below 2.5Å.

**Analysis of equilibration of the system**

We also checked the equilibration of CDK2-5-FU complex and the TP-5-FUcomplex structures by analyzing temperature (Fig. 3),total energy, kinetic and potential energy of the system as a function of simulation time period (Fig. 4). From these plots we can see that the structure of CDK2-5-FU complex and the TP-5-FU complex have reached equilibration. From the Fig. 4, we can clearly infer that CDK2-5-FU complex exhibits stability similar to that of human thymidine phosphorylase-5-FU complex.

To check the local deformability in CDK2-5-FU complex and the TP-5-FU complex, we analyzed the Root mean square fluctuation (RMSF) for the entire C-α atoms in the backbone. The RMSF for the backbone C-α atoms were calculated from MD simulation trajectory and are plotted against their residue index (Fig. 5A & B). From the RMSF plot, we see in the case of the CDK2-5-FU complex, the C-α atom of the residues are observed to show relatively higher fluctuations from their equilibrium positions than the C-α atoms in theTP-5-FU complex.

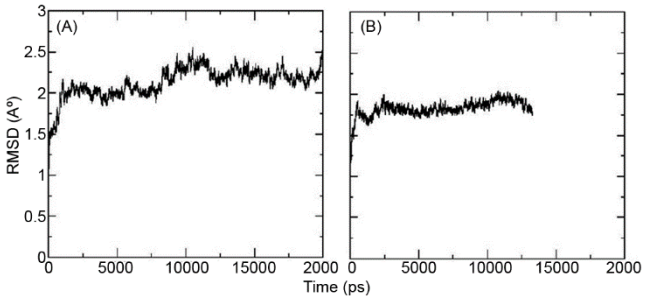


Fig. 2—Root mean square deviation (RMSD) of 5FU complex structure with A) Human CDK2 and B) Human TP as a function of simulation time.

**Binding Free energy analysis using MM-GBSA/PBSA**

The free energy of ligand binding for CDK2/TP-5-FU complexes was then determined to ascertain the accuracy of study. Different computational methods

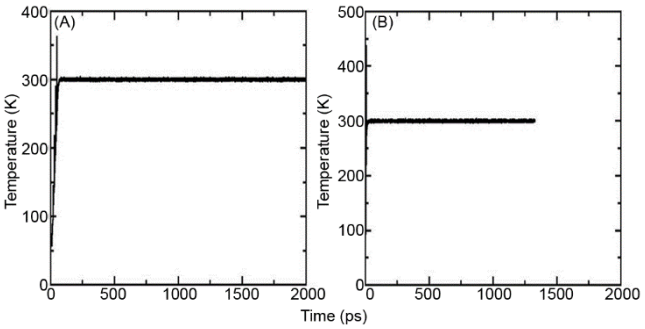


Fig. 3—Temperature as a function of simulation time for A) human CDK2-5FU complex and B) Human TP-5FU complex.

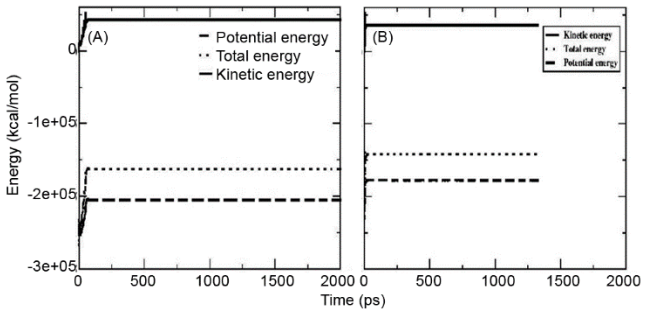


Fig. 4—Energy as a function of simulation time for A) human CDK2-5FU complex and B) Human TP-5FU complex.

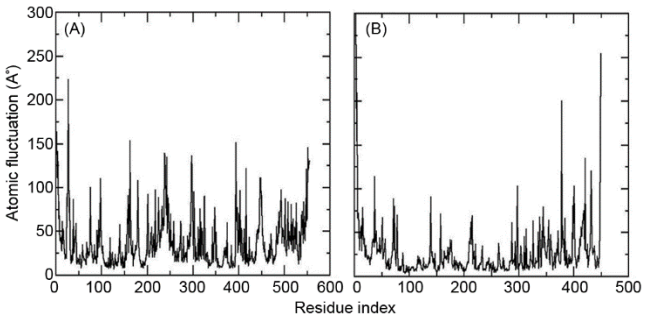


Fig. 5—Atomic fluctuation as a function of Residue Index for A) human CDK2-5FU complex and B) Human TP-5FU complex.

with varying degree of accuracy and efficiency are available to determine the free energies of binding[30]. But most of the methods or rather the software are expensive and require a hefty amount of money to be paid as a license fee. And so we opted for MM-PBSA/GBSA method[23]. This method provides accurate results at comparatively low cost[31]. Another advantage of this method is that it gives information about the various contributions to the free energy, such as the van der Waals, electrostatic, and solvation energy contributions. The details of the MM-GBSA/PBSA[23] calculations have been shown in the Table 3. We can also see the binding free energy ($\Delta G_{bind}$) results for the CDK2/human thymidine phosphorylase-5-FU complexes (Table 3). In Table 3, PBTOT and GBTOT indicate the total binding free energies using the PBSA and GBSA[23]. The negative total binding free energies (PBTOT/GBTOT=−13.08/−16.12 kcal $mol^{-1}$)for CDK2-5-FU and (PBTOT/GBTOT=−13.95/ −14.29 kcal $mol^{-1}$) for TP-5-FU complex show that both the complexes are favorable.

## Conclusion

Primarily we screened 15 molecular targets from protein structure database for an anti-cancer drug, 5-FU, based on binding site analysis of targets as well as ligand similarity with 5-FU. These 15 targets were further screened by molecular docking to find out the potential putative molecular targets for 5-FU. In this study, we identified CDK2 as a putative human molecular target for 5-FU among all available human protein targets in a protein database. The ligand binding affinity of CDK2 towards 5-FU was found to be very similar with the well-studied 5-FU target; human thymidine phosphorylase. Finally, ligand-binding affinity and stability of both the complexes were validated by molecular dynamics simulations. This study suggested that CDK2 can bind with 5-FU with significant affinity and this study could help in elucidating the exact mechanism of action of 5-FU in cancer treatment.

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