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Abstract:
Natural products, like emodin, are quickly gaining relevance as effective anticancer agents, and emodin possess inhibitory activity against a broad spectrum of kinases. Herein, we investigate the interaction of emodin with serine/threonine kinases, one subclass of kinases, with protein-ligand docking, which are implicated in phosphorylation cascades; thus, competitive inhibition can reduce the expression of a wide range of proteins required for essential cell function, which will result in cell death. Because serine/threonine kinases have similar active sites, we have successfully created a blueprint for emodin binding and interactions with active site residues, which include many hydrophobic interactions and minimal amounts of hydrogen bonding. Although emodin’s biological targets are over expressed in cancer, serine/threonine kinases are also important regulators of normal cellular function. We also show through molecular dynamics that emodin forms favorable interactions with chitosan and chitosan-PEG copolymers, which can aid in loading the drug into nanoparticles for targeted drug delivery to cancerous tissue. Both polymers demonstrated reasonable entrapment efficiencies, which encourages experimental exploration of emodin through targeted drug delivery vehicles and their anticancer activity.

Author Comments:
We were motivated to find a way to quickly simulate nanoparticles and estimate its properties for our wet lab work, so we tried to use computational tools designed for proteins to simulate nanoparticles as the ligand-molecule interactions are similar. Interestingly, everyone was captivated by the movies we generated of the nanoparticles forming in-silico. It was difficult to repurpose a tool, but exploring the field led to unrelated, but interesting bits of knowledge for everyone in the group, and it was an enriching experience to contribute to the field.
In-Silico Modelling of Emodin’s Interactions with Serine/Threonine Kinases and Chitosan Derivatives

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Summary

Natural products, like emodin, are quickly gaining relevance as effective anticancer agents, and emodin possess inhibitory activity against a broad spectrum of kinases. Herein, we investigate the interaction of emodin with serine/threonine kinases, one subclass of kinases, with protein-ligand docking, which are implicated in phosphorylation cascades; thus, competitive inhibition can reduce the expression of a wide range of proteins required for essential cell function, which will result in cell death. Because serine/threonine kinases have similar active sites, we have successfully created a blueprint for emodin binding and interactions with active site residues, which include many hydrophobic interactions and minimal amounts of hydrogen bonding. Although emodin’s biological targets are overexpressed in cancer, serine/threonine kinases are also important regulators of normal cellular function. We also show through molecular dynamics that emodin forms favorable interactions with chitosan and chitosan-PEG copolymers, which can aid in loading the drug into nanoparticles for targeted drug delivery to cancerous tissue. Both polymers demonstrated reasonable entrapment efficiencies, which encourages experimental exploration of emodin through targeted drug delivery vehicles and their anticancer activity.

Introduction

Emodin is a natural derivative of anthraquinone (a polycyclic aromatic hydrocarbon derived from anthracene used to manufacture dyes) and is typically found in various medicinal plants such as rhubarb, buckthorn, moulds, and lichen (1) (Figure 1). It has various therapeutic roles as a tyrosine kinase inhibitor, a laxative, and an anticancer agent (2).

Emodin binds to proteins that have been found to have anticancer properties including serine/threonine kinases, dual specificity kinases, GTPases, and proteases (3, 4, 5). Kinases are central to maintaining proper cellular function via turning on protein functions through the phosphorylation of specific amino acids. Serine/threonine kinases act specifically on serine and threonine amino acid residues on regulatory proteins, which leads to widespread effects through phosphorylation cascades. Dual specificity kinases act as tyrosine kinases as well as serine/threonine kinases. Due to their role in promoting cell proliferation and anchorage-independent growth, the overexpression of protein kinases are typically associated with oncogenesis (6). Dual specificity kinases are generally also overexpressed in multiple cancers as they increase cell proliferation and anchorage-independent growth of cells — critical characteristics for tumor growth and metastasis (7). GTPases also play a key role in the regulation of cellular function such as cell differentiation, proliferation, and motility, and thus are a prime target for anticancer therapies (8). In this study, the specific characteristics of emodin binding to serine/threonine kinases were
identified via a variety of in silico methods to aid in identifying emodin’s mechanism of action as an anticancer agent.

The use of nanoparticles is emerging as a promising method of small molecule delivery in cancer therapeutics. Traditionally, molecular dynamics (MD) has been utilized to model protein-drug complex interactions on time course, and has more recently also been used to model nanoparticles, and their interactions with surrounding media. Inorganic nanoparticles and carbon-based nanoparticles have primarily been studied, whereas polymer nanoparticles have not been able to be accurately predicted through in silico approaches. In one study exploring the production of solid lipid nanoparticles, drug entrapment efficiency was found to be more than 87% and proved to show long-term physical stability (9). In another study, chitosan nanoparticles were investigated to deliver ciprofloxacin hydrochloride (10). Drug entrapment efficiency was found to increase with increasing amounts of drug in polymer:drug ratios.

Previous studies have investigated the binding mechanisms behind emodin and tyrosine kinase interactions. Due to the similarity between tyrosine kinases and serine/threonine kinases, we hypothesize that emodin has strong binding interactions to these kinases as well. This study aims to identify the specific interactions that allow emodin to bind to serine/threonine kinases. Furthermore, because emodin consists of a hydrophobic core surrounded by polar functional groups, we hypothesize that emodin will form strongly favorable interactions with chitosan, which will lead to higher entrapment efficiencies at lower drug to polymer ratios that decrease as more drug molecules are added.

Results

Molecular Docking

Serine/threonine kinases were found to bind well to emodin with binding scores averaging approximately -8.6 whereas caspase-3, a cysteine protease, only scored -5.8. Binding in these proteins was facilitated by emodin’s nonpolar interactions with the amino acid residues leucine and valine (Figure 2a). Additionally, binding was also facilitated by hydrogen bonding between the phenols on emodin (Figure 1) and the polar regions of the protein. Dual specificity kinases did not bind well to emodin to the lack of interaction between the polar regions of the kinases and the nonpolar regions of emodin (Figure 2b). When bound to MAPK-9 and S/THPK, emodin was found to interact with various residues including leucine, valine, glycine, phenylalanine, and threonine (Figure 2c, Figure 2d). Proteins with low magnitude binding scores did not display specificity to any amino acid residues. Overall proteins with high magnitude binding scores had interactions with emodin primarily via leucine and valine residues. Specific proteins used are identified in Table 1. Additionally, emodin was found to bind to serine/threonine kinases in the same location as the adenosine of ATP as opposed to where the triphosphate binds (Figure 3). Due to the
competitive inhibition of ATP by emodin, the downstream effects of serine/threonine kinases such as cell proliferation and differentiation are blocked.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Protein Class</th>
<th>PDB ID</th>
<th>PDB ID for Protein-Drug Complex</th>
<th>Score for Emodin</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEK1 (12)</td>
<td>dual specificity kinase</td>
<td>3E8N</td>
<td>3E8N</td>
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</tr>
<tr>
<td>MAPK-9 (13)</td>
<td>serine/threonine kinase</td>
<td>3DTC</td>
<td>3DTC</td>
<td>-9.7</td>
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<td>S/THPK (14)</td>
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<td>6BDN</td>
<td>6BDN</td>
<td>-9.7</td>
</tr>
<tr>
<td>MAPK-10 (13)</td>
<td>serine/threonine kinase</td>
<td>2B1P</td>
<td>2ZDU</td>
<td>-9.6</td>
</tr>
<tr>
<td>MAPK-12 (13)</td>
<td>serine/threonine kinase</td>
<td>5CEN</td>
<td>5VO2</td>
<td>-9.5</td>
</tr>
<tr>
<td>MAPK-2 (13)</td>
<td>serine/threonine kinase</td>
<td>3R2B</td>
<td>3KGA</td>
<td>-9.3</td>
</tr>
<tr>
<td>PIM-1 (15)</td>
<td>serine/threonine kinase</td>
<td>3C4E</td>
<td>3C4E</td>
<td>-9.2</td>
</tr>
<tr>
<td>PI3K (16)</td>
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<td>3APC</td>
<td>1E7V</td>
<td>-9.1</td>
</tr>
<tr>
<td>MTOR (17)</td>
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<td>5FLC</td>
<td>5FLC</td>
<td>-8</td>
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<tr>
<td>PRK1 (18)</td>
<td>serine/threonine kinase</td>
<td>4OTD</td>
<td>4OTI</td>
<td>-8.5</td>
</tr>
<tr>
<td>RAC1 (19)</td>
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<td>1HH4</td>
<td>4GZM</td>
<td>-7.4</td>
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<tr>
<td>Casein kinase 2 (20)</td>
<td>serine/threonine kinase</td>
<td>4MD7</td>
<td>4GRB</td>
<td>-6.6</td>
</tr>
<tr>
<td>MAPK (21)</td>
<td>serine/threonine kinase</td>
<td>3S4E</td>
<td>4BID</td>
<td>-6.5</td>
</tr>
<tr>
<td>Aryl hydrocarbon receptor (22)</td>
<td>transcription factor</td>
<td>5NJ8</td>
<td>5V35</td>
<td>-8.3</td>
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<tr>
<td>MAP2K-4 (13)</td>
<td>dual specificity kinase</td>
<td>3VUT</td>
<td>3ALN</td>
<td>-7.8</td>
</tr>
<tr>
<td>DSP-7 (23)</td>
<td>dual specificity kinase</td>
<td>6IB0</td>
<td>6IB0</td>
<td>-7.8</td>
</tr>
<tr>
<td>VEGFR (24)</td>
<td>tyrosine kinase</td>
<td>2VPF</td>
<td>4AGC</td>
<td>-7.9</td>
</tr>
<tr>
<td>HSP90 (25)</td>
<td>kinase regulator</td>
<td>1US7</td>
<td>2QF6</td>
<td>-7.8</td>
</tr>
<tr>
<td>MTH1 (26)</td>
<td>pyrophosphatase</td>
<td>5OTM</td>
<td>5MZG</td>
<td>-7.8</td>
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</table>
Table 1: Names and class of protein targets emodin was bound to during docking. RCSB PDB ID for both the protein as well as the protein-drug complex used during docking is identified. Docking scores for emodin bound to protein included in column 5. References for protein bioactivity included in column 6.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Class of Protein</th>
<th>RCSB PDB ID Protein</th>
<th>RCSB PDB ID Complex</th>
<th>Docking Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl-2 Protein (27)</td>
<td>antiapoptotic protein</td>
<td>7CA4</td>
<td>4AQ3</td>
<td>-7.6</td>
</tr>
<tr>
<td>Topoisomerase (28)</td>
<td>topoisomerase</td>
<td>1LPQ</td>
<td>1SEU</td>
<td>-7.5</td>
</tr>
<tr>
<td>Ras Protein (29)</td>
<td>GTPase</td>
<td>4M21</td>
<td>1X1R</td>
<td>-7.2</td>
</tr>
<tr>
<td>Matrix metalloproteinase-2 (30)</td>
<td>proenzyme</td>
<td>1CXW</td>
<td>1HOV</td>
<td>-5.9</td>
</tr>
<tr>
<td>Caspase-3 (31)</td>
<td>Cysteine protease</td>
<td>3KJF</td>
<td>3KJF</td>
<td>-5.8</td>
</tr>
</tbody>
</table>

Molecular Dynamics

Emodin remained stable in the binding pocket and throughout the simulation and stabilized the protein backbone as well (Figure 4). Consequently, emodin would be an effective inhibitor as the protein was more stable when emodin was bound than when no ligands were present.

The fourth and fifth oxygen (located on the third carbon ring of emodin) are closest to MAPK-9 within a range of 3.6 to 4.0 angstroms, whereas the first, second, and third oxygens range from 5.2 to 7.0 angstroms away from the protein. The carbons of emodin are facing toward the solvent, while the oxygens and hydrogens are interacting with MAPK-9 through hydrogen-bonding (Figure 5).

In both the chitosan and copolymer trials, a clear maximum EE occurred below a drug:polymer ratio of 0.5. Overall, the copolymer trials had lower EEs compared to the chitosan. Specifically, the chitosan NP had a maximum EE of 65.7% while the copolymer NP was 60.9%, and this trend is consistent throughout the majority of the drug:polymer ratios. Furthermore, there was not a linear relationship between the drug:polymer ratio and EE, which was contrary to our hypothesis; instead, the EE quickly climbed to a maximum as the ratio was increased, then slowly decreased to a plateau (Figure 6). The equation was chosen to model the initial increase in entrapment efficiency as well as the plateau that follows, and because it accurately predicted the EE of emodin, the model is valid.

Downstream PyMOL analysis indicated that emodin relies on both hydrogen bonding and hydrophobic interactions to remain near the chitosan. This is consistent with emodin’s structure as it has a hydrophobic anthraquinone core, which is surrounded by the phenols. Thus, the core participates in hydrophobic interactions, and the phenols and ketones, which are both polar, hydrogen bond with the alcohols and amines on chitosan (Figure 7). Because a small number of monomer units were used in the
simulation, all the polymers aggregated together to form the NP, but the PEG in the copolymer moved to
the exterior of the NP (Figure 8). Furthermore, the copolymer adopted a more flexible structure that was
able to integrate emodin more thoroughly while the normal chitosan remained rigid, which caused the
copolymer to have an irregular shape and the chitosan created a spherical NP (Figure 9).

Discussion

The docking studies presented within this work show that emodin is capable of binding to a wide
variety of serine/threonine kinases in the same location as current inhibitors due to the presence of a
nonpolar binding pocket, which is assisted by hydrogen bonding. Because emodin is able to act as a
competitive inhibitor to ATP, emodin suppresses serine/threonine kinase activity by inhibiting the
phosphorylation of protein substrates. Despite this, dual specificity kinases also use ATP as their source of
phosphate for phosphorylation, but because their pockets contain more polar residues, emodin is unable to
strongly bind in the pocket. Our results also show that there is a lack of nonpolar residues, which was
essential for emodin binding to serine/threonine kinases; therefore, emodin could not form stable
interactions with dual specificity kinases. Kinases function at higher levels of signal transduction pathways;
as a result, inhibition affects a multitude of downstream functions at the molecular level, which results in
the cell no longer being able to sustain function. Because serine/threonine kinases and tyrosine kinases are
both generally overexpressed in tumorous cells, a broad spectrum inhibitor such as emodin would be
effective in promoting cell death of cancerous cells, but normal somatic cells also rely on kinase function;
therefore, it is important to develop alternative formulations that minimize off target effects.

We have successfully demonstrated that emodin can be efficiently loaded into chitosan
nanoparticles in order to aid in the targeting of emodin to cancerous tissue. Both regular chitosan and
chitosan-PEG polymers peak in entrapment efficiency when the drug:polymer ratio is about 0.4. Our
hypothesis relies on the assumption that emodin-polymer interactions were favorable, but our
experimentation suggests that emodin’s interaction with the polymers only leaned to one side of the
equilibrium. As a result, not all of the emodin molecules tried to interact with the chitosan. During the in
silico synthesis of NPs, the polymer strands bent around emodin molecules, which increased the number of
emodin-polymer interactions. This was further stabilized by pi-pi stacking between emodin molecules.
Consequently, at lower drug:polymer ratios, the EE is more polarized toward lower percentages because
fewer emodin-emodin interactions can form, and the formation of interactions is closer to random. As the
drug:polymer ratio increases, the EE levels off and drops toward 0 due to a lack of polymer molecules to
interact with emodin. The chitosan-PEG produced a slightly lower EE because emodin was unable to
efficiently interact with the PEG, which reduced the amount of polymer available for emodin. PEG is
moderately polar due to the ether linkages, but emodin consists of distinct nonpolar and polar regions,
which results in unfavorable interactions. Despite this, the EEs between the two polymers were similar, yet
the chitosan-PEG polymer increases NP stability, which outweighs the slight loss of EE.

With the use of computational tools including docking and MD, we have demonstrated that emodin
effectively binds to a broad spectrum of kinases, showing its cytotoxic activity. As a result, future studies
can look into the design and testing of serine/threonine kinase specific inhibitors based upon an emodin
scaffold. This can be mediated through the use of NPs because chitosan is able to favorably interact with
emodin, which will likely lead to a high EE and stable release rate, which can be quickly synthesized and
tested in biological solutions.

Methods

Docking

First, emodin was built in Avogadro (32), and optimized with the MMFF94 force field for 10,000
steps before being fully optimized with TD-DFT in Orca using the B3LYP and def2-TZVP functionals with
CPCM(Water) as the solvent model.

UCSF Chimera, a molecular visualization tool, was used to construct proteins based on its PDB ID
taken from RCSB. All non standard residues, subunits, and ligands were deleted and emodin was loaded
onto the receptor file. The receptor and emodin were prepped prior to performing surface binding analysis
using the Autodock Vina extension on Chimera (33). A box was used to encapsulate the ligand and receptor
and blind docking was performed. From the DockViewer, the top binding score was recorded. The complex
was then loaded into PyMOL, another leading molecular visualization tool, to compare with other protein-
drug complexes (34).

A PDB ID for the protein in complex with a leading drug was obtained from RCSB. The protein-
drug complex was then aligned through PyMOL with the protein-emodin complex. The ligands listed under
the small molecules section on the RCSB profile for the protein-drug complex were then highlighted in
PyMOL. The binding locations of the selected ligands were compared to the binding location of emodin on
the protein.

A script was made to locate all of the residues within 2.75 Angstroms of each non-hydrogen atom
in emodin. This script was run on all of the docked conformations, and a heatmap was created to represent
the amino acid frequency near each amino acid for groups of proteins including serine/threonine kinases,
dual specificity kinases, and low-scoring proteins (35).

Molecular Dynamics

The docked structure of emodin to MAPK-9 was chosen for further analysis through molecular
dynamics (GROMACS version 2020.4) (36) because it possessed a high binding score, and it has high
biological relevance. The structure was solvated in water using the TIP3 model and EM, NVT, NPT equilibration were conducted until the system was stable. Afterwards, a 10 ns simulation was performed, and the RMSD of the backbone chain was plotted over time.

A base polymer of chitosan with 6 monomer units of glucosamine was constructed using Avogadro, and optimized with the MMFF94 force field for 10,000 steps. Similarly, to construct the copolymer, the base polymer was taken, and 3 units of ethylene glycol were directly attached onto the amine of the last glucosamine monomer on chitosan. Parameters for GROMACS were generated through the CHARMM webserver hosted by the University of Maryland (37, 38, 39, 40, 41). Next, a system of 6 polymers and a predetermined number of emodin molecules were added to a box without water or ions, which has been previously reported to accurately simulate NP formation (42) An energy minimization (EM) was performed for a maximum of 5,000 steps. Afterward, the system was optimized for 75,000 steps under a constant number of molecules, volume, and temperature (NVT) at 300 Kelvin. A PME mesh was used in the system and LINCS was used to constrain the bond lengths and hydrogen bonds, which increased the speed of the simulation without a large impact on accuracy. For a similar reason, a Verlet cutoff scheme was used.

After the simulation was completed, a script was made to find the shortest distance between any atom on emodin and any atom on the nearest chitosan polymer. The number of emodin molecules less than 2.75 Å from chitosan was averaged over the last 25,000 steps of the simulation and divided by the total emodin molecules in the simulation to find the entrapment efficiency (43). After the entrapment efficiency was plotted, Equation 1 was used to draw the line of best fit with least squares minimization and predict the optimal drug:polymer ratio.

\[
\eta(x) = a x^3 + b x^2 + c x + d
\]

**Equation 1:** Entrapment efficiency formula. \(x\) represents the drug:polymer ratio, and the constants \(a, b, c, d,\) and \(f\) were optimized with least squares minimization. The function returns the entrapment efficiency.

**References**


Acknowledgements

We would like to thank the Olive Children’s Foundation for providing us with the funding to conduct the research presented in this work. Furthermore, we gratefully acknowledge the Aspiring Scholars Directed Research Program for providing us access to the server to run our simulations on.

Captions

**Figure 1**: Chemical structure of emodin. Emodin is composed of three six-membered carbon rings with three hydroxyl groups, a methyl group, and two carbonyl groups attached.

**Figure 2**: Residues interacting with emodin. Heatmap of residues interacting with emodin in serine/threonine kinases (a) and dual specificity kinases (b). Positive values signify nonpolar interactions while negative values signify polar interactions like hydrogen bonds. Residues within 4 angstroms of emodin in MAPK-9 (c). Residues within 4 angstroms of emodin in S/THPK (d).

**Figure 3**: Emodin bound to S/THPK. S/THPK bound to only emodin (a) and (b). S/THPK bound to emodin and ADP (c). Emodin binds at the same position in the binding pocket as the adenosine in ATP.

**Figure 4**: RMSD of the protein backbone of MAPK-9 over 1 nanosecond. The free protein had relatively large fluctuations in RMSD (a) compared to when emodin was inhibiting the active site (b).
Figure 5: Distance in angstroms between oxygens on emodin and MAPK-9 (a), hydrogens on emodin and MAPK-9 (b), carbons in the 1st ring of emodin and MAPK-9 (c), carbons in the middle ring of emodin and MAPK-9 (d), and carbons in the 3rd ring of emodin and MAPK-9 (e).

Figure 6: EE of chitosan (green) and chitosan-PEG NP (blue). The equation showed reasonable correlation coefficients of 0.9972 and 0.8857 respectively. Both show clear maximums and plateau off as the drug to polymer ratio is increased.

Figure 7: Chitosan system before (a) and after (b) MD simulation. Many emodin molecules aggregate with each other and some interact with chitosan.

Figure 8: Chitosan-PEG system before (a) and after (b) MD simulation. Emodin, assisted by pi-pi stacking, solely interacts with the chitosan component of the copolymer.

Figure 9: Surface morphology of chitosan (a) and chitosan-PEG (b) NP. Although the chitosan NP exhibits more spherical shapes, more emodin is aggregated while the chitosan-PEG forms a less tight NP, which allows emodin to interact with the polymer rather than other emodin molecules.