Scientific Research Publishing

Advances in Biological Chemistry, 2024, 14, 87-102
https://www.scirp.org/journal/abc
ISSN Online: 2162-2191
ISSN Print: 2162-2183

Synthesis and Characterization of Naproxen-Salicylate Derivatives as Potential Dual-Targeted Inhibitors of Dihydrofolate Reductase

Syon Schlecht*, Emily Gunderson, Ruthie Fowler, Takara Aguilar

Biotechnology Research and Development, Empower College and Career Center, Jefferson, Georgia
Email: *director@syonlabs.org

How to cite this paper: Schlecht, S., Gunderson, E., Fowler, R. and Aguilar, T. (2024) Synthesis and Characterization of Naproxen-Salicylate Derivatives as Potential Dual-Targeted Inhibitors of Dihydrofolate Reductase. *Advances in Biological Chemistry*, **14**, 87-102.
https://doi.org/10.4236/abc.2024.144008

Received: May 9, 2024
Accepted: July 13, 2024
Published: July 17, 2024

Abstract

Dihydrofolate reductase (DHFR) is an enzyme that catalyzes the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF). Chemotherapy drugs such as methotrexate help to slow the progression of cancer by limiting the ability of dividing cells to make nucleotides by competitively inhibiting DHFR. Non-steroidal anti-inflammatory drugs (NSAIDs) have been previously reported to exhibit competitive inhibition of DHFR, in addition to their primary action on cyclooxygenase enzymes. This interaction interferes with the enzymatic reduction of dihydrofolate to tetrahydrofolate, thereby impeding the folate metabolism pathway essential for nucleotide synthesis and cell proliferation. This activity stems from their structural resemblance to the p-aminobenzoyl-l-glutamate (pABG) moiety of folate, a substrate of DHFR. It has been established that NSAIDs containing a salicylate group (which has structural similarities to pABG), such as diflunisal, exhibit stronger DHFR-binding activity. In this study, we synthesized salicylate derivatives of naproxen with the aim of exploring their potential as inhibitors of DHFR. The interactions between these derivatives and human DHFR were characterized using a combination of biochemical, biophysical, and structural methods. Through polyacrylamide gel electrophoresis (PAGE) analysis, enzymatic assays, and quantitative ELISA, we investigated the binding affinity and inhibitory potency of the synthesized salicylate derivatives towards DHFR. The findings of this study suggest the potential of salicylate derivatives of naproxen as promising candidates for the inhibition of DHFR, thereby offering novel therapeutic opportunities for modulating the inflammatory process through multiple pathways. Further optimization of these derivatives could lead to the development

of more efficacious dual-targeted analogs with enhanced therapeutic benefits.

Keywords

Dihydrofolate Reductase, DHFR, Chemotherapy, Nonsteroidal Anti-Inflammatory Drugs, NSAIDs, Folate Metabolism Pathway, Anti-Folate, Novel Therapeutic Development

1. Introduction

DHFR plays a pivotal role in folate metabolism, catalyzing the conversion of dihydrofolate to tetrahydrofolate via a NADPH-dependent reduction reaction. This enzymatic process is indispensable for the synthesis of purines, pyrimidines, and amino acids, rendering DHFR a critical regulator of DNA synthesis and cellular proliferation [1]. DHFR inhibition holds significant implications in cancer therapeutics, particularly in the context of chemotherapy. Cancer cells exhibit elevated rates of proliferation and DNA synthesis, rendering them highly dependent on folate metabolism for nucleotide biosynthesis and cellular growth. Chemotherapy drugs often exploit this dependency on folate metabolism by targeting DHFR activity, thereby disrupting DNA synthesis and inducing cytotoxic effects in rapidly proliferating cancer cells [2]. Methotrexate, a classical chemotherapeutic agent, functions as a potent inhibitor of DHFR by competitively binding to its active site and blocking the conversion of DHF to THF. This inhibition deprives cancer cells of essential nucleotide precursors, leading to impaired DNA replication and eventual cell death.

The catalytic mechanism of DHFR is based on its binding interaction with the p-aminobenzoyl-L-glutamate (pABG) moiety of folate. This structural component of the folate molecule comprises a para-aminobenzoic acid (pABA) group linked to a glutamate residue via an amide bond [3]. Within the DHFR active site, the pABG moiety forms key interactions, anchoring the folate substrate and facilitating the enzymatic reduction of DHF to THF. Specifically, residues within the pABG binding site of DHFR engage in hydrogen bonding, hydrophobic interactions, and electrostatic interactions with the pABA and glutamate moieties of folate, orchestrating a precise molecular recognition event essential for catalysis [4].

It is understood that salicylate-containing NSAIDs, such as diflunisal, exhibit structural similarities to pABA, which enables them to mimic this molecule and effectively bind to the active site of DHFR [5]. This binding is facilitated by the ability of these compounds to form stabilizing hydrogen bonds and electrostatic interactions with key amino acid residues in the DHFR enzyme. Consequently, salicylate-containing NSAIDs are implicated in potentially inhibiting DHFR activity due to this structural resemblance. The inhibition of DHFR by salicylates arises from their similarity to the pABG moiety of folate, which is the natural

substrate of DHFR. By mimicking pABG, salicylates can competitively bind to the DHFR active site, thereby inhibiting the enzymatic reduction process essential for the conversion of DHF to THF [6]. This critical step in the folate metabolism pathway is necessary for nucleotide biosynthesis and cellular proliferation.

By disrupting folate metabolism, salicylates can impede the synthesis of nucleotides, leading to reduced DNA replication and cell division. This mechanism suggests a potential anti-cancer effect of salicylate-containing NSAIDs, as they can inhibit the growth of rapidly proliferating cancer cells [7]. Furthermore, the ability of salicylates to interfere with DHFR activity highlights their potential role in therapeutic strategies aimed at targeting folate-dependent pathways in cancer cells. These promising implications signal the importance of further experimental studies to elucidate the precise extent and significance of DHFR inhibition by salicylates. Such research would help determine the therapeutic potential and safety of using salicylate-containing NSAIDs in cancer treatment. Additionally, understanding the molecular interactions and structural determinants that govern DHFR binding will aid in the design of more effective inhibitors with improved specificity and reduced side effects. Overall, the investigation of DHFR inhibition by salicylates represents a promising area of research with significant implications for cancer therapeutics [8].

Naproxen possesses pharmacological properties attributed to its ability to inhibit cyclooxygenase enzymes. However, its carboxylic acid moiety presents an opportunity for chemical modification to enhance its therapeutic effects. Considering that NSAIDs containing carboxylate groups have been reported to inhibit DHFR, the incorporation of a salicylate moiety into naproxen could offer synergistic pharmacological benefits [9]. By combining the anti-inflammatory properties of naproxen with the potential DHFR inhibitory effects of salicylates, naproxen salicylate derivatives may exhibit enhanced efficacy in modulating inflammatory pathways while concurrently disrupting folate metabolism [10]. This dual mechanism of action could provide a rationale for the development of novel NSAID formulations with improved therapeutic profiles, offering broader applications in the treatment of inflammatory conditions and potentially extending to diseases where folate metabolism plays a role, such as cancer [11]. Further exploration of the structure-activity relationships and pharmacological properties of naproxen salicylate derivatives is warranted to elucidate their potential clinical utility and optimize their therapeutic efficacy.

2. Methods

2.1. Synthesis of Naproxen-Salicylate Derivatives

Naproxen (2.0 g, 8.7 mmol, Sigma-Aldrich, purity ≥ 98%) and salicylic acid (1.7 g, 12.4 mmol, Sigma-Aldrich, purity ≥ 99%) were utilized as starting materials for the synthesis of naproxen salicylate derivatives. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl, 3.1 g, 16.2 mmol, Sigma-Aldrich,

purity ≥ 99%) and 4-dimethylaminopyridine (DMAP, 0.5 g, 4.1 mmol, Sigma-Aldrich, purity ≥ 99%) served as coupling reagents (Figure 1). Dichloromethane (DCM, 50 mL, Fisher Chemicals, purity ≥ 99.8%) was employed as the solvent. In a round-bottom flask with a magnetic stir bar, naproxen and salicylic acid were dissolved in dry DCM. EDC·HCl and DMAP were added to the reaction mixture as coupling reagents. The reaction proceeds through the activation of the carboxylic acid group of naproxen by EDC·HCl to form an O-acylisourea intermediate. This intermediate then undergoes nucleophilic attack by the hydroxyl group of salicylic acid, facilitated by DMAP as a catalyst, to form the desired ester linkage. The resulting mixture was stirred at room temperature under a nitrogen atmosphere for 24 hours until TLC analysis indicated completion of the reaction. Upon completion, the reaction mixture was filtered to remove the dicyclohexylurea byproduct, and the filtrate was concentrated under reduced pressure. The crude product was purified by column chromatography using a silica gel column and eluted with a gradient of hexane/ethyl acetate (3:1) to yield the naproxen salicylate as a white solid (2.3 g, yield 76%). Quenching with saturated sodium bicarbonate solution, extraction with DCM, and purification of the organic layer yielded the desired naproxen salicylate derivative as a white solid (2.1 g, yield 70%). Characterization of the synthesized compounds was performed using IR spectroscopy (Figure 2).

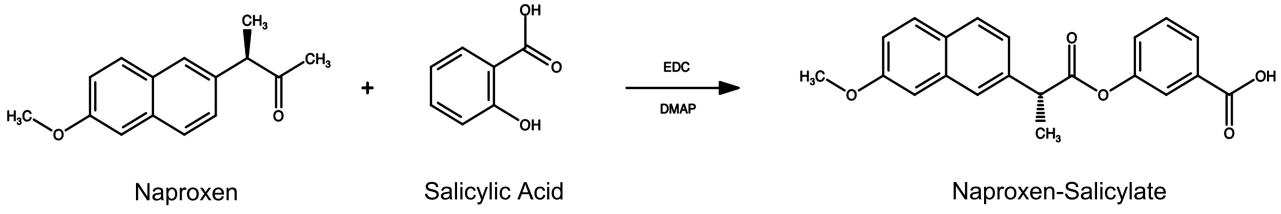

Figure 1. Reaction scheme.

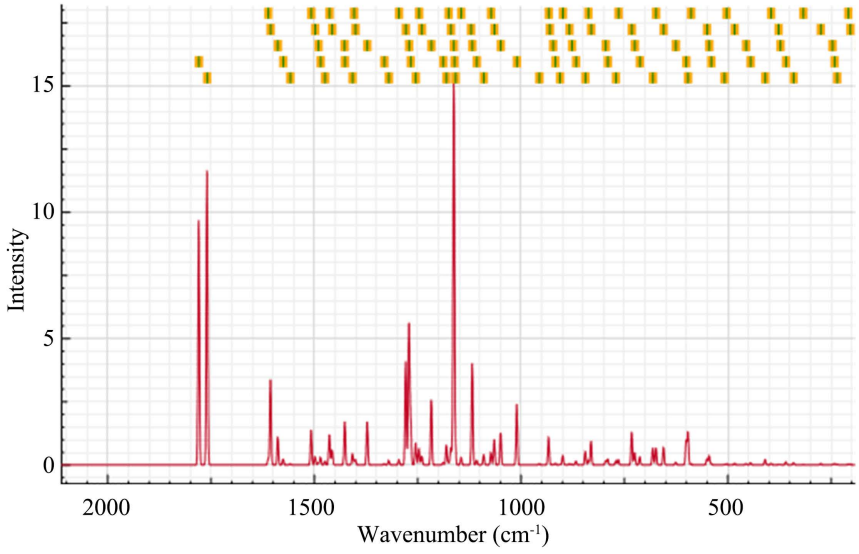

Figure 2. IR spectrum of Naproxen-Salicylate derivative.

IR spectrum analysis of the synthesized naproxen salicylate molecule reveals several characteristic absorption peaks. A strong absorption peak is seen around 1700 cm^{-1}, corresponding to the carbonyl group (C=O) stretching in the salicylate group [12]. Medium peaks in the region of 1200 - 1500 cm^{-1} are seen, corresponding to C-C and C=C stretching vibrations within the aromatic rings of naproxen and salicylate. The C-O linkage for the ester linkage between the two moieties is seen at about 1240 cm^{-1}. Peaks around 1000 - 1300 cm^{-1} represent C-O stretching vibrations present in both ester and alcohol functional groups. Finally, a complex set of peaks is found below 1000 cm^{-1} forming the fingerprint region. Overall, the IR spectrum aligns with the expected functional groups present in the naproxen salicylate, confirming its successful synthesis [13].

2.2. Growth and Expression of DHFR

Equipment for the growth and purification of DHFR-producing *E. coli* was obtained from Bio-Rad (Hercules, CA, USA). The pET21a plasmid, containing the T7 promoter, is used with BL21(DE3) *E. coli*, where induction with lactose or IPTG triggers T7 RNA polymerase expression and subsequent gene expression. Similarly, the pDHFR plasmid system in BL21(DE3) involves tight regulation via the lac repressor protein, allowing controlled transcription of the inserted GST-DHFR-His gene upon induction with lactose or its analog. The lyophilized BL21(DE3) *E. coli* containing the pDHFR plasmid was rehydrated and plated on selective Luria-Bertani (LB) agar plates with 50 μg/mL ampicillin. The plates were placed at 37˚C overnight, until colonies of bacteria were present. A single colony was taken from the dish and 11 mL of LB/amp broth was inoculated. The broth also contains 1% glucose to maintain repression of the lac operon, preventing the expression of T7 RNA polymerase or GST-DHFR-His. This was incubated overnight until mid-log phase until OD600 of 0.6. This was further subcultured into 50 mL of LB broth and the expression of the recombinant protein was induced at this stage by the addition of 115 μL of 100 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG) to the medium. The 50 mL culture was left 24 hours at 37˚C. The cells were then ready to be lysed to release the DHFR protein. To pellet the induced cells, two microcentrifuge tubes were filled with 2 ml of induced cell culture each. Centrifugation at 16,000 ×g for 2 minutes was performed, followed by removal of the supernatant. For lysing induced cells, 250 μl of lysis buffer (20 mM sodium phosphate, 300 mM NaCl and 0.5 mg/ml lysozyme) was added to each of the cell pellets and resuspended. The tube was then subjected to freeze-thaw cycles by placing it at −20˚C for 24 hours, followed by complete thawing, and repeated two more times. After the final freeze-thaw cycle, 500 μl of imidazole solution (38 ml dH_2O, 10 ml 10× PBS, 2 ml Imidazole) was added to the tube, and thorough mixing was performed. The soluble and insoluble fractions were then separated by centrifugation at 16,000 ×g for 20 minutes. The supernatant containing the soluble fraction was carefully transferred to a clean microcentrifuge tube.

2.3. Chromatographic Purification of DHFR

To prepare the chromatography setup, 200 µl of profinity IMAC Ni-charged resin slurry was added to a Micro Bio-Spin column. The IMAC resin selectively binds polyhistidine-tagged proteins while all other biomolecules will flow through the column. This was followed by centrifugation at 1000 ×g for 2 minutes to remove the packing. The column was washed with 200 µl of distilled water and then with 500 µl of equilibration buffer, the buffer contains NaCl that prevents the non-specific binding of charged molecules in the *E. coli* lysate soluble fraction from binding to the column. The imidazole has a similar structure to histidine which prevents non-specific binding of any *E. coli* proteins which contain multiple histidine residues, but the imidazole is not at a high enough level to prevent the binding of the polyhistidine tag on the GST-DHFR-His to the Ni-IMAC beads. The washed column was then used for sample binding by adding 600 µl of the soluble lysate fraction, and mixing for 20 minutes. The flowthrough fraction was collected by centrifugation, and the column was washed with wash buffer. After washing, the eluate fraction was collected by adding elution buffer to the column and centrifuging. The eluate was desalted by addition to a Bio-Gel P-6 column with a fractionation range of 1000 to 6000 Da, centrifuging, and collecting the desalted eluate. GST-DHFR-HIS has a molecular weight of 52 KDa and will be excluded, and any remaining salts or small proteins will be fractionated. Approximately 500 µl of desalted eluate was obtained.

2.4. Quantification of DHFR

The concentration of GST-DHFR-His in the desalted eluate fraction was determined using a spectrophotometric method based on the Beer-Lambert law. The extinction coefficient (ε) of the entire GST-DHFR-His construct is 75,540 $M^{-1}\cdot cm^{-1}$. Rearrangement of the Beer-Lambert Law results in Equation (1) to calculate the molarity of DHFR. The molarity of DHFR was converted to a concentration in mg/mL using the molar mass in Equation (2). 150 µl desalted eluates were quantified and the concentration of DHFR was found to be 0.92 mg/mL.

$$[\mathrm{DHFR}](\mathrm{M}) = \frac{\text{Absorbance at 280 nm}}{75540} \tag{1}$$

$$[\mathrm{DHFR}](\mathrm{mg/mL}) = [\mathrm{DHFR}](\mathrm{M}) \times 50361\,\mathrm{g/mol} \tag{2}$$

2.5. Polyacrylamide Gel Electrophoresis (PAGE)

To examine the purity of purified DHFR, 50 µl of Laemmli sample buffer was mixed with 50 µl of protein sample and heated at 95˚C for five minutes. SDS-PAGE was performed using a 4% - 20% TGX gel in a Mini-PROTEAN system. Samples ran at 200 V for 35 minutes. Proteins were visualized by Coomassie Brilliant Blue staining. The GST-DHFR-His protein runs on SDS-PAGE gels at an apparent MW of approximately 43 kDa, despite the actual protein molecular weight of 52 kDa [14]. The PAGE conducted in this study (**Figure 3**) is consistent with this result.

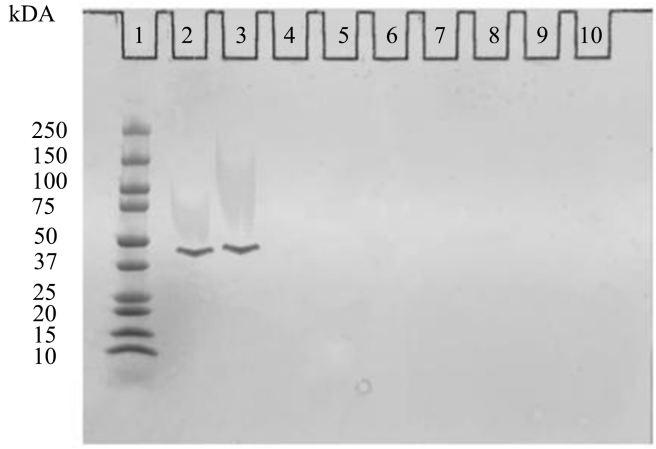

Figure 3. PAGE of DHFR.

2.6. DHFR Activity Assay

To assess the enzymatic activity of DHFR, an assay was conducted employing a spectrophotometric method. NADPH is the cofactor of DHFR, and is reduced to NADP+ in an oxidation-reduction reaction by DHFR (**Figure 4**). For every molecule of NADPH that is converted to NADP+ by DHFR, one molecule of THF is produced from the substrate DHF. By measuring the rate at which NADPH decreases, the activity of DHFR could be calculated. This would be done by measuring the change in absorbance of NADPH at 340 nm.

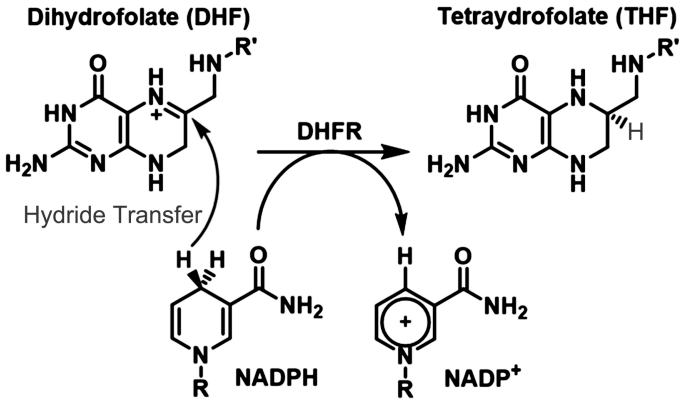

Figure 4. DHF to THF conversion by DHFR.

An ultra-micro cuvette containing 985 µl of 1× PBS was blanked at 340 nm to establish a baseline absorbance reading of 0.000. Subsequently, the enzyme sample consisting of GST-DHFR-His and its cofactor, NADPH, was prepared by adding 6 µl of 10 mM NADPH to the cuvette, followed by the 15 µl of the enzyme sample. Since none of the DHF substrates was present, the absorbance at 340 nm should not change because NADPH is not being used and this would provide us with our control values. After 5 minutes, the enzymatic reaction was initiated by adding 5 µl of 10 mM DHF to the cuvette. Measurements at 340 nm were again taken over 5 minutes. This would provide the baseline activity for

DHFR with no inhibition. The same process was repeated with the naproxen-DHFR and naproxen-salicylate-DHFR complexes. Each assay would generate a graph of the absorbance at 340 nm over 5 minutes (Figure 5). This data could be used to calculate the change in optical density (ΔOD) for each reaction. The change in the optical density would be used to calculate the change in concentration of NADPH (ΔC). ΔC is the activity of the DHFR in terms of how many mol/min of NADPH it can convert to NADP+ per ml of reaction volume. This would be determined from Equation (3), a form of Beer's Law that relates absorbance values to concentration values shown below. The activity of DHFR was fitted to Michaelis-Menten kinetics where ΔC was used as the velocity of the reaction. The kinetics of DHFR were used to create Lineweaver-Burk and Dixon plots (Figures 6-8).

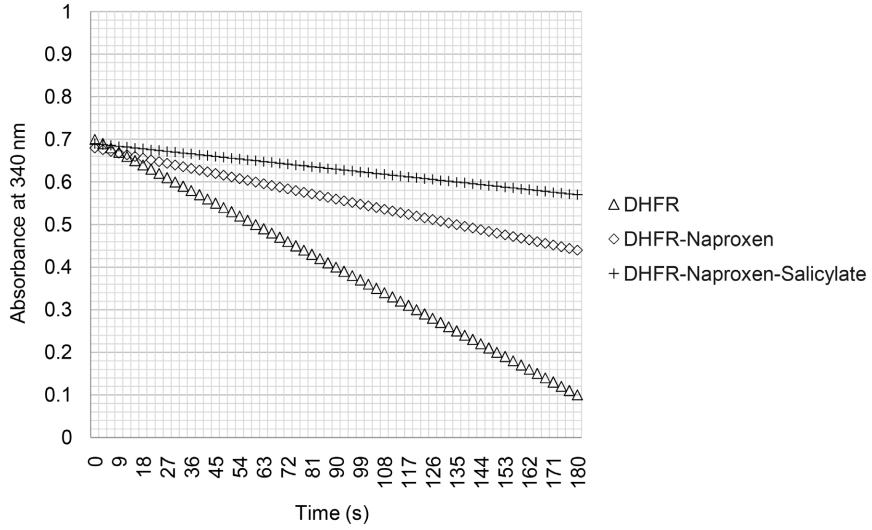

Figure 5. Enzyme activity assay, absorbance of NADPH over time.

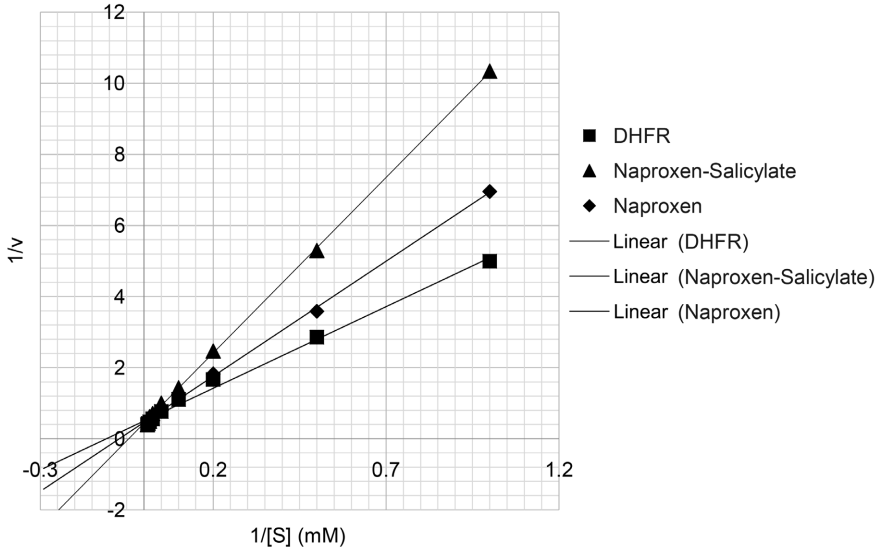

Figure 6. Lineweaver-Burk plot.

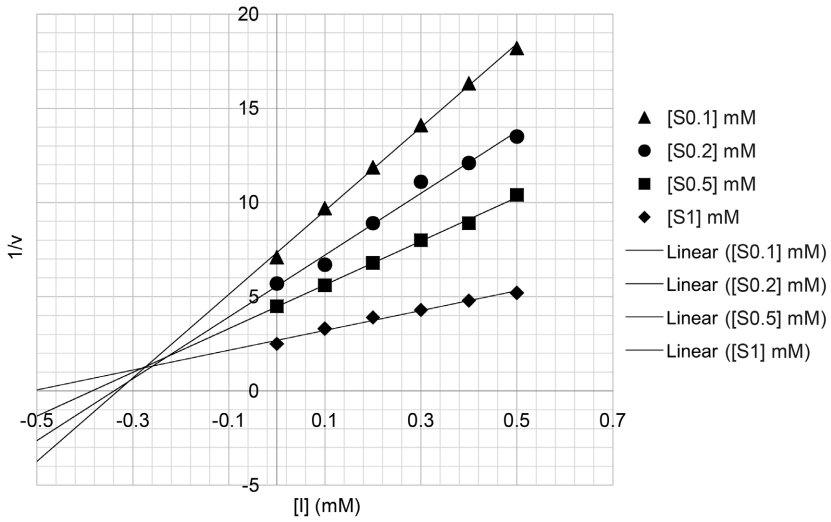

Figure 7. Dixon plot for Naproxen.

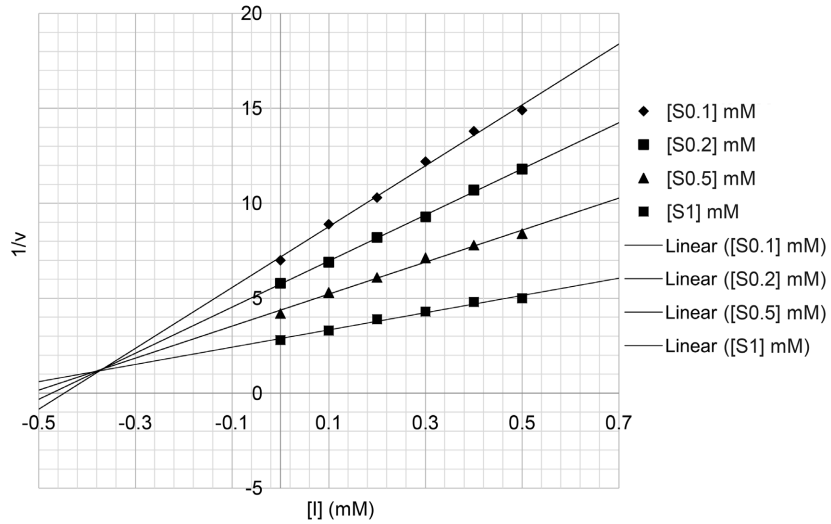

Figure 8. Dixon plot for Naproxen-Salicylate.

$$\Delta OD_{\text{Of Reaction}} = \frac{A_{300} - A_0}{300} * 60 \tag{3}$$

where A_{300} and A_0 represent absorbance values at 0 seconds and 300 seconds:

$$\Delta OD_{\text{Total}} = \left|\Delta OD_{\text{Of Reaction}}\right| - \left|\Delta OD_{\text{Control}}\right|$$

$$\Delta C = \Delta\left[\text{NADPH}\right]$$

where ΔC is given in $\frac{\text{mol/L}}{\text{min}}$,

$$\Delta C = \frac{\Delta OD_{\text{Total}}}{\varepsilon * I}$$

$$\varepsilon\left(\text{extinction coefficient}\right) = 6220\ \text{M}^{-1} \cdot \text{cm}^{-1} \text{ for NADPH}$$

$$I\left(\text{path length}\right) = 1\ \text{cm}\ .$$

2.7. Quantitative ELISA for THF

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted to measure the concentration of THF using a polyclonal antibody specific to THF (Cloud-Clone, PAG411Ge01). Firstly, the ultra-micro cuvette from the activity assay was coated with the polyclonal antibody diluted in Tris-buffered saline (TBS) with 0.1% Tween 20 and incubated at room temperature for 2 hours to facilitate antibody binding. After washing to remove unbound antibodies, the microplates were blocked with 1% bovine serum albumin in 1× PBS, to prevent non-specific binding. Subsequently, samples containing THF were added to the wells and allowed to incubate, enabling the binding of THF to the immobilized polyclonal antibodies. Following another washing step to remove unbound THF and other contaminants, a secondary antibody conjugated to HRP was added to the wells. This secondary antibody is specifically bound to the primary polyclonal antibody-THF complex. After washing to remove excess secondary antibodies, TMB (3, 3', 5, 5'-tetramethylbenzidine) was added to initiate the enzymatic reaction catalyzed by the bound enzyme. The reaction was halted by adding 0.16 M H_2SO_4, and the absorbance of each well was measured spectrophotometrically at 450 nm. Standard curves were generated using known concentrations of THF to determine the concentration of THF in the samples. The stoichiometry of the reaction in the activity assay means that the concentration of THF can be no more than the DHF (limiting reagent), at a concentration of 0.022 mg/mL. The standard curve was generated in increments of 5 μg/mL (Figure 9) and the results were used to characterize the degree of DHFR inhibition by the synthesized salicylate derivatives of naproxen.

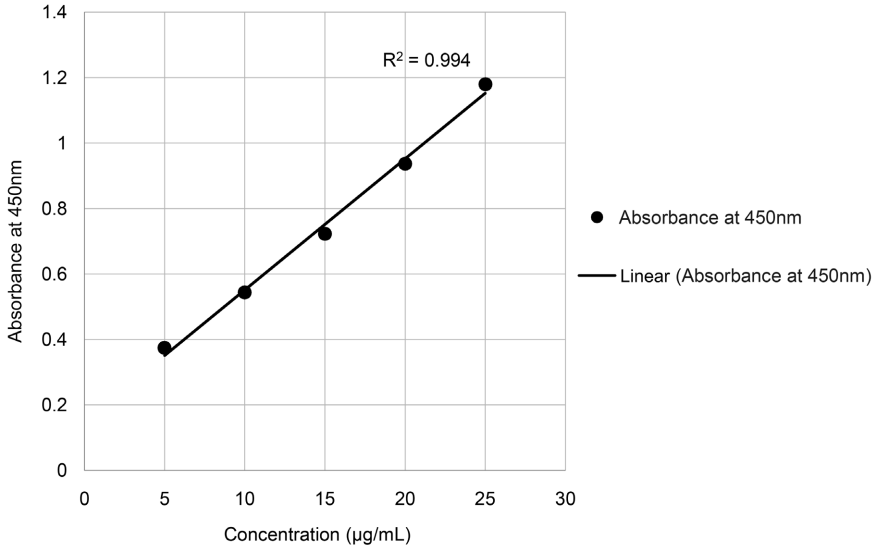

Figure 9. ELISA standard curve.

3. Data

See Tables 1-3 and Figures 5-9.

Table 1. Enzyme activity values for DHFR and Naproxen.

Tested Enzyme	ΔOD	ΔC
DHFR	$\Delta OD = 0.6$	$\Delta C = 3.21 * 10^{-4}$
DHFR-Naproxen	$\Delta OD = 0.24$	$\Delta C = 1.28 * 10^{-5}$
DHFR-Naproxen-Salicylate	$\Delta OD = 0.12$	$\Delta C = 0.64 * 10^{-5}$

Table 2. Inhibition constant values.

Tested Enzyme	Ki Value
DHFR-Naproxen	3.87
DHFR-Naproxen-Salicylate	2.59

Table 3. THF produced from standard curve.

Tested Enzyme	THF Present (μg/mL)
DHFR	20.97
DHFR-Naproxen	15.68
DHFR-Naproxen-Salicylate	10.48

4. Results and Discussion

4.1. Analysis

The enzymatic activity of DHFR was evaluated in the presence of naproxen and naproxen-salicylate derivatives. Our findings demonstrate a reduction in enzyme activity upon binding with both compounds. The decrease in ΔOD suggests an inhibition of DHFR activity by naproxen and its salicylate derivative. Furthermore, we quantified the inhibitory potency of the synthesized compounds by determining the enzyme activity (ΔC) and performing Michaelis-Menten kinetics studies through Lineweaver-Burk and Dixon plots (**Figures 5-7**). The decrease in ΔC observed with both naproxen and naproxen-salicylate means that the DHFR-naproxen complexes can convert a lower concentration of NADPH to NADP+ compared to free DHFR, demonstrating their inhibitory activity (**Table 1**). Analysis of the Linewaver-Burk plots demonstrates intersections of the y-axis for both inhibitors, indicating competitive inhibition by both NSAIDs with DHF. The competitive binding characteristics indicate that inhibition occurs through direct interference with substrate binding. Dixon plots were used to calculate the inhibition constants (K_i values) through the intersection of reciprocal initial reaction rates at varying inhibitor concentrations. The calculated K_i values further establish the inhibitory action of naproxen-salicylate. The K_i values for naproxen-DHFR and naproxen-salicylate-DHFR were determined as 3.87 and 2.59, respectively (**Table 2**). This indicates a more potent inhibition of DHFR activity by the naproxen-salicylate derivative compared to naproxen alone.

The ELISA performed in this study examined the concentration of THF and its correlation with the inhibition of DHFR activity by the synthesized salicylate derivatives of naproxen. Standard curves generated using known concentrations of THF enabled the quantification of THF in the samples, where analyzing the absorbance values at 450 nm allowed for determination of THF present in the samples. These results were then utilized to characterize the extent of DHFR inhibition by the naproxen-salicylate derivatives. The ELISA data revealed a dose-dependent decrease in THF concentration with increasing concentrations of the naproxen-salicylate derivatives, indicating their inhibitory effect on DHFR activity (**Table 3**). This inhibition suggests the potential of these derivatives as promising candidates for modulating folate metabolism pathways and subsequently influencing cell proliferation, offering novel therapeutic opportunities for managing inflammatory processes and potentially targeting cancer progression. Further analysis and optimization of these derivatives could enhance their efficacy as dual-targeted inhibitors with significant clinical implications.

4.2. Implications for Drug Development

The observed decrease in enzymatic activity of DHFR upon binding with naproxen highlights the potential of NSAIDs to interfere with folate metabolism. This finding signifies the need for a deeper understanding of the structural interactions between NSAIDs and target enzymes like DHFR in drug development. By elucidating the molecular mechanisms underlying NSAID-mediated inhibition of DHFR activity, we can inform the design of more potent and specific therapies while minimizing potential side effects associated with folate metabolism disruption [15].

The enhanced inhibition observed with the DHFR-naproxen-salicylate complex suggests that the presence of salicylate groups can augment the inhibitory effect of NSAIDs on DHFR activity. This indicates a potential synergistic relationship between naproxen and salicylate derivatives, offering opportunities for combination therapy or the development of novel NSAID-based formulations with enhanced efficacy in modulating inflammatory processes and potentially targeting cancer progression [16]. This knowledge can guide the rational design of NSAIDs with improved pharmacokinetic and pharmacodynamic properties, enhancing their therapeutic potential while minimizing off-target effects.

Moreover, understanding the structural basis of NSAID-DHFR interactions may facilitate the development of selective inhibitors that specifically target inflammatory pathways while sparing essential folate metabolism processes [17]. Such inhibitors could offer significant therapeutic advantages, particularly in conditions characterized by chronic inflammation and dysregulated cell proliferation, such as autoimmune diseases and certain cancers [18]. The structural and functional characteristics of NSAID interactions with DHFR can pave the way for the design of next-generation NSAIDs with improved efficacy, safety, and specificity. These efforts hold promise for the development of innovative thera-

pies that effectively modulate inflammatory processes while minimizing the risk of adverse effects associated with folate metabolism disruption.

5. Conclusions

The findings presented offer significant insights into the mechanistic connections between folate metabolism and inflammatory processes, shedding light on the pharmacological actions of salicylate groups within NSAIDs. Folate metabolism plays a pivotal role in chronic inflammatory conditions, and drugs targeting folate-metabolizing enzymes, such as methotrexate and sulfasalazine, are commonly used in the treatment of arthritis. The current research demonstrates that NSAIDs containing salicylate groups act as competitive inhibitors of DHF by binding to the substrate binding site of dihydrofolate reductase DHFR. The salicylate group mimics the flexible pABG moiety by adopting a conformation and positioning within the binding site that resembles how pABG connects Glu to the p-aminobenzoyl ring. This structural relationship highlights salicylate continuing NSAIDs as having potential anti-folate characteristics, with pharmacokinetic characteristics that result in sustained plasma levels capable of significantly interfering with folic acid metabolism.

The ability of naproxen and its salicylate derivative to effectively inhibit DHFR highlights their potential as lead compounds for the development of novel therapeutic agents targeting folate metabolism-related diseases, including cancer and inflammatory disorders. Furthermore, elucidating the structural basis of their inhibitory effects provides valuable insights for rational drug design, facilitating the development of more potent and selective inhibitors with improved efficacy. This research opens promising avenues for the design of next-generation NSAIDs with enhanced therapeutic profiles, offering new opportunities for addressing unmet medical needs in the treatment of various diseases associated with folate metabolism.

Acknowledgments

Words cannot express our gratitude towards Dr. Meri Blackburn and Mr. John Uesseler for their invaluable contributions to this research. While it is often said that the highest academic honor is being listed as an author on a paper, we feel compelled to emphasize the profound honor it has been to collaborate with these esteemed individuals. Dr. Blackburn and Mr. Uesseler have exemplified unparalleled dedication, expertise, and mentorship throughout the course of this study. Their unwavering support and guidance have been instrumental in shaping the direction of our research. Their insights, wisdom, and encouragement have consistently driven us forward, inspiring us to strive for excellence in our endeavors. Although these individuals may humbly downplay their role in this work, their contributions are immeasurable. It is through their mentorship that we have been able to overcome challenges, expand our knowledge, and ultimately, achieve meaningful outcomes. Their impact on this research and our

academic journey is profound and enduring, and we are truly grateful for the privilege of working alongside them.

The first author wishes to express a sincere thanks to Ms. Emily Gunderson, whose remarkable qualities as both an educator and a scientist have contributed profoundly to this research. Ms. Gunderson's dedication, expertise, and unwavering support have been instrumental in shaping the direction of this work. Beyond her role as a teacher, she has become a cherished friend, and the opportunity to collaborate with her has been a true privilege.

The authors would like to extend their heartfelt thanks to a number of individuals who have contributed significantly to this work. The authors extend their appreciation to Ms. Anna Crowe and Ms. Lisa Cauthen, healthcare science educators whose guidance and passion were invaluable. The authors are sincerely grateful for their unwavering support and are thankful for the opportunity to work with them. Additionally, the authors would like to thank Dr. Andrew Fabich for his guidance and insight, which has played a crucial role in shaping the direction and scope of this work. A special thanks goes out to Ms. Christina Curtis whose unwavering support and guidance have been instrumental in every step of this journey. Her dedication to our success and willingness to go above and beyond have truly made a difference and have greatly contributed to this work. Finally, the authors also wish to express their gratitude to Mr. Steven Bowles, a dedicated educator whose support has been invaluable throughout this endeavor. The authors deeply appreciate Mr. Bowles' guidance and encouragement, which has contributed significantly to their academic and personal growth.

Lastly, the authors would like to thank the Empower College and Career Center and the Jackson County School System for their unwavering support and belief in the students and the significance of their work. The authors are profoundly grateful for the opportunities provided and the encouragement extended, which have empowered students to pursue research and contribute to the advancement of knowledge.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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