

Coumarins with Xanthine Oxidase Inhibiting and Radical Scavenging Properties: Tools to Combat Oxidative Stress in Cells

E. Hofmann, J. Webster, T. Kidd, R. Kline, M. Jayasinghe, and S. Paula

Abstract—Certain representatives from the large natural compound class known as coumarins are known to inhibit the enzyme xanthine oxidase (XO) and are capable of absorbing reactive oxygen species (ROS) produced by XO and other enzymes. These dual properties make coumarins a promising scaffold for the development of agents against reperfusion injuries, which are caused to a large extent by ROS and occur once blood circulation has been restored after ischemic events. A selection of eighteen coumarins was tested for XO inhibition and radical scavenging activities in cell-free assays. The most effective XO inhibitors carried a hydroxyl group in the C7 position of the coumarin scaffold whereas the best radical scavengers were coumarins with two hydroxyl groups in neighboring positions at the phenyl ring. Molecular docking confirmed the essential role of hydroxyl groups for effective enzyme/inhibitor interactions. The coumarins were further investigated in cell-based assays that determined their ability to reduce oxidative stress. As anticipated, the *in vivo* test results showed that the most effective compounds were those that were both potent XO inhibitors and good radical scavengers, thereby illustrating the potential of coumarins with dual activities for future development.

Index Terms—Reperfusion injuries, hyperuricemia, enzyme inhibition, oxidative stress.

I. INTRODUCTION

For decades, inhibitors of the enzyme xanthine oxidase (XO) have been of therapeutic value for the treatment of hyperuricemia, a condition that can cause cardiovascular and chronic kidney disease as well as gout [1], [2]. The latter is caused by high levels of uric acid in the blood, which leads to the deposition of crystals in joints that can trigger inflammatory arthritis [3]. Therapeutically used XO inhibitors suppress the production of uric acid, which allows for the renal excretion of uric acid precursors such as xanthine or hypoxanthine. The prototype XO inhibitor allopurinol, a purine analog, has been in use since 1966 [1]. Although efficacious, the relatively low potency of allopurinol requires dosages that can cause unwanted side effects that range from mild gastrointestinal upset to more severe hypersensitivity

reactions and renal toxicity [4]-[6]. As a result, the development of alternative XO inhibitors with novel chemical structures is an active field of current research, as evident from the recent approval of the non-purine XO inhibitor febuxostat [7], which is significantly more potent than allopurinol and more effective in lowering uric acid levels in patients [8].

In addition to their traditional use against hyperuricemia, XO inhibitors have been proposed to be useful for the prevention of reperfusion injuries. This condition can occur after ischemic events or surgery once the blood circulation to oxygen-deprived tissue has been restored. The causes of reperfusion injuries are multi-faceted and several factors, such as inflammation or the production of reactive oxygen species (ROS) like the superoxide anion, are believed to be involved [9]-[11]. Whereas the inflammatory component of reperfusion injuries can be counteracted by immunosuppressants, there is currently no pharmacological treatment for the damaging effects of ROS. In order to gain access to agents that could serve in this capacity, the development of compounds with dual properties that are capable of preventing the generation of ROS and of absorbing ROS has been proposed [12], [13]. Since a major source of ROS is radical production by XO, suitable compounds should be XO inhibitors. Moreover, they should be able to absorb ROS already generated by XO or other sources. Whereas these two properties have been investigated separately for diverse compound classes, few efforts have been made to date to combine them in a single molecule, thereby optimizing versatility and effectiveness [14].

Coumarins (Fig. 1) are natural products found in plants with a remarkable range of bioactivities, including the abilities to inhibit XO [15] and – unlike most other XO inhibitors including allopurinol and febuxostat – to act as radical scavengers and directly absorb harmful ROS already present or produced by enzymes other than XO [13]. Although the potential of coumarins as XO inhibitors has been appreciated for several decades [16], [17], no systematic efforts have been undertaken to date to develop these compounds into medicinally useful agents against hyperuricemia or the prevention of reperfusion injuries. Current structure-activity relationship (SAR) knowledge on XO inhibition is limited to two studies [13], [15] with relatively small selections of coumarins. Other studies focused in detail on the *in vitro* and *in vivo* properties of single compounds and are therefore not useful for the establishment of SARs [14], [18], which require information on an ensemble of molecules. The resultant lack of fundamental knowledge about the molecular determinants of coumarin-mediated XO

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inhibition and ROS absorption has thus far prevented this compound class from being developed into medicinally useful agents.

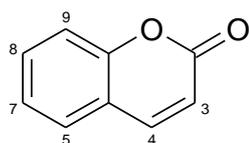


Fig. 1. The coumarin scaffold.

In this study, we evaluated the inhibitory potencies and the radical scavenging abilities of a selection of eighteen coumarins. The compounds varied in the substituents attached in positions 3-9 at the coumarin scaffold (Fig. 1). For the visualization of the XO/inhibitor interactions at the molecular level, we performed molecular docking with a previously published X-ray crystal structure of the enzyme. Lastly, the coumarins were evaluated in a cell-based viability assay that quantified a compound's ability to reduce oxidative stress in neurons, an effect believed to be the combined result of XO inhibition and ROS absorption.

II. MATERIALS AND METHODS

A. Materials

Coumarin derivatives listed in Table I and Fig. 2 were purchased from the following vendors: **1**, **4**, **5**, **7**, **14**, and **15** were from Sigma Aldrich (St. Louis, MO); **2**, **3**, **6**, **12**, and **13** were from Fisher Scientific (Pittsburg, PA); and **8-11** and **16-18** were from Indofine (Hillsborough, NJ). Bovine XO, xanthine, potassium phosphate, and the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical were received from Sigma Aldrich. Dimethylformamide (DMF) and phosphate-buffered saline (PBS) were purchased from Fisher Scientific. Murine Neuro-2A cells were received from Eton Bioscience (San Diego, CA) whereas growth medium, serum, trypsin, and antibiotics were from Atlanta Biologicals (Atlanta, GA). β -amyloid peptide (25-35) was from Abbtotec (San Diego, CA).

B. Determination of Inhibitory Potencies against XO Activity

The rates of XO-catalyzed conversion of xanthine to uric acid were measured spectroscopically by monitoring the concomitant absorbance changes at 295 nm for several minutes [2]. Commercially available bovine XO (140 units/ μ g) was suspended in phosphate buffer (20 mM phosphate, pH 7.5) and the reaction was initiated by mixing the enzyme buffer (final concentrations: 39 μ g/mL XO) with xanthine (final concentration: 46 μ M) dissolved in the same buffer. Experiments were conducted in the absence and presence of potential inhibitors at 11 different concentrations. 200 μ L samples were placed in 96-well plastic plates capable of transmitting UV light. The absorbance changes over time in the wells were measured with a plate reader (SpectraMax 190, Molecular Devices, Sunnyvale, CA) and reaction rates were obtained by linear regression of the absorbance versus time traces. Rates measured at varying inhibitor concentration were fit to a three-parameter logistic equation and inhibitory

potencies were expressed as IC₅₀ values [19], the inhibitory concentration that reduced XO activity by 50%.

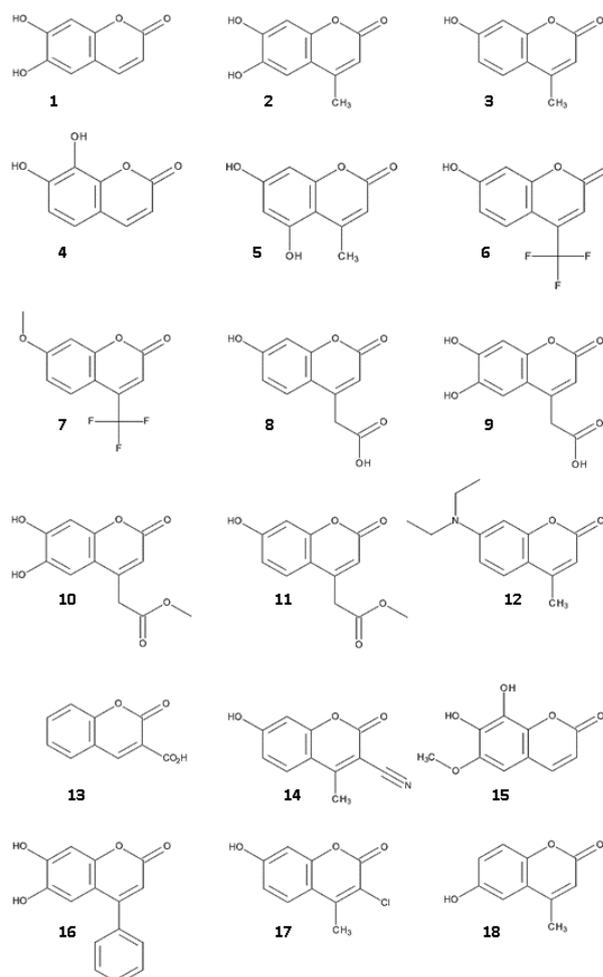


Fig. 2. Chemical structures and ID numbers of coumarins tested in this study.

C. Prediction of Inhibitor Binding Poses by Computational Ligand Docking

Docking of esculetin (**1**) was performed with the program GOLD (version 5.1; Cambridge Crystallographic Data Centre, UK) [20], [21] and the X-ray crystal structure of the XO/lumazine complex (Protein Data Bank entry 3ETR) [22]. The protein structure was prepared in GOLD for docking by adding hydrogen atoms and deleting all water molecules and lumazine. All other non-protein entries were kept since some of them were prosthetic groups that were part of the active site. The scoring function used for docking was ChemScore [23], [24] and the genetic algorithm of GOLD was executed at the default settings, performing 30 independent and identical repeats. The docking sphere had a radius of 10 Å, which was centered in the middle of the bond connecting the two central carbon atoms of the (deleted) lumazine molecule. The structure of esculetin was modeled in MOE (version 2012.10; Chemical Computing Group, Montreal, Canada) and energy-minimized with the MMFF94s force field. For visualization, docking results were imported into MOE.

D. Measurement of Radical Scavenging Activity

The ability of compounds to scavenge radicals was determined by mixing 50 μ L of a freshly prepared solution of

the radical DPPH in ethanol (0.2 mM) with 150 μ L of the test compound in ethanol at several concentrations ranging from 5 to 50 μ M [25]. Samples were incubated for 30 minutes at room temperature in the dark in a 96-well plate. Potential scavenging of the DPPH radical was apparent by the disappearance of the DPPH absorbance, which was measured at a wavelength of 517 nm with a plate reader.

E. Assessment of Ability to Protect Cells from Oxidative Stress

The ability of coumarins to rescue cells from oxidative stress was measured according to an established protocol using neuroblastoma (Neuro-2A) cells that were subjected to oxidative stress by exposure to β -amyloid peptide [12]. Cells were grown according to the supplier's guidelines in Dulbecco's minimum essential medium complemented with 10% of fetal bovine serum and 100 units/mL penicillin-streptomycin at 37 $^{\circ}$ C in an atmosphere of 95% air and 5% carbon dioxide. For seeding, cells were washed with PBS, treated with trypsin for no longer than one minute, and then placed in 100 μ L aliquots in a 96-well plate at a density of 2,000 - 4,000 cells/mL. After 6 hours of incubation, a 10 μ L aliquot of test compound (250 μ M), 15 μ L of β -amyloid peptide (833 μ M), and 25 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) buffer (5mg/mL in PBS) were added to each well [26]. After six hours of incubation, 100 μ L of extraction buffer (prepared by addition of 2.5% v/v of a mixture composed of four parts of acetic acid and one part of 20% w/v SDS prepared in a 50:50 mix of DMF and water, pH 4.7) was added. After another six hours on an incubating shaker (25 rpm) in the dark at 37 $^{\circ}$ C, the absorbances of the samples were determined at 570 nm with a microplate reader.

III. RESULTS AND DISCUSSION

A. Structure-Activity Relationships of Inhibitory Potencies of Coumarins against XO Activity

Of the 18 coumarins shown in Fig. 2, 13 displayed measurable inhibition of XO activity with IC₅₀ values ranging between 7.5 μ M and 2 mM (Table I). Fig. 3 illustrates the good quality of the results by showing representative activity assays for two active compounds performed at varying inhibitor concentrations along with the curve fits.

Inspection of the inhibitory potencies listed in Table I identified **1** (esculetin) as the most potent inhibitor by a large margin, which was in agreement with earlier reports [13], [15]. The results also highlighted the importance of a hydroxyl group at C7. Almost every active coumarin in Table I displayed this feature and comparisons of **2** with **18**, **6** with **7**, and **3** with **12** showed a drastic drop in or disappearance of potency if the hydroxyl group at C7 was removed or chemically altered. In contrast, the presence of a hydroxyl group in position 6 – even though beneficial for potency – appeared to be of lesser importance, as can be seen by the rather modest decline in potency upon its removal (**2** versus **3**) or modification (**4** versus **15**). The placement of bulky, non-polar trifluoromethyl or phenyl groups at C4 was in general detrimental to potency, as evident from the lower

potencies of **2** and **16** relative to **1**. Similarly, the presence of large polar groups (acetyl or acetyl methyl ester groups) at C4 resulted in poor inhibitors or inactive compounds, as could be seen for compounds **8–11**. The introduction of a cyano group or a chlorine atom at C3 (**14** and **17**) did not induce a significant potency change in comparison to parent compound **3**, suggesting that this part of the inhibitor did not interact with the enzyme or might have been solvent exposed. The potencies of **4** and **5** indicated that moving the hydroxyl group at C6 to the C8 or C5 positions resulted in reduced potencies compared to parent compounds **1** and **2**.

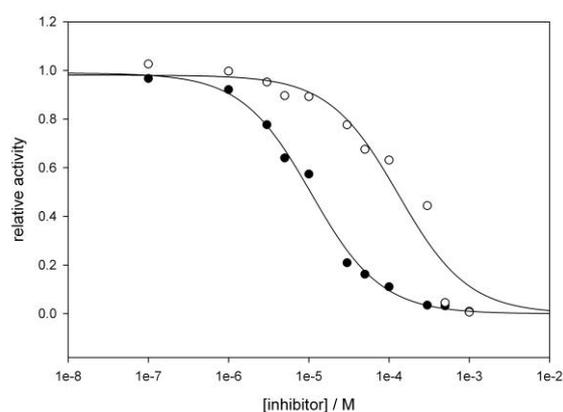


Fig. 3. Representative XO activity inhibition assay for compounds **1** (●) and **16** (○). The lines represent curve fits to the data points.

TABLE I: COUMARINS' INHIBITORY POTENCIES, RADICAL SCAVENGING CAPABILITIES, AND ABILITIES TO PROTECT CELLS FROM OXIDATIVE STRESS. IC₅₀ VALUES, RADICAL SCAVENGING PERCENTAGES, AND CELL VIABILITY PERCENTAGES (AVERAGES OF AT LEAST THREE TRIALS WITH STANDARD DEVIATIONS) WERE CALCULATED AS DESCRIBED IN THE BODY OF THE MANUSCRIPT

compound #	IC ₅₀ / μ M	% radical scavenging	% cell viability
1	7.49 \pm 5.57	98 \pm 1	91 \pm 5
2	203 \pm 49	82 \pm 3	87 \pm 6
3	360 \pm 69	4.9 \pm 0.9	50 \pm 6
4	527 \pm 104	88 \pm 1	50 \pm 6
5	740 \pm 174	15 \pm 3	47 \pm 7
6	554 \pm 72	3.4 \pm 0.8	42 \pm 7
7	inactive	4.1 \pm 0.9	44 \pm 4
8	1070 \pm 70	8.7 \pm 1.8	40 \pm 2
9	inactive	89 \pm 1	75 \pm 6
10	inactive	90 \pm 2	76 \pm 10
11	809 \pm 38	7.3 \pm 1.0	41 \pm 6
12	inactive	9.3 \pm 0.3	33 \pm 4
13	inactive	5.3 \pm 2.0	49 \pm 6
14	322 \pm 19	3.2 \pm 1.3	43 \pm 5
15	1610 \pm 160	82 \pm 3	66 \pm 4
16	126 \pm 3	89 \pm 1	59 \pm 4
17	553 \pm 34	1.8 \pm 1.0	59 \pm 5
18	2010 \pm 310	1.0 \pm 0.9	44 \pm 5

B. Docking-Predicted XO/Inhibitor Interactions

Even though several high resolution X-ray crystal structures of bovine XO in complex with various small molecules have been published [22], [27]-[30], none of these structures contained a coumarin derivative, which leaves the molecular details of coumarin-binding to XO elusive. In the

absence of crystallographic information, computational docking can provide valuable information about critical enzyme/inhibitor interactions in the active site [31]. Among numerous commercial and academic docking routines, the program GOLD has ranked consistently under the top-performing packages and was therefore used to explore coumarin binding to XO in this study [21], [32].

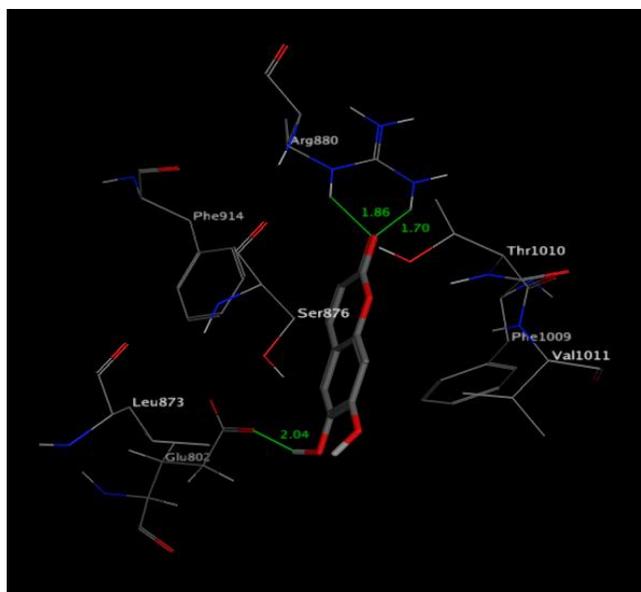


Fig. 4. Docking-predicted binding pose of esculletin (**1**) in the active site of XO. Hydrogen bonds are shown as green lines and distances are in Å.

Docking was limited to the compound esculletin (**1**), the most potent inhibitor among the coumarins tested in this study and thus presumably the one with the strongest enzyme/inhibitor interactions most reliably detected by GOLD. As the receptor, the crystal structure of XO in complex with lumazine was chosen from the sizeable number of published XO structures because of the structural resemblance of lumazine to the coumarins (two condensed six-membered rings) [22]. 30 docking runs with esculletin under identical conditions generated the same ligand pose, indicating excellent consensus and reproducibility. It is noteworthy that the esculletin binding pose was virtually identical to the one obtained by Lin and coworkers in an earlier study [13] using a different crystal structure (PDB code: 1FIQ) and another docking program (AutoDock).

Analysis of the interaction-specific energy contributions to the overall docking score and inspection of docking-predicted inhibitor poses showed that coumarin binding to XO was primarily driven by hydrogen bonding and hydrophobic interactions. In comparison, unfavorable energy terms arising from steric clashes and the loss of conformational freedom upon ligand binding were minor. The binding site accommodated esculletin snugly, which explains the inactivity of some coumarins carrying larger substituents which likely made the molecules too large to fit into the binding pocket. As depicted in Fig. 4, one hydrogen bond occurred between the hydroxyl group at esculletin's C6 and the side chain of Glu802, whereas a second, bifurcated one involved the ligand's carbonyl oxygen and the guanidino group of Arg880. While the docking results were in agreement with the experimentally determined general need for hydroxyl groups for good inhibitory activity, they did not reveal the important role of

esculetin's hydroxyl group at C7. Given that computational docking has imperfections and sometimes suffers from limitations such as restricted conformational flexibility of the active site, a high resolution X-ray crystal structure of XO in complex with a coumarin would undoubtedly be beneficial for the final elucidation of critical enzyme/inhibitor interactions at the molecular level.

C. Structure-Activity Relationships for Radical Scavenging by Coumarins

Only seven of the eighteen tested coumarin derivatives were capable of scavenging the radical DPPH to a noticeable degree. A typical result of the radical scavenging test is displayed in Fig. 5, which shows DPPH absorbance after 30 minutes of incubation at coumarin concentrations (compound **1**) up to 100 μ M. To enable a convenient comparison of compound activities, Table I lists only the radical scavenging activity observed at 25 μ M coumarin concentration, calculated from the absorbance values as follows (the control was a sample containing no radical scavenger):

$$\% \text{ radical scavenging activity} = 100 - \left(\frac{A_{517}(\text{coumarin})}{A_{517}(\text{control})} \times 100 \right)$$

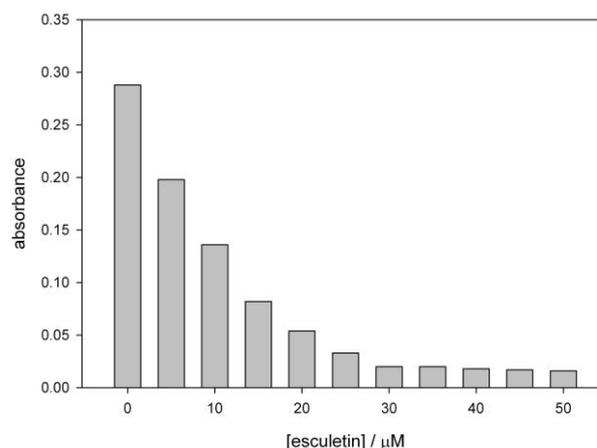


Fig. 5. Representative DPPH radical scavenging assay for **1**. The absorbance of the DPPH radical was measured at 517 nm.

Under the experimental conditions of this assay, the coumarins could be clearly divided into two groups with unique behavior. They either had activities of greater than 80% (**1**, **2**, **4**, **9**, **10**, **15**, and **16**) or were essentially inactive with observed activities below 10% (all other compounds). The obvious structural feature that all active compounds shared was the presence of two hydroxyl groups located at neighboring carbon atoms at the phenyl ring of the coumarin scaffold. This observation was in agreement with the results of a study on phenolic compounds that showed that two hydroxyl groups in *ortho* or *para* position of each other were optimal for radical scavenging activity [33]. Compounds **1-3** were also included in the study by Lin and coworkers and the reported findings agreed well with the ones reported here [13].

D. Protection of Cells from Oxidative Stress by Coumarins

In order to be useful for the prevention of reperfusion injuries after ischemic events, a compound needs to be able to

suppress oxidative stress imposed by ROS in living cells. To evaluate this property, coumarins were added to cultured Neuro-2A cells that had been exposed to β -amyloid peptide, an agent known to promote the formation of ROS upon self-aggregation, presumably through activation of the NADPH oxidase [34]. A colorimetric MTT-based cell viability assay then permitted the study of the effect of the added coumarins on cell survival rates. Cell viability percentages (Table I) were obtained by dividing the MTT absorbance at 570 nm by the absorbance of a control sample not subjected to oxidative stress.

The results in Table I showed that coumarins **1** and **2**, clearly provided the best level of protection, followed by **9**, **10**, and **15**, that offered limited protection. Interestingly, **1** and **2** were both good XO inhibitors and excellent radical scavengers. The protective effects of **9**, **10**, and **15** could be attributed mostly to these compounds' radical scavenging abilities since **9** and **10** had no measurable potencies against XO activity and **15** was a rather poor inhibitor. In summary, the data suggested that the best protection against oxidative stress was afforded by compounds that combined high inhibitory potencies with good radical scavenging abilities within the same molecule.

IV. CONCLUSION

In this study, we have systematically evaluated the ability of eighteen coumarin derivatives to protect cells from oxidative stress *via* inhibition of XO, scavenging of radicals, or both. SARs derived from *in vitro* assays with the isolated enzyme identified 6,7-dihydroxylated coumarins as the most effective XO inhibitors. The radical scavenging assays showed that two hydroxyl groups at neighboring positions at the phenyl ring were a prerequisite for good scavenging activities. The two properties combined were evaluated employing an *in vivo* assay with neurons subjected to oxidative stress. Consistent with the *in vitro* results, esculetin (**1**) and 4-methyl coumarin (**2**) proved to be suited best for the suppression of oxidative stress. They may therefore be considered prime candidates for further structural optimization with the ultimate goal of obtaining novel drugs for an effective prevention of reperfusion injuries.

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Jonathan Webster is an undergraduate student at Northern Kentucky University pursuing a BS in biology with a minor in chemistry. His research focuses primarily on experimental testing of potential enzyme inhibitors of the enzymes xanthine oxidase and sarco/endoplasmic reticulum calcium ATPase. Jonathan is expected to graduate in 2015 and hopes to continue his education in medical school.



Taylor Kidd is an undergraduate student of chemistry at Northern Kentucky University and has participated in undergraduate biochemistry research since early 2012. Her area of expertise compasses enzyme activity assays and the synthesis of novel enzyme inhibitors. She plans on graduating with a bachelor's degree in chemistry in 2015 and intends to pursue a career in secondary chemistry education.



Reid Kline is an undergraduate sophomore at Northern Kentucky University majoring in biochemistry. His research project focuses on the use of computational methods to study interactions between drugs and their protein receptors. He is also the treasurer of the Student Affiliates of the American Chemical Society and a member of the honors community at NKU.



Manori Jayasinghe is an assistant professor at the Department of Physics, Mathematics, and Computer Science at the University of Cincinnati, Blue Ash campus. She obtained her Ph.D. in physics from University of Cincinnati in 2007 and then pursued post-doctoral training in computational biophysics, studying structure and mechanisms of protein-based biomolecular machines at University of Cincinnati. In 2010, Dr. Jayasinghe continued her postdoctoral work in computational drug design, specializing in computational docking and free energy calculations of interactions between drug-like compounds and proteins at Northern Kentucky University. Dr. Jayasinghe authored and co-authored several research articles published in peer-reviewed journals. Her current research interests are primarily in two areas: 1) generating physical descriptions of how biomolecular machines act as nano-machines to perform specific functions using computer simulation methods such as molecular dynamics and normal mode analysis; 2) modeling of interactions between drug-like small molecules and proteins to estimate the absolute binding free energies based on statistical mechanical free energy perturbation molecular dynamics methods.



Stefan Paula is a native of Germany. He obtained his diploma in chemistry at the University of Kaiserslautern, Germany, in 1992. He then spent one year as a Fulbright fellow in the United States at the University of California at Davis. He received his Ph.D. in chemistry in 1998 from the University of California at Santa Cruz. After postdoctoral training at the Max Planck Institute for Biochemistry, Germany, and the University of Cincinnati College of Medicine in the United States, he became a faculty member at Northern Kentucky University (Highland Heights, Kentucky) in 2004, where he is currently an associate professor of chemistry.

His research interests are in the area of computer-assisted drug design. His group uses a combination of experimental and computational methodologies to gain an understanding of how small bioactive molecules interact with their protein receptors. The ultimate goal is the design of novel molecules with improved bioactivities. Several enzyme/inhibitor systems are currently under study, including inhibitors of the sarco/endoplasmic reticulum calcium ATPase, the sodium/potassium ATPase, xanthine oxidase, and aromatase. The preferred approach entails a computational analysis of the interactions between a compound class and the receptor, followed by virtual screening of large compound libraries with predictive models and experimental testing of selected molecules in bioassays. To gain access to specific molecules, his research group collaborates with several synthetic organic chemists.

Dr. Paula is a member of the American Chemical Society, the Biophysical Society, and the Council on Undergraduate Research.