Implementing Environmentally Responsible Biomedical Laboratory Practices and Developing a Nonradioactive Alternative Pharmacological Research Technique

Participants
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Project Summary

Aim 1: Creating a Nonradioactive Alternative to [^{3}H]Steroid Binding Assay

While glucocorticoid receptor (GR) ligand binding has historically been measured using tritiated steroid receptor agonists, such as [^{3}H]dexamethasone, recent approaches have sought to utilize fluorescently conjugated ligands as more sustainable alternatives. Steroid binding assays allow researchers to assess the direct chemical interaction between a steroid, such as dexamethasone, and the glucocorticoid receptor (GR). This binding assay allows us to model the steroid-GR biophysical interaction that is established as the first step in facilitating the body's cellular response to a multitude of physiological endocrine processes (e.g., stress and the sleep-wake cycle) as well as the corticosteroid-based drug therapies used for treating multiple immuno-inflammatory disorders (e.g., treatments for asthma, inflammation, and cancers). A key purpose of our study was to develop a nonradioactive, fluorescence polarization (FP)-based assay for measuring GR binding activity that was suitable for use in undergraduate research environments, and then apply this method to evaluating the extent to which specific agonists and antagonists of the GR and hsp90 chaperone machinery affect ligand binding.

While steroid binding assays have historically relied on using radioactively labeled steroids, which are well established and easy to perform, they result in the production of radioactive waste with suboptimal environmental implications. The first aim of our project was to establish a non-radioactive alternative research protocol for assaying glucocorticoid binding at Seattle University. This was the chief focus of the work completed by Nicky Manlove and myself, with additional involvement of a second SU undergraduate physics major, Jane Walden. Nicky, Jane, and I used dexamethasone fluorescin (Dex-Fl, Figure 1) to devise a fluorescence polarization (FP)-based glucocorticoid binding assay, which did not rely on radioactivity.

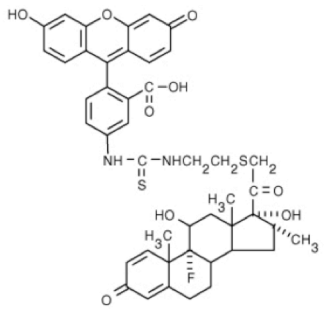

Figure 1. Chemical structure of dexamethasone fluorescein (Dex-FL). Dex-Fl was the nontoxic, nonradioactive fluorescently labeled steroid we desired to use as an alternative to radioactive [^{3}H]dexamethasone. The flourescein label (*top 4-ring structure*) covalently linked to dexamethasone (*bottom 4-ring structure*) can be detected using a spectrophotometer and provide an indicator of ligand binding.

We derived our technique from work originally described by Pfaff & Fletterick (2010). Although we initially presumed this would be a relatively straightforward assay application and development, we ended up completing a substantial about of time trouble-shooting, optimizing, and modifying the originally reported technique. The technique is based on spectroscopic principle that Dex-Fl not bound to the GR will be freely rotating in solution, and thus less likely to emit fluorescent light in a single polarized plane, whereas Dex-Fl that is bound to the much larger molecules of GR will be relatively stationary, sterically hindered, and more likely to return planar fluorescent light. We are able to assay FP using our lab's newly acquired Molecular Devices SpectraMax i3 spectrophotometer equipped with an FP detection cartridge.

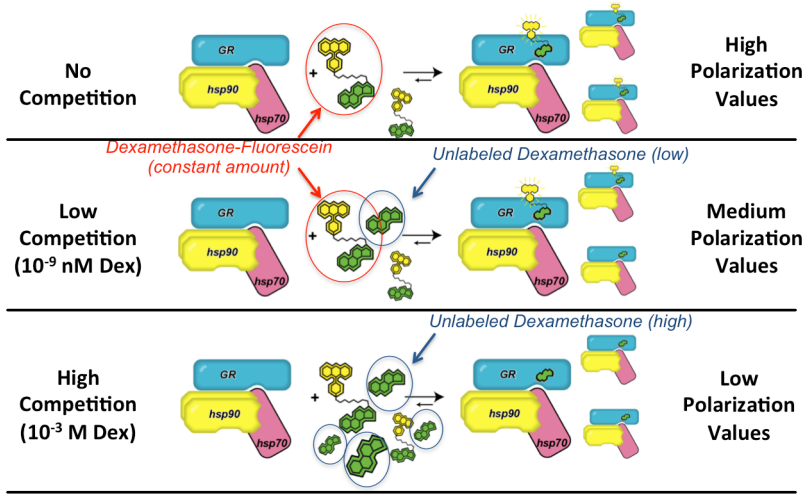

Figure 2. Theoretical illustration of our nonradioactive steroid binding assay utilizing fluorescence polarization, fluorescently labeled dexamethasone (Dex-Fl), and competitive steroid binding between Dex-Fl and unlabeled (i.e., non-fluorescent) dexamethasone (Dex). Samples containing glucocorticoid receptor (GR) and Dex-Fl were incubated with increasing concentrations of unlabeled Dex and assayed for fluorescence polarization (FP). Since polaraization values correlate to GR:Dex-Fl binding, we would expect to see a decrease in polarization as the concentration of unlabeled Dex is increased.

In order to verify the polarization signal is a specific indicator of GR:Dex-Fl binding, increasing concentrations of unlabeled dexamethasone (Dex) were added to GR:Dex-Fl (Figure 2). If the GR:Dex-Fl binding is specific, then we would expect Dex-Fl to bind to the GR both competitively and reversibly in the presence of unlabeled Dex. As described above, high polarization values would be expected when Dex-Fl is incubated with the GR in the absence of unlabeled Dex. If the assay works as anticipated and unlabeled Dex is able to compete with Dex-Fl for GR binding, then as the concentration of unlabeled Dex is increased, the amount of Dex-Fl bound to the GR should decrease, leading to lower polarization values.

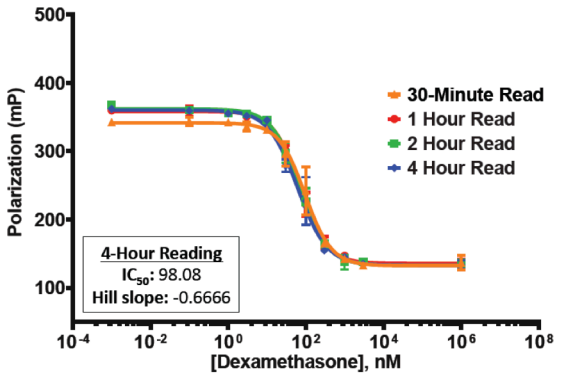

Figure 3. Demonstration of fluorescence polarization (FP)-based nonradioactive steroid binding assay. Samples containing GR and Dex-Fl were incubated with 10^{-4}–10^{6} M unlabeled Dex and assayed for FP signal (mP). The assay conditions were stable enough to maintain unaltered GR:Dex-Fl binding over the course of 4 h.

Aim 1 Key Activities and Findings

We worked extensively over the summer and into the academic year accomplishing this aim, and our efforts produced a qualified success in generating a functional assay. The protocol we created allowed us to observe spectrophotometric differences between and diminution of polarization signal from GR incubated with Dex-Fl and and increasing concentrations of unlabeled Dex (Figure 3). We were able to

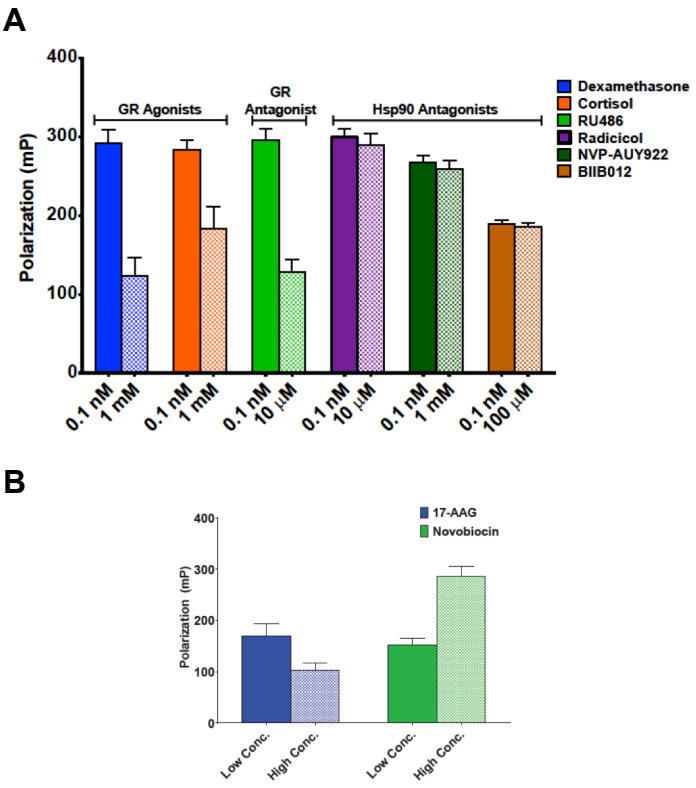

Figure 4. GR agonists and antagonists produce similar competition effects, while hsp90-based chaperone machinery inhibitors were mixed. Multilog dose-response ssays for each Dex-Fl compeitor were completed, and competitor concentrations representing the low competitor concentration maximum (solid bars) and high competitor concentration minimum (shaded bars) mP values observed were evaluated for relative GR binding efficacy. A, The GR agonists dexamethasone and cortisol produced nearly equivalent Dex-Fl competition for GR binding as the GR antagonist RU486. The purported hsp90 antagonists radicicol, NVP-AUY922, and BIIB012 did not appear to affect Dex-Fl binding. B, The hsp70 inhibitor novobiocin showed an unexpected increase (i.e., potentitation) of Dex-Fl binding, pointing to an intriguing finding worthy of future investigation.

optimize the reaction conditions to enable GR-Dex-Fl binding to be sustained for a >4 h time window, which is experimentally significant because the GR-hsp90 multiprotein complex necessary to facilitate steroid binding typically dissociates during a prolonged incubations at room temperature. We concluded that our FP assay was versatile and provided comparable results to the industry-standard radiation-based method.

We next sought to apply our FP technique to evaluating the extent to which specific agonists and antagonists of the GR and the molecular chaperone protein hsp90 affect ligand binding. This was a clinically significant step as the hsp90 chaperone machinery has been shown to be essential for facilitating GR ligand binding (Murphy et al., 2011), and structurally unrelated hsp90 inhibitors are currently in various phases of clinical investigation. Using the developed FP method to evaluate steroid binding can be applied to broader studies examining clinically relevant GR and hsp90-based drug therapies.

Interestingly, GR agonists dexamethasone and cortisol produced equivalent Dex-Fl competition to the GR antagonist RU486, and did so in a concentration-dependent manner (Figure 4). Data from hsp90 and hsp70 inhibitors showed mixed results, with hsp90 antagonists radicicol, NVP-AUY922, and BIIB012 having limited effects (Figure 4A), while the hsp70 antagonist novobiocin showed a potentiation of FP-detectable steroid binding (Figure 4B).

Aim 1 Dissemination

These data were presented as invited podium presentation at two regional conferences (i.e., The Murdock College Science Research Conference in Vancouver, WA, presented by Nicky, and the Conference for Undergraduate Women in Physics, presented by Jane) and at the national Experimental Biology/American Association of Biochemistry & Molecular Biology conference, where we co-presented the work. Jane and Nicky also presented their research intramurally as invited speakers for the SU Natural Sciences Seminar Series. This assay is becoming a staple of our research repertoire, and our current NIH/NSF grant applications and biochemistry research manuscript in preparation describe it in substantial detail.

Aim 2: Implementing Environmentally Responsible Biomedical Laboratory Practices and Developing a Model for Use in Student-Faculty Research at Seattle University

The second aim of our project was to devise and implement a best practice for ensuring responsible environmental stewardship and sustainability in our research laboratory. Beginning with an audit and continuing on through implementation, reporting out, and assisting others in replicating our results, we sought to redevelop our approach to waste generation, sample size selection, and consumable reagent usage in order to minimize our environmental footprint. We began to construct a simple framework for developing and implement environmentally responsible biomedical research practices in our laboratory. An immediate goal of this project was to model time, material, chemical, energy, and cost-savings using the sustainably focused framework; whereas an anticipated future goal of this work was to subsequently engage Seattle University's life sciences faculty and research labs in a meaningful dialogue in order to add an element of sustainability awareness into our laboratory-based research education.

Aim 2 Key Activities and Findings

I completed this work collaboratively with Gabe Kaemingk, who worked fulltime over the summer and then extended his efforts through the full academic year. Gabe rephrased our research question as follows: "What efforts will succeed in instigating behavioral changes that lead to waste reduction, energy savings and tangible gains indicating our labs are performing research sustainably?". This ultimately led to Gabe coining the phrase "EESI [ee-zee] lab practices for sustainable research" (Figure 5) in the hope that the framework we developed could be articulated as a meaningful, accessible, and effective pneumonic.

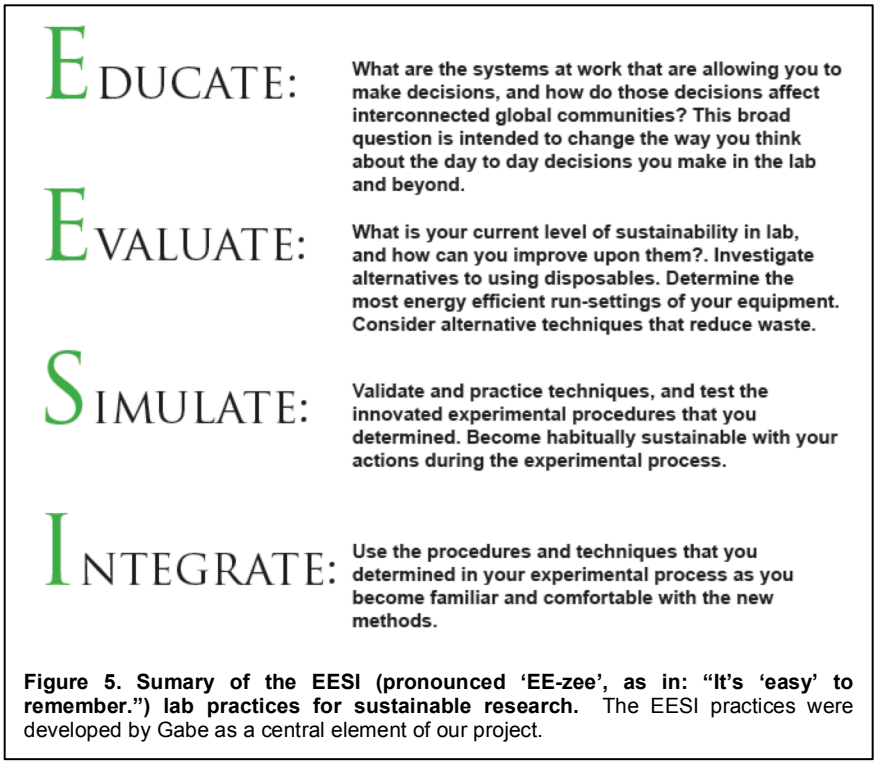

Figure 5. Sumary of the EESI (pronounced 'EE-zee', as in: "It's 'easy' to remember.") lab practices for sustainable research. The EESI practices were developed by Gabe as a central element of our project.

We were able to take advantage of the meaningful resources available here at SU, most notably the University's Chemical Hygiene Officer (Shelia Lockwood), who was particularly enthusiastic about being an actively engaged collaborator in this project. We initially planned to not only restructure our protocols, but ensure all lab members were adequately trained in these improved practices. The literature on the subject suggested that making these revisions would result in a net positive for both laboratory productivity and cost-savings, which we sought to quantify.

My initial thought for completing this aim is that we would simply adapt some of the excellently prepared materials on green lab practices to our SU research environment. In fact, Shelia provided a never-ending cornucopia of best practices for us to consider. However, this approach did not resonate at all with Gabe, which I found highly informative and telling. In this sense, Gabe was both researcher and test subject: If a motivated (and financially invested) undergraduate student could not be excited about sustainability and waste reduction, how could we expect to compel other students who were less predisposed to taking actionable steps?

In order to investigate this process and decision making further, I had Gabe complete a controlled series of our lab's most commonly used—and most waste-generating—experiments and instructed simply to log the produced waste, and then repeat the identical experiments while trying to minimize waste without adding to the research cost (i.e., no 'green' purchases). What made all the difference to Gabe was seeing the impact of making sustainable choices in his research practice and design firsthand. More than any white paper or explicit instructions, Gabe needed to see the environmental effects of the choices he made, and the more that he saw how his decisions produced positive outcomes (i.e., reduction in waste, materials, and energy), the more motivated he was to continue those practices and seek out additional approaches to sustainability. My perception is that there was an element of discovery research and gamification that were the key drivers of his efforts.

Gabe identified multiple material savings when our EESI lab practices were applied to the experimental process investigating the nucleotide dependency of hsp90 and hsp70 on the biochemical production of a GR-hsp90 heterocomplex capable of steroid binding activity. Several notable examples are described below.

- Conduct a pilot run using protein standards with known molecular weights to determine the fractionation window of interest. This reduces the fraction collection to a specified window.
- Use 4 mL reusable borosilicate glass culture tubes to collect fractions of interest instead of our current stock of disposable 9 mL tubes. This is an example of breaking 'anecdotal habits'. We had originally used 9 mL tubes because we were collecting 8 mL fractions; however, as we adjusted the collection volume, we had not broken the habit of using the larger culture tube (even though the larger size was no longer necessary).
- Reduce the number of culture tubes consumed by decreasing the collection window. This translates into less using fewer culture tubes for sample preparation, and less pipette tips for transferring liquids from fraction tubes to culture tubes.
- Limit the 'power on' of water baths and heat blocks to when they are actually in need of being used. Energy will be saved by loading samples efficiently into an electric boiler and setting a timer for five minutes so as not to waste time in retrieving samples; whereas currently the heat blocks and water baths are left on for hours before and after they are needed.

Two logistical elements that were critical for allowing us to complete this sustainability work were (1) having the means for Gabe to dedicate himself fulltime to his research for the duration of the summer and (2) being able to map out the project in detail, from beginning to end. As the waste piled up, literally speaking, the significance of our efforts in sustainable practices became all the more real. Often during the school year, experiments are stretched out to fit between classes and protracted over weeks or months and the net accumulation of waste

and substandard sustainable practices are removed from the lab and students' minds with the trash. This was profoundly impactful, as I would have not considered how his behavior was constructively modified. *A major consequence of this aim is that I now have all students begin a new experiment by estimating the consumption of disposable reagents, with the challenge to ensure that they are utilizing the smallest amount of consumables without adversely impacting the likely success of the research.* This has the pedagogical value, environmental benefits, and cost-savings.

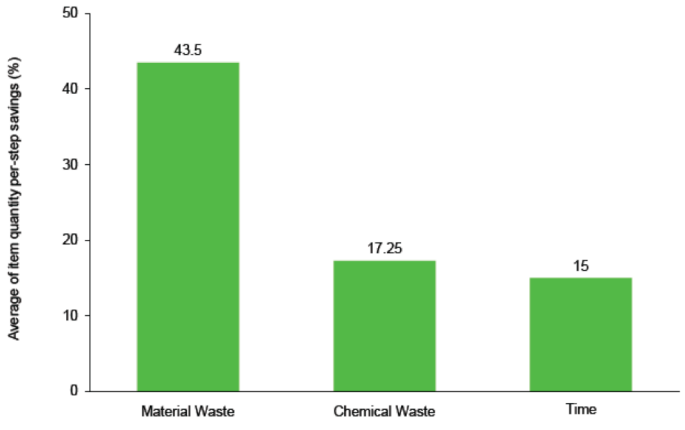

Figure 6. Waste reduction achieved by application of behavioral changes to a typical series of five FPLC experimental processes. Significant and tangible gains in sustainability were achieved while reducing time and cost. Material waste consisted primarily of

Gabe completed these experiments multiple times in order to quantify a consistently achievable waste reduction (Figure 6). Solid material and liquid chemical waste were measured in volumes of waste produced. The major sources of material waste included disposable pipets, pipet tips, and tubes. The major source of chemical waste was buffers used in the FPLC process. As Gabe was able to decrease the number and volume of samples were decreased, the amount of time necessary for processing the samples and completing the experiments was decreased by 15% (~60 h to ~51 h). Chemical waste reduction was consistently >15%, and material waste production was >40% of the standard volume consumed. At this stage, we did not attempt to calculate financial cost-savings associated with our decrease in material waste in part due to the extensive conversion costs to convert from use of primarily disposable plasticware consumables to primarily reusable glassware re-useables. While there are substantial theoretical savings to be gained from reusable materials, are overall research and consumption amounts did not make the switch cost-neutral or produce a cost-savings at this time. As research productivity increases (i.e., funding for extensive research beyond the summer, I plan to revisit this calculation.

Aim 2 Dissemination

Gabe and I presented this work at the Seattle University Undergraduate Research Association spring symposium. We are continuing to share our data with Shelia, and there is the potential for developing our experience into a case study measuring outcomes of implementing EESI and "Shut the Sash" sustainable lab practices, in which we utilize stickers showing appropriate lab practices that save energy, reduce waste, and promote safety (e.g., lowering the sash level for fume hoods). We have developed a curriculum for improved lab practice training that I have incorporated into my wet lab research course, which can also be transferrable to other life science research labs across campus. In addition to benefits these practices have towards promoting environmental justice, the new approaches provide meaningful training and a marketable skill for students planning to apply to graduate schools and to work as laboratory technicians after graduation. We plan to continue to work with Shelia to make our findings (both the audit and improved sustainable practices) available to other members of the SU community, and have already begun to challenge other labs to match our green lab practices. Our future lab publications will include description of our improved techniques and reference their sustainability benefits in the manuscripts' Methods sections.