A) Program Overview. My research program is dedicated to the study of cancer research. Currently, our focus resides on the treatment of melanoma—a highly aggressive form of skin cancer—via the exploration of the functional properties and application of ssDNA aptamers.

B) Background Information. *i) Societal implications of melanoma.* Melanoma originates in the skin, eye, inner ear and leptomeninges: metastatic mutations in the melanocytes (pigment-producing cells) cause the most aggressive and deadly form of skin cancer currently known (Domingues et al., 2018). Although the disease is relatively uncommon in comparison to other forms of skin cancers, melanoma continues to be difficult to treat—the 10-year survival rate for patients is less than 10% (BHATIA et al., 2009). Dacarbazine chemotherapy remains the standard care, despite significant drawbacks: a) it was approved in 1974 and is therefore an extremely outdated form of treatment; b) its long-term potential side effects—such as serious blood and liver disorders—are far too dangerous to ignore; c) despite b), dacarbazine is relatively ineffective as only about 16% of patients had a 5-year survival rate from melanoma (Kim et al., 2010). In 2020, a worldwide total of 325,000 new melanoma cases were reported, along with 57,000 resultant deaths. These numbers are projected to rise to 510,000 and 96,000 respectively (Arnold et al., 2022); that is, of course, unless we find a more effective treatment than the ones in the status quo.

ii) ssDNA aptamers. At the base level, aptamers are nucleic acid molecules that bind specific molecular targets. DNA molecules, which exist mostly in single-stranded (ss) form have been shown to function as aptamers, which use defined structures to bind non-nucleic acid targets such as small molecules and proteins. Although ssDNA aptamers are not known to exist in nature, they can be selected from random-sequence pools of DNA (libraries) through In Vitro Selection (IVS). Hence, strands with desired properties can be isolated from the rest using specific experimental conditions. The first DNA aptamers were developed in 1992 with the selection of strands that bind and inhibit human thrombin (Bock et al., 1992) Since then, the field has produced aptamers capable of binding to a variety of targets while demonstrating usage as a diagnostic tool and potential as a therapeutic agent. Notable aptamer targets include those that bind ATP (Huizenga & Szostak, 1995), human thrombin (Bock et al., 1992), and live leukemia cells (Shangguan et al., 2006).

iii) Melanocortin 1 Receptor. The Melanocortin 1 Receptor (MC1R) is a melanocytic G_S protein coupled receptor, and is involved in the regulation of skin pigmentation and UV responses expressed mainly in melanocytes. It uses cyclic adenosine monophosphate (cAMP) signaling to up-regulate the production and deposition of melanin (in order to limit UV penetration and increase the DNA repair rate). Thus, MC1R is heavily associated with melanoma risk—it is polymorphous, and the melanoma-prone phenotype results in defective epidermal melanin formation and DNA repair (Wolf Horrell et al., 2016). MC1R overexpression is strongly associated with malignant melanoma—it was detected in 83% of all melanoma cell lines, which far exceeded the detection of other commonly expressed melanoma markers such as S-100mAb (Salazar-Onfray et al., 2002).

C) OBJECTIVES. The long-term objective of our program is to develop a treatment for melanoma via the use of ssDNA aptamers as a "drug delivery" vehicle to induce cell death. This will be broken down into 3 short-term aims in order to simplify and organize the steps towards achieving the objective.

Aim 1: isolating DNA aptamers that bind selectively to MC1R. Currently, there has been little exploration towards the development of MC1R-specific aptamers. In order to further our research, this is the most fundamental step.

Aim 2: testing for non-promiscuous binding. We will test the binding affinity of the MC1R aptamer (MC1Ra) to both melanoma and non-melanoma cell lines.

Aim 3: delivery of cell death-inducing drugs via MC1R. We speculate that the combination of aptamers and previously discovered cell death-inducing treatment will allow for the selective targeting of metastatic melanoma cells; this novel method not only have a higher success rate than current treatments, it will also evade their life-threatening side effects including low WBC and RBC count, low platelet count, and even a risk of developing other cancers (eg. sarcoma, fibrosarcoma, angiosarcoma) (Arnold et al., 2022).

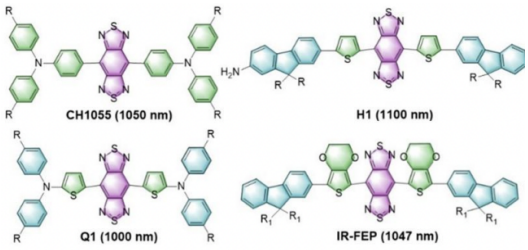
D) RESEARCH PLAN. Here are the proposed research plans to accomplish each aim.

Aim 1: isolating DNA aptamers that bind selectively to MC1R. *Rationale.* MC1R is the melanoma marker that we have chosen to work with due to its presence on most melanoma cell lines (>80%). ssDNA aptamers will be used as the diagnostic detector over other traditional ones (MC1R-AB antibody) due to their small size, which facilitates manufacturing and administration. DNA aptamers—since their conception in 1992—have already been proven to be able to selectively bind to proteins (Bock et al., 1992); thus, we should have no issue isolating an MC1R-specific aptamer. *Methodology.* In vitro selection (SELEX) will begin with a pool of 10^{15} random sequence DNA oligonucleotides. They will then be partitioned based on their binding affinity to MC1R proteins bound to Ni-NTA magnetic beads, where nickel ions will bind to the polyhistidine-tags present—this method will be repeated for 3 cycles. Next, the binding affinity of aptamers will further be tested using native gel-based diffusion methods for 10 cycles. In between SELEX cycles, strands will be removed from the aptamer-protein complexes via polyacrylamide gel electrophoresis (PAGE) and enriched using PCR. The 32 aptamers with the highest binding affinity will be isolated, enriched, and sequenced; a deletion analysis will be conducted to find consensus sequences that suggest the location of the binding site. Ultimately, we will use the 10 most successful MC1R-specific aptamers (MC1Ra[1-10]) for further stages of experimentation.

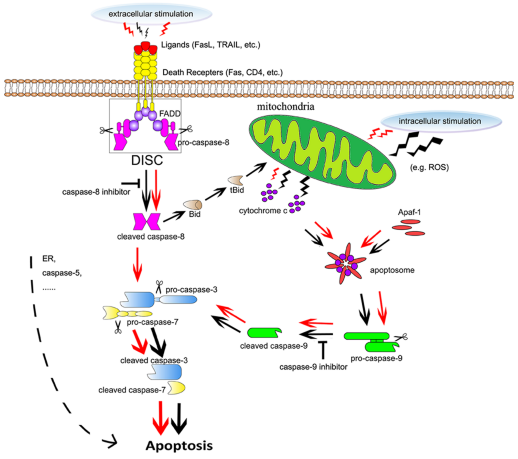
Aim 2: testing for non-promiscuous binding. *Rationale*. We need to ensure that the isolated MC1Ra binds selectively to melanoma cell lines in order to prove its effectiveness as a melanoma marker. MC1R has already been shown to be overexpressed on the surfaces of 83% of common melanoma cell lines; therefore, our aptamers should be able to selectively bind to their desired target and thus be used as a diagnostic tool for the presence of metastatic melanoma. By the same principle, previous researchers have developed diagnosis methods by using selective MC1R antibodies (MC1R-Ab) (Seenivasan et al., 2015).

Methodology. A dot-blot assay will be conducted in order to compare the MC1Ra interactions between melanoma and non-melanoma cell lines. The aptamers will be labeled with γ-[^{32}P] ATP by mixing 2 µl of 1 µM of them with 2 µl of γ-[^{32}P] ATP, 1 µl of 10× PNK reaction buffer A, 1 µl OF PNK, and 4 µl of water. For the dot-blot analysis, 5 µl of aptamer solution and 15 µl of MC1R protein solution (10 different concentrations ranging from 0-500 nM) will be placed into the dot-blot apparatus assembled with a nitrocellulose membrane, a nylon membrane, and a wetted Whatman paper. The radioactivity of the membrane will be measured using phosphor storage, which will allow for the evaluation of the concentration of aptamer-protein complexes and subsequent analysis of binding affinities. This process will be repeated with other proteins commonly expressed near the melanocytes (eg. Melan-A/MART-1, LAMP2, HPS1) to ensure that MC1Ra binds non-promiscuously (Boissy et al., 2005). We will detect the prevalence of our aptamers in both melanoma and non-melanoma cell lines via flow cytometry. Predicting

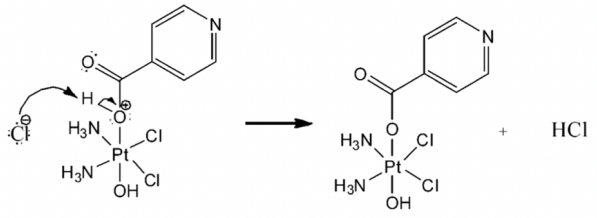
that the MC1Ra aptamers will all be relatively simple oligonucleotides (~50-100 mer), small fluorophores that emit bright wavelengths in the NIR-II (1000-1700 nm) will be tested. Probes with benzo-bis thiadiazole cores (shown on the right) such as CH1055, H1, Q1, and IR-FEP are small and organic molecules and potential aptamer dyers (Tu et al., 2019). Fluorescent aptamers will be cultured with 20 common melanoma cell lines that were found to overexpress MC1R and will be subsequently analyzed using the flow cytometer (Salazar-Onfray et al., 2002); the same will be done with regular melanocyte cell lines.



Aim 3: delivery of cell death-inducing drugs via MC1R. *Rationale.* As characteristic of all cancers, melanoma cells evade apoptosis by inhibiting pro-apoptotic signals and upregulating anti-apoptotic ones. Specifically, the Ras/Raf/MAPK pathway—which is directly involved in the activation of growth, differentiation, and division genes—was found to be the key contributor. The BRAF gene (which codes for the B-Raf protein) was mutated in over 60% of tested melanoma cell lines, thereby causing kinases to activate downstream signalling transcription factor substrates to activate (including NF-kB, c-Jun and ATF2). As such, the death receptor pathway of apoptosis is unable to activate caspase-3, 8, and 10, which are protease enzymes that are normally released to dismantle the cell (Ivanov et al., 2003). Conventional chemotherapy treatment has already identified a viable method for inducing apoptosis in melanoma cells: the mitochondrial pathway of apoptosis (shown on the left) begins with the permeabilization of the mitochondrial outer membrane; water then enters through the permeability transition pores (PT), which causes the swelling then subsequent rupture of the membrane; a host of proteins are released, notably APAF-1, cytochrome C, and pro-caspase-9, which collectively form an apoptosome that activates the caspase-9 pathway (*Apoptosis: Mitochondrial and Death Receptor Pathways | Abcam*, n.d.). Currently, cis-diamminedichloroplatinum(II) (CDDP) performs its cytotoxic functions by applying consistent oxidative stress and hence inducing mitochondrial pathway apoptosis (Mattia et al., 2018). Since CDDP is a much smaller molecule than an antibody (with a molecular weight of 301.1 g/mol), it creates the possibility of a plausible mechanism for melanoma treatment, in which our strongest aptamer MC1Ra will be used as a selective "drug delivery vehicle" for CDDP via MC1R on the melanoma cell surfaces, hence avoiding the negative repercussions of standard chemotherapies.



Methodology. The end goal is to form MC1Ra-CDDP conjugates as a means of targeted therapy. In order to facilitate this process, a modified isonicotinic linker will be used (shown on the right): the linker will perform a nucleophilic attack on the cisplatin while a chloride ion leaves the new complex (forming nicoplatin), ultimately allowing for a bio-metal-organic framework (bio-MOF) (Rosari et al., 2019). Upon the synthesis of aptamer-nicoplatin complexes, we will perform a flow cytometric



analysis: using one specific melanoma cell line out of the 24 most common ones, we will incubate (at 37℃) one cell line sample of 10^5 cells with 100 μl of phosphor-labelled MC1R-nicoplatin conjugate, and one cell line of 10^5 cells with 100 μl of solely cisplatin. This is to ensure that the bio-MOF can passively diffuse into the cells just as functionally as bona fide cisplatin. Cytotoxicity assays will be performed using both regular melanocytes and metastatic melanoma cells.

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