METABOLIC REPROGRAMMING IN HIGH-GRADE SARCOMAS, REPURPOSING ANTI-CHOLESTEROL AGENTS AS A NOVEL THERAPEUTIC STRATEGY

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Introduction

Soft tissue sarcomas (STS) are a diverse group of mesenchymal tumors with over 50 histologically distinct subtypes (1). STS commonly develop in extremities or the retroperitoneum. Undifferentiated pleomorphic sarcoma (UPS), leiomyosarcoma (LMS) and liposarcoma (LPS), are three of the most common subtypes and comprise >50% of adult sarcoma (1). UPS are heterogeneous tumors that develop from an unknown cell of origin, LMS arise from smooth muscle cells, and LPS are derived from an adipocyte lineage. Current treatment includes chemotherapy, radiation and/or surgery and despite this multimodal therapy survival rates have remained largely unchanged for the last decade (2). Radiation and chemotherapy are often offered to patients who have advanced disease – locally aggressive or metastatic. UPS and LMS metastasize in over 50% of cases, commonly to the lungs or liver (3). In contrast, LPS metastasizes in only 10-20% of cases but recurs locally in 50% of cases after treatment (3). Outcomes for patients with advanced disease are dismal as the response rates to the current standard of care chemotherapies, doxorubicin and ifosfamide, are only 20-30% (2). Recent data from The Cancer Genome Atlas (TCGA) suggests that sarcomagenesis is driven by copy number alterations rather than high mutation burdens, which limits the potential for targeted therapy (4). Since the main patterns of failure are metastatic disease and local recurrence, development of more efficacious systemic therapy is essential to improve disease outcomes (5).

A hallmark of cancer is metabolic reprogramming: alteration of cellular metabolism to increase energy and metabolite production required to sustain proliferation and cell growth (6). Cancer cells are able to exploit proliferative signaling pathways such as the phosphoinositide 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway, which regulates glucose uptake, glycolysis, protein production and the mevalonate (MVA) pathway, which is involved in anabolism (7). The PI3K/AKT/mTOR pathway has been documented to be dysregulated in many STS subtypes. Our lab has previously shown that PI3K/mTOR inhibitors are effective therapies for LMS and rhabdomyosarcoma (RMS), however selective agents that target such a critical signaling pathway have challenges in vivo, such as toxicity due to off target effects, and currently have limited utility (8, 9). The MVA pathway produces cholesterol that is required for proliferating cells as it is an integral component of cellular membranes, and other metabolites that are required for post-translational modifications (7, 10). When MVA pathway metabolites are low, the transcription factor sterol regulatory element binding protein (SREBP) is activated and increases the transcription of genes that will increase the amount of cholesterol in the cell. This includes those involved in endogenous cholesterol production and those that produce the low density lipoprotein receptor, which takes up cholesterol. The PI3K/AKT/mTOR pathway can upregulate the MVA pathway to meet the increased cholesterol demand by increasing the expression of SREBP (11, 12). The MVA pathway is also able to influence the PI3K/AKT/mTOR pathway, possibly through metabolites like geranylgeranyl pyrophosphate that
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is require for isoprenylation - a post-translational modification that is required for cellular localization of proteins such as small guanosine triphosphate enzymes (GTPases) (13).

Taken together, metabolic reprogramming is a key hallmark in high-grade sarcoma and may be targetable to allow for improved patient outcomes.

Rationale:
To identify novel therapies for sarcoma, we screened four patient derived UPS cell lines (STS 148, STS 235, STS 309, STS 162), and one fibroblast cell line as a control, with >3,300 compounds from four libraries: OICR Kinome, NIH Clinical, Prestwick, and Tocris (Figure 1A). From the screen we identified the current standard of care chemotherapies (ex. doxorubicin), receptor tyrosine kinase inhibitors, PI3K/mTOR inhibitors (previously studied in our lab in LMS) and statins (Figure 1B). We reviewed the hits that reduced viability in 3/4 of the UPS cell lines with a sarcoma oncologist and determined that simvastatin should be further validated. Statins inhibit the rate limiting enzyme, 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) in the mevalonate pathway. Cancer cells can exploit this pathway through PI3K/AKT/mTOR signaling to enhance proliferation and survival (7). Statins are commonly used to treat hypercholesterolemia and are generally well tolerated. The use of statins as an anticancer agent has been tested in other solid tumors, such as breast cancer and colon cancer; however it has not been investigated in sarcoma (14-16). Thus, goals of our study are to define the mechanism(s) responsible for statin sensitivity in UPS and determine if LMS and/or LPS are also statin sensitive.

Methods:
Hit validation with EC₅₀ curves validated and confirmed that both UPS and LMS, but not LPS, are sensitive to statins (Figure 1C). To investigate the mechanism of action of statins in sarcoma cell lines, we performed immunoblots to assess PI3K/AKT/mTOR effectors (phosphorylated (p)-AKT and phosphorylated (p)-4EBP1) and apoptosis (PARP cleavage) and flow cytometry to assess cell death (Annexin V and propidium iodide (PI)) and apoptosis (active caspase-3). To analyze if simvastatin could be successfully combined with doxorubicin, the current standard of care, as a future therapeutic strategy, we performed a Bliss analysis, used to assess the interaction of two drugs that target different pathways, which demonstrated that these two agents are synergistic in vitro.

To determine the best route of administration of simvastatin in vivo, we performed a pharmacokinetic study comparing oral versus intraperitoneal routes. Mice were treated with simvastatin once a day for five days via oral gavage (n=5) or intraperitoneal (IP) injection (n=5). An hour after the last treatment mice were euthanized and tumor and plasma were used to assess simvastatin concentrations by high pressure liquid chromatography (HPLC). To assess the in vivo efficacy and toxicity of simvastatin alone and in combination with doxorubicin, we treated STS 148 xenografts in NSG mice with vehicle (n=9), simvastatin (n=10), doxorubicin (n=9), or simvastatin + doxorubicin (n=10). Necropsies were performed when mice reached endpoint and the tumors were used for subsequent analysis to determine treatment effect and the mechanism of action of simvastatin in vivo.

Results:
To determine if dysregulation of the PI3K/AKT/mTOR pathway renders sarcoma cells statin sensitive, we assayed the activity of downstream effectors of PI3K/AKT/mTOR. In UPS, we found a decrease in the levels of p-AKT and p-4EBP1 indicating that simvastatin downregulates PI3K/AKT/mTOR signaling in a dose dependent manner (Figure 2A). This effect was rescued with the addition of MVA, an effector immediately downstream of HMGCR (the enzyme that
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Statins inhibit (Figure 2B). We also observed a cleavage of PARP with simvastatin treatment, that was also dose dependent (Figure 2C). We are currently investigating if PI3K/AKT/mTOR signaling is similarly dysregulated in LMS cell lines to determine if this is a potential mechanism of simvastatin sensitivity. Simvastatin alone and simvastatin in combination with doxorubicin significantly increase cell death in 3/4 sensitive UPS cell lines (Figure 2D&E). Simvastatin in combination with doxorubicin showed an increase in caspase-3 positive cells, which was not seen with simvastatin alone in some sensitive UPS cell lines. In vitro, the combination of simvastatin + doxorubicin therapy also produced a greater increase than doxorubicin alone. This is currently being assessed to determine significance. In LMS the mechanism of the reduction of viability caused by statins appears to be different: there was no increase in dead cells positive for caspase 3 with either simvastatin or simvastatin + doxorubicin treatment. There does appear to be a reduction in proliferation with both simvastatin treatments, which is under investigation.

Importantly, as our laboratory has a panel of UPS and LMS xenografts, we investigated if statin inhibition is an effective therapy in vivo and are currently working on mechanistically dissecting the basis for tumor growth inhibition. We performed a preliminary pharmacokinetic study to assess the bioavailability of simvastatin in UPS xenografts and found that simvastatin is present in the tumors treated by IP injections but not oral gavage. Thus, in our pre-clinical murine studies we administered simvastatin by IP injections both alone and in combination with doxorubicin to assess efficacy and toxicity (Figure 3A). These tumors were confirmed to be UPS by H&E assessment, with majority being of the pleomorphic spindle-epithelioid morphology (Figure 3B). There was a significant reduction in tumor volume in mice treated with simvastatin + doxorubicin when compared to control mice (p<0.05) (Figure 3C). Mice that received doxorubicin experienced significant toxicity, assessed by >20% weight loss since the start of treatment leading to humane early endpoints, which complicated the assessment of disease specific survival (p<0.05). The tumors in mice treated with simvastatin + doxorubicin had a significantly higher concentration of simvastatin present than the tumors of any other group, including simvastatin alone (p<0.05). This may indicate that there is synergy occurring between the two drugs in vivo.

Conclusion:
Taken together, we report the promising findings that primary UPS and LMS cells are highly sensitive to simvastatin. This work suggests that two common high-grade sarcomas rely on metabolic reprogramming to support the increased energy demand and proliferation through the PI3K/AKT/mTOR pathway. Simvastatin, a repurposed and well tolerated drug, is able to inhibit this process which may lead to treating sarcomas with this novel therapy. Future studies will focus on elucidating connections between major metabolic pathways and preclinical drug studies of UPS, LMS and other sensitive sarcoma models.

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Letter of Submission:

Dear Gallie Day 2020 Adjudicators,

I am a second year Master’s Student in Dr. Rebecca Gladdy’s laboratory and I am the first author of the research described in this abstract. Dr. Gladdy is a Clinician Scientist at the Lunenfeld-Tanenbaum Research Institute and Surgical Oncologist at Mount Sinai Hospital. She is the primary investigator of this project. Majority of the in vitro work and all of the in vivo work was done at the LTRI. I am responsible for performing a panel of immunoblots on the sarcoma cell lines. I solely performed the flow cytometry assessing caspase-3. I designed and ran the preclinical mouse trial and performed the subsequent analysis.

In the Gladdy lab at LTRI, Dr. Yael Babichev performed the original screen and flow cytometry assessing cell death and Rosemarie Venier performed a panel of western blots on the sarcoma cell lines.

Dr. Richael Marcellus and Dr. Rima Al-Awar are at the Ontario Institute for Cancer Research (OICR) and helped analyze the results of the original drug screen.

Dr. Linda Penn, a Senior Investigator at the Princess Margaret Cancer Centre, is a member of my program advisory committee and has expertise in statins and cancer. She has provided expert input in the conduct of my studies.

Dr. Albiruni A. Razak, a Medical Oncologist at Princess Margaret Cancer Centre, prioritized the hits from the original drug screen to be validated.

Dr. Brendan Dickson is a dedicated Sarcoma Pathologist at Mount Sinai Hospital and reviewed the slides of the sarcoma xenografts.

Dr. Eric Chen, a Medical Oncologist at Princess Margaret Cancer Centre, performed the assessment of simvastatin concentrations in the sarcoma xenografts.

Dr. Irene Andrulis, a Senior Scientist at LTRI, provided expert input in the project.

Dr. Jay Wunder, an Orthopedic Surgeon at Mount Sinai Hospital, is a member of my program advisory committee and has expertise in sarcoma and sarcoma genetics. He has helped guide the direction of this project.

Sincerely,

Jen Dorsey
Institute of Medical Science, MSc. Candidate, University of Toronto
Figure 1. A) Schematic of high-throughput screen work flow. 4 patient-derived UPS cell lines screen with >3,300 compounds. Hits reduced viability by 3 SD in 3/4 UPS cell lines, with therapeutic range <1uM, and not toxic to normal fibroblasts. B) Hits were characterized and prioritized based on FDA-approval status, mechanism of action, and toxicity profiles. C) Two statins that were hits, simvastatin and pitavastatin were validated with EC\textsubscript{50} curves that confirmed sensitive to statins in 3/4 UPS cell lines (STS 148, STS 162, STS 235).
Figure 2. (A) UPS cells were treated with increasing doses of simvastatin (Simva), or Simva (1 μM) + MVA (200 μM). p-AKT (Ser 473) and p-4EBP1 (Thr 37/46) were reduced in a dose-dependent manner. The effect was reversed in the presence of MVA. (B) Simva induced cleavage of PARP in a dose dependent and on-target manner. (C) Representative flow cytometry plots of STS 548, (D) quantified apoptotic events of all cell lines. Cells were treated with Simvastatin (1 μM), Simva + MVA (200 μM), Doxorubicin (Dox) (500 nM) and Simva + Dox then stained with Annexin V and PI. Simva increased the quantity of Annexin V+/PI+ cells compared to vehicle or Simva + MVA. Simva + dox treated cells may indicate some drug additivity. *p<0.05 compared to Vehicle, Dox, or Simva + MVA.
Figure 3. A) Work flow of preclinical murine UPS model to assess the efficacy and toxicity of simvastatin alone and in combination with doxorubicin. B) Representative H&E of STS 148 xenograft at 10x magnification. C) The tumors of mice treated with Simva + Dox are significantly smaller than those from the control mice (p<0.05). There was no significance found between the other treatment groups. *p<0.05 compared to vehicle, ns = not significant.
References: