Proteasomes are multi-subunit protein recycling machines of cells turning damaged and no longer needed proteins into building blocks to make new proteins [1]. Rapid growth and elevated rates of protein synthesis in cancer cells increase the production of abnormal proteins increasing the load of the proteasome. Proteasomes are also involved in the specific destruction of regulatory proteins such as transcription factors. These proteins are essential for cancer cell proliferation and survival. Taken together, these factors create a therapeutic window for proteasome inhibitors in various cancers. Inhibiting the proteasomes of the cancer cells by blocking the active site(s) of the proteasome causes selective apoptosis of malignant cells. FDA-approved proteasome inhibitors, bortezomib, carfilzomib, and ixazomib, are used for the treatment of multiple myeloma, a bone marrow cancer. However, the usage of proteasome inhibitors in solid tumors is limited by their poor tumor penetration and toxicity to normal tissues. By packaging FDA-approved proteasome inhibitors into liposome nanoparticles, selective delivery may protect drugs from metabolic inactivation, improve efficacy, tumor penetration, target engagement, and reduce toxicity to normal tissues [2]. To test this, tumor growth and the target engagement by the drug were evaluated in a murine model of triple-negative breast cancer. These cancers lack targeted therapies and are highly dependent on proteasome activity [3]. However, clinically achievable concentrations of FDA-approved proteasome inhibitors are not high enough to inhibit the growth of these tumors [4].

A remote loading technique [5] was used to encapsulate Carfilzomib (Cfz) into pre-formed liposome nanoparticles. We created two types of nanoparticles, conventional nanoparticles with a non-modified lipid envelope and pegylated-long-circulating nanoparticles, which carried polyethylene glycol (PEG) on their surface. PEG is a biocompatible and biodegradable polymer used to extend the half-life of nanoparticles in vivo because it prevents liposomes from being recognized and cleared by the innate immune system. We have compared the efficacy of conventional liposomes and PEG-liposomes of Cfz in a murine model of triple negative breast cancer. Tumors were created by injecting $10^5$ 4T1 cells into a mammary fat pad. This murine breast cancer cell line closely resembles human triple negative breast cancer in its molecular properties. When tumors became palpable, mice were treated with free, liposome, or PEG-liposome encapsulated Cfz twice weekly on consecutive days to match the clinical schedule of Cfz in myeloma patients. Tumor volume was measured with a caliper over the course of experiment. Mice were sacrificed when they lost more than 15% body weight as per approved IACUC protocol.

As can be seen on Fig. 1, traditional formulation of Cfz did not block growth of these tumors although Cfz was used at doses close to the maximal tolerated dose in mice [6]. On
the other hand, tumor growth in mice treated with the same dose of Cfz in the liposomal formulations was dramatically delayed. Thus, liposomal formulation dramatically improved the efficacy of Cfz in this model of triple negative breast cancer. We also showed that the liposome formulations had less weight loss, compared to free drug. This suggests that these formulations were less toxic to healthy tissue compared to free Cfz. In a separate experiment (Fig. 2), we have measured intratumoral inhibition of proteasome by Carfilzomib. Tumor tissue was harvested 2 and 24 hours after a single dose of Cfz and lysed. The samples were incubated with activity based fluorescent probes that affinity label the β1, β2, β5 active sites of Cfz targets of the 20S proteasome [7]. Cfz is an irreversible inhibitor, and its binding to proteasome active sites prevents their labeling with the probes. After labeling, extracts were fractionated in polyacrylamide gel in the presence of lithium dodecyl sulfate to separate subunits (LDS-PAGE), followed by visualization of subunits by fluorescent imaging (Fig. 2). Darker bands indicate activity while the absence of the bands indicates proteasome inhibition. The β5 site is the prime target of Cfz, but our laboratory has previously determined that, in order for Cfz to kill triple-negative breast cells, it must co-inhibit either β1 or β2 sites [4]. As can be seen on Fig. 2, in the non-liposomal formulation of Cfz and empty nanoparticles caused little to no inhibition of the proteasome. Contrary to it, strong to near-complete inhibition of all active sites was observed in tumors that were exposed to inhibitors for 24 hours. Thus, the liposomal formulations of carfilzomib dramatically improved intratumoral target engagement.

Subsequent Coomassie staining was used as a loading control. Tumors were harvested at times indicated after treatment with a single 3mg/kg does of Cfz.

**Statement of Research Advisor**

Laura Downey played a very important role in this project conducting all *in vitro* analysis of animal tissues. We will use her results in the NIH grant application, and eventually in a publication. This project was carried out under very difficult circumstances after Dr. Andrey Maksimenko, who created nanoparticles and conducted animal experiments, died unexpectedly. We were unable to hire a replacement essentially depriving us of opportunity to conduct additional experiments that would have generated additional samples for Laura to analyze. Laura presented these findings at the Student Research Symposium, where she won the Second prize in COSAM poster competition, and at the recent Inaugural Harrison College of Pharmacy Research showcase, where she got an award for the second-best poster in Cancer Research.

- Alexei Kisselev, Harrison College of Pharmacy

**References**


Authors Biography

Laura E. Downey is a junior-year student pursuing a B.S. degree in Biomedical Sciences with a Pre-medical concentration at Auburn University. She is also currently double minoring in Public Health and Dance. She played key research roles in quantifying tissue samples and testing multiple cell lines with various proteasome inhibitors. She received second place for University-Wide Undergraduate Student in Science, Technology, Engineering and Mathematics in the Auburn Research Symposium as well as Outstanding Presentation Cancer Focus at the HCOP Research showcase this April.

Andrey V. Maksimenko received his M.S. and Ph.D. in Chemistry from M.V. Lomonosov Moscow State University in Russia. After graduation, he moved to France, where he has held a variety of positions in industry and academia, and where he developed a strong interest in drug delivery. Dr. Maksimenko has been a visiting scientist at the Harrison College of Pharmacy from January 2018 till his untimely passing in March 2021.

Robert D. Arnold, Ph.D., is a Professor in the Department of Drug Discovery and Development. He completed a B.S. in Biochemistry and a Ph.D. in Pharmaceutical Sciences. After completing postdoctoral training at the University at Buffalo and Roswell Park Comprehensive Cancer Center, he was at the University of Georgia. He joined Auburn University and the Harrison College of Pharmacy in 2012. His research focuses on development of composite lipid-based nanoparticles and use of alternate dosing schedules to improve the treatment of primary cancer and metastatic disease.

Alexei F. Kisselev has been an Associate Professor of Drug Discovery and Development at the Harrison College of Pharmacy since 2017. He received his M.S. and Ph.D. in Chemistry from M.V. Lomonosov Moscow State University in Russia. Dr. Kisselev has conducted post-doctoral research at Harvard Medical School, and has served on the faculty of the Geisel School of Medicine at Dartmouth College for 12 years, where he was also a member of the Norris Cotton Cancer Center, an NCI-designated Comprehensive Cancer Center.