STATEMENT

By: Prof. Dr. Ivanka Georgieva Tsacheva,

Department of Biochemistry, Sofia University "St. Kliment Ohridski",
member of the scientific jury appointed by order No 121-OB/27.05.2026
of the Director of the Institute of Molecular Biology, Bulgarian Academy of Sciences

regarding the dissertation of ALEXANDER EMILOV ALEXANDROV entitled: "**Synergistic induction of extracellular vesicle and DNA secretion by autophagy inhibitors and DNA-damaging agents**" for awarding the educational and scientific degree "PhD" in a professional direction 4.3. Biological Sciences
Research supervisor: Assoc.Prof. Dimitar Iliev, PhD

Renewed interest in extracellular vesicles is linked to updated scientific understanding of their physiological role and their involvement in various processes that maintain human homeostasis. A more comprehensive characterization reveals them to be structures with dynamic compositions that carry valuable information regarding the state of the cells that release them, molecular communication between different cell types, and—consequently—pathological conditions that are either being triggered or are already established. For this reason, extracellular vesicles serve as diagnostic markers in autoimmune, oncological, cardiovascular, metabolic, and neurodegenerative diseases, enabling the detection and monitoring of these pathological conditions.

Autophagic molecular markers such as LC3 (microtubule-associated protein light chain 3) and p62 are found in the membrane domains of endosomes and multivesicular bodies. Autophagy, an evolutionarily conserved catabolic process, is linked to the secretion of extracellular vesicles; the two processes complement each other and ensure the maintenance of homeostasis by eliminating harmful or excess material.

Autophagy inhibition is frequently employed in anticancer therapies, rendering cells with dysregulated apoptosis sensitive to metabolic stress. Examples of such inhibitors include chloroquine (CHQ) and Bafilomycin A1 (BafA1).

Temozolomide (TMZ) is an alkylating agent included in the standard therapeutic approach following glioblastoma multiforme resection. However, TMZ therapy has a transient effect due to the frequent development of resistance, with elevated expression of autophagy markers associated with a poorer prognosis. Consequently, combination therapy with an autophagy inhibitor would likely yield a superior therapeutic outcome. All of this makes the topic relevant not only for fundamental research but also promising in terms of clinical application.

The aim of the presented dissertation is to investigate the interplay between autophagy inhibitors and DNA-damaging agents, on one hand, and the release of extracellular vesicles and

extracellular nucleic acids, on the other. The specific objectives comprise a coherent, logical sequence of experiments designed to achieve this aim.

The goal was achieved through a complex experimental approach, which included the analysis of the glioblastoma cell lines U87-MG and U138-MG, as well as the endocervical adenocarcinoma line HeLa Kyoto (CVCL_1922) upon treatment with TMZ and CHQ. The cytotoxic activity of CHQ and TMZ, the composition and size of the secreted extracellular vesicles upon treatment with CHQ and TMZ, the composition and amount of the secreted nucleic acids, the amount of damaged DNA in cells under combined treatment, the inhibition of autophagy by ATM kinase inhibitors, the effect of ATM inhibitors on extracellular vesicle secretion, and the interaction of ATM inhibitors with DNA-damaging agents regarding the extracellular vesicle secretion induced by them.

The study demonstrated that CHQ significantly enhances the cytotoxic effect of TMZ against TMZ-resistant cells, with a synergistic action of TMZ and CHQ observed in the induction of secreted extracellular vesicles containing autophagy markers and caveolin-1 in U87-MG and U138-MG cell cultures. It has been found that TMZ increases Bafilomycin A1-induced extracellular vesicle secretion, while CHQ and etoposide synergistically induce the secretion of autophagic markers in the large extracellular vesicle fraction. CHQ enhances the TMZ-induced DNA damage response without increasing the level of DNA damage in U87-MG cells, and CHQ and TMZ synergistically induce the secretion of extracellular nucleic acids in cultures of the same cells. It has also been established that the ATM inhibitors KU-60019 and KU-55933 induce the secretion of small and large extracellular vesicles—bearing both autophagic markers and markers typical of classical exosomes—and trigger the accumulation of LC3B-II and p62 in HeLa Kyoto cells. Treatment with etoposide synergistically enhances the KU-60019-induced secretion of large and small extracellular vesicles, as well as extracellular DNA.

To prepare the dissertation, a comprehensive and in-depth analysis of the scientific literature on the subject was conducted, citing 99 sources. The experimental results obtained during the study conclusively demonstrate the pathological potential of the secreted extracellular vesicles and show the capacity of combined treatment with autophagy inhibitors and DNA-damaging agents to synergistically induce the secretion of extracellular vesicles and nucleic acids. The analysis of the dissertation results in the concluding section makes an excellent impression, particularly where the findings are discussed in the context of data previously published by other authors. This discussion reinforces my conviction that the work on this dissertation makes an original, fundamental contribution and that the newly acquired knowledge will expand future therapeutic applications.

Regarding the technical formatting, I would recommend avoiding the use of loanwords. It is advisable to place figures as close as possible to the text where they are introduced (for example, Fig. 1 appears on page 5 but is introduced in the text on page 3; Fig. 2 appears on page 8 but is introduced in the text on page 4).

The results of the dissertation have been published in two articles in impact-factor journals. Furthermore, the results have been presented at one international and two national scientific forums.

CONCLUSION

I would like to highlight my excellent impressions of the dissertation presented. A substantial amount of experimental work has been carried out with a level of precision and strategic planning that reveals Alexander Alexandrov to be a researcher possessing an analytical approach and a wide range of practical skills. The written presentation of the dissertation, characterized by a scholarly style, reinforces the excellent impression made by Alexander's work as a whole. The dissertation and its related publications exceed the requirements for the educational and scientific degree of Doctor.

Because of the above, I confidently give my positive assessment of the conducted research, the achieved results and contributions, and I propose to the honorable scientific jury to award the educational and scientific degree "PhD" to ALEXANDER ALEXANDROV.

13.07.2026 Prof. Dr. I. Tsacheva