OPINION

by Assoc. Prof. Anastas Gospodinov, PhD
Institute of Molecular Biology "Acad. Roumen Tsanev", Bulgarian Academy of Sciences on the dissertation entitled: "Synergistic Induction of Extracellular Vesicle and DNA Secretion by Autophagy Inhibitors and DNA-Damaging Agents" submitted for the acquisition of the educational and scientific degree "Doctor" by Alexander Emilov Alexandrov, doctoral student in the Department of Gene Activity Regulation at the Institute of Molecular Biology "Acad. Roumen Tsanev", Bulgarian Academy of Sciences, in Professional Field 4.3. Biological Sciences.

The dissertation given to me for evaluation was authored by Alexander Emilov Alexandrov and prepared under the scientific supervision of Assoc. Prof. Dimitar Iliev, PhD.

The dissertation comprises a total of 71 pages and cites 99 references, all in English. The work follows the conventional structure, with the aim and objectives presented at the beginning. The literature review comprises 13 pages, "Materials and Methods" 5 pages, "Results" 26 pages, and "Discussion" 8 pages. The dissertation is illustrated with a total of 25 figures.

Glioblastoma multiforme remains one of the most difficult malignancies to treat, and resistance to temozolomide is a major obstacle to its successful therapy. In recent years, extracellular vesicles have emerged as an important factor in tumour biology. They participate in intercellular communication and in the development of drug resistance and are therefore increasingly considered as diagnostic markers. Degradative autophagy and the secretion of extracellular vesicles represent functionally interconnected and, to some extent, alternative mechanisms for the maintenance of cellular homeostasis. A point of convergence between the two pathways is represented by amphisomes—hybrid organelles formed by the fusion of autophagosomes with multivesicular bodies—which may be directed either towards lysosomal degradation or towards fusion with the plasma membrane and release of their contents. Therefore, blocking late-stage autophagy may redirect the accumulating cellular cargo towards a secretory pathway and increase the release of extracellular vesicles and extracellular nucleic acids. It is precisely this insufficiently studied balance between intracellular degradation and the extracellular elimination of cellular material that lies at the core of the dissertation and determines its relevance and practical significance.

The aim and objectives are presented at the very beginning of the text, which I consider an advantage. They are formulated clearly and specifically.

The literature review is logically structured and guides the reader from the general properties of extracellular vesicles, through autophagy and the relationship between the two processes, to glioblastoma multiforme and ATM kinase. The topics selected are precisely those required for

understanding the experimental part, and the review avoids unnecessary detail. The concise and accurate presentation demonstrates that the author is theoretically well prepared and capable of identifying and focusing on the essential issues.

The “Materials and Methods” chapter is written with precision. The author demonstrates command of a broad range of contemporary techniques, including differential centrifugation and PEG precipitation for vesicle isolation, immunoblotting and dot blotting, determination of particle-size distribution by dynamic light scattering, fluorescence and confocal microscopy, flow cytometry, and alkaline comet assay. Particularly commendable is the combination of methods from different fields, ranging from classical cell biology to biophysical particle-size measurements, which enables the same question to be addressed from several independent perspectives. The inclusion of appropriate controls, such as monitoring the mitochondrial marker cytochrome C1 to assess sample purity, demonstrates the doctoral candidate’s mature experimental approach.

The experimental logic of the work may be summarised in two consecutive lines of investigation. The first used the glioblastoma cell lines U87-MG, which are sensitive to temozolomide and do not express MGMT, and U138-MG, which express MGMT and are more resistant to temozolomide. The cytotoxicity of temozolomide and chloroquine was initially assessed both individually and in combination. Under the selected conditions, chloroquine sensitised the resistant U138-MG cells to temozolomide but did not substantially increase its cytotoxicity in U87-MG cells. Large and small extracellular vesicles were then isolated and their composition analysed. The temozolomide–chloroquine combination increased the secretion of fractions containing the autophagic markers LC3B-II and p62, the extracellular-vesicle markers CD63, syntenin-1, and ALIX, as well as caveolin-1. The sizes of the isolated particles were examined by dynamic light scattering. Microscopic analyses demonstrated the accumulation of LC3B in CD63-positive amphisomal/autolysosomal structures and the colocalisation of LC3B with caveolin-1, supporting the existence of a point of convergence between the autophagic and endosomal pathways. The increase in secretion following chloroquine treatment was verified using another late-stage autophagy inhibitor, bafilomycin A1, and another DNA-damaging agent, etoposide. Brefeldin A was included as a cytotoxicity control. At a comparable degree of cell death, brefeldin A did not cause a similar increase in autophagic and vesicular markers in the extracellular-vesicle fraction. Together with the absence of the mitochondrial marker CYC-1 from the extracellular-vesicle fractions examined, this supports the conclusion that the observed effect was not the result of passive release of cellular debris. It was additionally shown that chloroquine enhanced the temozolomide-induced γH2AX response, although the comet assay did not detect a corresponding increase in the overall amount of DNA damage.

The second experimental line investigated the relationship between ATM signalling, autophagy, and extracellular-vesicle secretion in HeLa Kyoto cells. The inhibitors KU-60019 and KU-55933 inhibited ATM kinase and induced the accumulation of LC3B-II and p62. KU-60019 also led to the accumulation and enlargement of LAMP1-positive lysosomes under serum-free conditions,

while the concentration used, 5 μM, did not significantly reduce cell viability under the same conditions. Treatment with KU-60019 or KU-55933 alone induced the secretion of small and large extracellular vesicles containing LC3B-II and p62, as well as CD63 and syntenin-1, without detectable CYC-1. A DNA-damaging agent is therefore not required for the increase in secretion itself. The addition of etoposide synergistically increased KU-60019-induced secretion of both large and small extracellular vesicles and extracellular DNA. In the final series of experiments, etoposide reduced the accumulation of p62 induced by KU-60019, whereas KU-60019 reduced the ubiquitination of γH2AX. These data outline a functional relationship between ATM, autophagic flux, DNA repair, and secretory processes. A significant contribution of the work is the demonstration that ATM inhibitors are capable of inducing extracellular-vesicle secretion and that this effect is enhanced by genotoxic stress.

The Discussion is written competently, and the results obtained are considered in the context of the literature published to date.

The dissertation presents ten conclusions that correspond to the experimental data, but the principal conclusions may be reduced to the following. Inhibition of degradative autophagy increases the secretion of extracellular vesicles and extracellular nucleic acids, while DNA-damaging agents further enhance this effect. The observation was reproduced using different combinations—TMZ with chloroquine or bafilomycin A1, and etoposide with chloroquine—supporting the existence of a general mechanism rather than a reagent-specific effect. The accumulation of LC3B-II and p62, together with the localisation of LC3B in CD63-positive structures, demonstrates a functional connection between autophagic and endosomal-vesicular trafficking. The data support a model in which impaired lysosomal degradation redirects part of the cellular cargo towards secretory pathways. The ATM inhibitors KU-60019 and KU-55933 independently disrupt the late stages of autophagy and induce the secretion of small and large extracellular vesicles. Etoposide further increases the secretion of extracellular vesicles and extracellular DNA, outlining a functional relationship between ATM signalling, autophagy, and the cellular response to DNA damage.

Two articles have been published on the subject of the dissertation, with Alexander Alexandrov as first author on both—a clear indication of his leading role in the work. One was published in the *International Journal of Molecular Sciences* and the other in the *European Journal of Biology*. The results have been presented at three scientific forums, including one oral presentation.

I agree with the submitted draft of the dissertation abstract. It presents the principal results of the dissertation with sufficient completeness.

Question to the doctoral candidate: Were there any additional methodological reasons, apart from the earlier studies cited in the Discussion, for conducting the experiments with the ATM inhibitors in HeLa Kyoto cells rather than in the U87-MG and U138-MG glioblastoma cell lines

used in the first part of the study, and to what extent do you consider that the results can be extrapolated to glioblastoma biology?

Conclusion: Alexander Alexandrov's dissertation is a comprehensive experimental study conducted at a high contemporary technical level. It identifies new interactions between the inhibition of degradative autophagy and DNA-damaging agents with regard to the secretion of extracellular vesicles and nucleic acids. The results achieved demonstrate that the author has developed into a highly qualified researcher with broad methodological expertise. The doctoral candidate fully meets the requirements of the Act on the Development of the Academic Staff in the Republic of Bulgaria and its implementing regulations for the acquisition of the educational and scientific degree "Doctor". On the basis of the above, I confidently recommend that the esteemed scientific jury evaluate Alexander Alexandrov's dissertation highly and award him the educational and scientific degree "Doctor" in Professional Field 4.3. Biological Sciences.

SIGNATURE:

/Assoc. Prof. Anastas Gospodinov, PhD/