REVIEW

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Regarding the dissertation by Alexander Emilov Alexandrov, INSTITUTE OF MOLECULAR BIOLOGY "ACADEMICIAN RUMEN TZANEV", SECTION "REGULATION OF GENE ACTIVITY", in professional field 4.3 Biological Sciences, speciality Molecular Biology, on the topic: "Synergistic induction of secretion of extracellular vesicles and DNA by autophagy inhibitors and DNA-damaging agents" for protection.

The dissertation is organised and presented in accordance with accepted national standards and includes: Introduction with aim and objectives - 2 pages; Literature review - 17 pages; Materials and methods - 6 pages; Results - 25 pages; Discussion - 8 pages; Conclusions – 1 page; Publications on the topic of the dissertation and participation in conferences – 1 page; Literature references, in which 99 sources are cited.

The theme of the dissertation, “Synergistic induction of secretion of extracellular vesicles and DNA by autophagy inhibitors and DNA-damaging agents”, is relevant and fully dissertable according to the Regulations of the Institute of Molecular Biology and Cell Biology of the Bulgarian Academy of Sciences for the implementation of the ZRASRB. Over the past decade, extracellular vesicles (EVs), which are secreted by almost all types of cells, have attracted the attention of the scientific community due to their diverse functions. In addition to their main role in intercellular communication, they participate in immune regulation, tissue repair and regeneration, the development of various diseases (including cancer), and the release of cellular waste. The composition and amount of secreted EVs vary greatly with cell state; therefore, they can serve as diagnostic markers in various diseases. Extracellular vesicles play an important role in the development of cancer by modulating the tumour microenvironment and immune response, participating in angiogenesis, and contributing to the development of resistance to applied therapies. Autophagy is also an important process in the development of cancer, albeit in dual ways. On the one hand, it participates in the suppression of tumour development; on the other hand, it can promote the survival of cancer cells, which can lead to resistance to anti-tumour therapies. Since there are interconnections between the processes of EV biogenesis and autophagy,

The aim of the present dissertation is to investigate the interaction between autophagy inhibitors and DNA-damaging agents with regard to the release of extracellular vesicles and extracellular nucleic acids induced by these agents.

The obtained data could be used to predict the potential therapeutic role, as well as the side effects, of the use of autophagy inhibitors and DNA-damaging agents in highly invasive brain tumour - glioblastoma multiforme.

Following a brief and clear Introduction that explains the core of the problem, the specific aim of this dissertation is outlined in the Aims and Objectives section, which states:

To study the interaction between autophagy inhibitors and DNA-damaging agents with respect to the release of EVs and extracellular nucleic acids induced by them

To achieve this aim, the following tasks were set:

1. Analysis of the cytotoxic activity of chloroquine (CHQ) and temozolomide (TMZ) in cultures of TMZ-sensitive (U87-MG) and TMZ-resistant (U138-MG) cells

2. Analysis of the composition and size of secreted EVs after treatment with CHQ and TMZ

3. Confirmation of the results with functional analogues – Bafilomycin A1 and etoposide

4. Analysis of the composition and quantity of secreted nucleic acids

5. Study of the amount of damaged DNA in cells during combined treatment

6. Study of the association of secreted nucleic acids with EVs by flow cytometric analysis

7. Study of the inhibition of autophagy by ATM inhibitors (ATMi)

8. Study of the influence of ATMi on EV secretion

9. Study of the interaction of ATMi with DNA-damaging agents in relation to EV secretion induced by them

The following is a concise literature review, which sequentially examines the main concepts and processes underlying the dissertation:

1. Extracellular vesicles - a review of their functions, classification by size, biogenesis and excretion is made. Attention is paid to their importance in the development of cancer. The main characteristics of the extracellular vesicle markers studied in this dissertation, including CD63, Syntenin-1, ALIX, and Caveolin-1, are described.

2. Autophagy – Three main types of autophagy and their role in the delivery and degradation of cellular material are described. Macrophagy is described in more detail, including the formation of specialised membrane structures, the activation of a cascade of autophagic proteins, the recruitment of cargo for autolysosomal degradation, and its degradation in lysosomes. Main attention is paid to the role of autophagy

3. Relationship between extracellular vesicle biogenesis and autophagy – Particular attention is paid to how these two complementary processes eliminate harmful or excess substances from the cell and maintain cellular homeostasis. Depending on the state of the cell, the contents of amphisomes (hybrid organelles formed by the fusion of autophagosomes and multivesicular bodies) can be degraded by lysosomes or excreted outside the cell. There is an equilibrium between these two processes, and if one is inhibited or defective, the other can be enhanced.

4. Glioblastoma multiforme – the main features and the applied therapy for this highly aggressive brain tumour are reviewed. The role of autophagy in disease progression and in the development of resistance to the applied therapy is considered. The relationship between autophagy and EV secretion is emphasised, as well as its modulation by the use of late autophagy inhibitors such as chloroquine and Bafilomycin A1.

5. ATM kinase and ATM inhibitors – the advantages of therapeutic strategies for combating cancer using inhibitors of enzymes involved in DNA repair, and of combining these inhibitors with genotoxic agents and applying radiation are described. The function of the enzyme ataxia-telangiectasia mutated (ATM) is described: it acts as a tumour suppressor by inducing cell cycle arrest and apoptosis, but can also support the survival of cancer cells by stimulating their

resistance to DNA damage. Two ATM inhibitors used in this dissertation (KU-55933 and KU-60019) are described and significantly increase the toxicity of double-strand break inducers. There is evidence of inhibition of autophagy by a different mechanism than these ATM inhibitors, which makes them an interesting subject for studying their influence on EVs secretion.

The literature review is well-structured, with the author competently summarising recent research in the field. However, it lacks depth, comprehensiveness and interconnectedness of the presented scientific data, which are needed to formulate hypotheses and determine the significance of the proposed study. Information on secretory autophagy is scarce, despite its potential connection to EV secretion. Including information on the immunomodulatory and neuroprotective effects of the inhibitors used would enrich the planned studies and provide additional depth. The review includes 6 informative figures; however, some are of low quality and poorly formatted in the text.

The Materials and Methods section correctly and in detail describes the materials used, including cell lines, common reagents, and antibodies. The methods used, such as cell viability analysis, EVs isolation, immunoblotting, DLS (Dynamic Light Scattering) analysis, fluorescence microscopy, extracellular nucleic acid analysis, and the comet assay, are described in detail.

The **Results** section includes:

I. Analysis of cytotoxic activity, composition, size and levels of EVs secretion from glioblastoma cells under the influence of autophagy inhibitors and chemotherapeutics

• Analysis of cytotoxic activity of chloroquine (CHQ) and temozolomide (TMZ) in cultures of TMZ-sensitive (U87-MG) and TMZ-resistant (U138-MG) cells

The study used TMZ-sensitive (U87-MG) and TMZ-resistant (U138-MG) glioblastoma cell lines to determine the potential interactions between TMZ and CHQ on EVs secretion. The effect of 10 μM CHQ on the cytotoxicity of 400 μM TMZ to U87-MG and U138-MG cell cultures after 48 hours of incubation in medium with 5% serum purified from EVs was compared. A sensitising effect of CHQ treatment on the cytotoxic effect of TMZ was observed only in the TMZ-resistant (U138-MG) glioblastoma cell line.

• Analysis of the composition and size of secreted EVs upon treatment with CHQ and TMZ glioblastoma cell lines

When examining the size of EVs secreted by U87-MG glioblastoma cells (TMZ-sensitive) treated with TMZ and CHQ (separately and in combination) by DLS analysis, only samples from cells treated with TMZ and CHQ showed measurable results. EVs with sizes of 50 to 150 (small EVs) and with sizes of 150-500 nm (large EVs) were detected. In the combined treatment, autophagic markers (LC3B-II and p62) and exosomal markers (alix, syntenin-1, and CD63) were significantly increased in large and small EVs, respectively. Caveolin-1 was detected in all samples of large EVs and was synergistically increased by the combination TMZ+CHQ. In cell lysates, the combined treatment also led to an increase in the autophagic markers p62 and LC3B-II.

In U138-MG (TMZ-resistant) glioblastoma cells, a synergistic effect of the combined treatment was also observed regarding the levels of LC3B-II and p62 in the large EVs fraction, as well as caveolin in that of small EVs. The same markers were also significantly increased in cell lysates.

In addition, CHQ treatment of U87-MG glioblastoma cells (TMZ-sensitive) was shown to induce LC3B accumulation within the lumen of CD63-positive amphisomes/autolysosomes and LC3B-caveolin-1 colocalisation within these structures.

• Confirmation of the obtained results upon treatment with functional analogues – Bafilomycin A1 and etoposide

As with CHQ, treatment of U87-MG cells with 50 nM BafA1 induced secretion of large EVs containing LC3B-II, p62, CD63 and caveolin-1, and these markers were significantly increased upon addition of TMZ. Combined treatment with BafA1 and TMZ significantly increased cytotoxicity. Addition of Brefeldin A (BFA) - an inhibitor of intracellular vesicular transport and EV secretion - did not change EV secretion from U87-MG cells.

To establish a potential synergistic effect of CHQ with other genotoxic agents with respect to increased EVs secretion, an experiment was performed with etoposide (a chemotherapeutic agent inducing DNA damage by inhibiting topoisomerase II). The results indicate that, like TMZ, etoposide significantly increases CHQ-induced secretion of autophagic markers by U87-MG cells.

II. Analysis of damaged DNA in cells, the composition and amount of secreted nucleic acids and their association with EVs

• Study of the amount of damaged DNA in cells upon combined treatment

It was found that CHQ, in combination with TMZ, significantly increased the levels of γH2AX in U87-MG cells. At the same time, no direct increase in the amount of damaged DNA in cells was observed.

• Study of the association of secreted nucleic acids with EVs by flow cytometric analysis

It was found that CHQ and TMZ synergistically induced secretion of extracellular nucleic acids in cultures of U87-MG cells. The samples contained molecules ranging from 200 to 20,000 base pairs, corresponding to a mixture of single-stranded DNA, double-stranded DNA, and RNA.

The association of secreted nucleic acids with EVs was investigated by flow cytometry. Combined treatment of U87-MG cells with CHQ and TMZ increased EV secretion and associated NK.

The data obtained from these subsections support the hypothesis that DNA damage (by TMZ and etoposide) in combination with lysosomal inhibition (CHQ and BafA1) leads to synergistically enhanced cytotoxicity and secretion of EVs and NK.

III. Study of the influence of ATM inhibitors, alone and in combination with DNA-damaging agents, on autophagy and EVs secretion

• Study of the inhibition of autophagy by ATM inhibitors (ATMi)

Use of two selective ATM inhibitors (KU-55933 and KU-60019) resulted in a dose-dependent accumulation of LC3B-II and p62 in HeLa Kyoto cells within autophagosomes or autolysosomes. In the absence of serum, KU-60019 induced co-localisation of the autophagy marker LC3B and the lysosomal marker LAMP1.

• Study of the influence of ATMi on EVs secretion

KU-60019 was found to induce a dose-dependent secretion of small and large EVs containing autophagic markers (LC3B-II and p62), as well as classical EV markers (syntenin and CD63). The presence of caveolin-1 was found only in large EVs secreted by HeLa Kyoto cells treated with KU-55933

• Study of the interaction of ATMi with DNA-damaging agents with respect to their induced secretion of EVs

Combined treatment of HeLa Kyoto cells with KU-60019 and etoposide leads to significant secretion of p62 and CD63 in large EVs, as well as LC3B-II, syntenin, CD63 and extracellular DNA in the fraction of small EVs.

The **Discussion** section discusses the main questions raised in the dissertation:

- ✓ The synergistic effect of the applied DNA-damaging agents and autophagy inhibitors on the induction of EVs and NA secretion occurs independently of the specifically applied DNA-damaging agents, as well as the applied inhibitors of late autophagy (chloroquine or bafilomycin) or ATM inhibitors. This suggests that the observed effect occurs universally upon DNA damage and inhibition of late autophagy, or autophagy in general.
- ✓ The induced active secretion of EVs is not predicted by high levels of apoptosis and release of apoptotic bodies. The reason for this is the lack of cytochrome C1 - a marker in EV samples.
- ✓ The colocalization of LAMP1 with the most intense foci of LC3 in the peripheries of cells treated with KU-60019 suggests that the possible mechanism for inhibition of autophagy is the likely inhibition of autolysosome formation.
- ✓ ATM inhibitors induce secretion of EVs from HeLa cells positive for LC3B-II and p62, which proves their autophagosomal origin.
- ✓ KU-60019-induced accumulation of p62 (autophagy marker) is the likely mechanism for inhibiting DNA repair.

In summary, the topic of this dissertation is aimed at clarifying the role of autophagy and the possible mechanisms involved in the secretion of extracellular vesicles and nucleic acids upon treatment of cancer cells with DNA-damaging agents, autophagy inhibitors and ATM.

The conclusions made are adequate and comprehensive. The emphasis is on the synergistic induction of EV and NAs secretion by autophagosomal inhibitors and DNA-damaging agents, which does not correlate with the proapoptotic activity of the combined treatment.

The synergistic secretion of EVs and NAs is probably due to DNA damage in the context of lysosomal inhibition. ATM inhibitors suppress autophagy at a late stage, inducing secretion of EVSs and DNA. ATM inhibitors in synergy with etoposide enhance the secretion of EVs and NAs. The presence of caveolin-1 in secreted EVs highlights their possible pathological potential in supporting tumour metastasis.

It would be very useful in the future to clarify the effects of increased EV secretion by cancer cells on angiogenesis and the immune response.

The dissertation follows a list of Publications related to the dissertation work and Participation in scientific conferences and seminars. From the lists presented, it is evident that the results of the dissertation work have been published in two articles with impact factors (both of which the doctoral student is the first author) and presented at national and international conferences.

The dissertation ends with a list of the literature used, and it is striking that brand-new titles were included, including current research on the topic of the dissertation.

On reviewing the material presented, it is clear that substantial research has been carried out in terms of both volume and quality, and that it fully meets the requirements of the Law on Scientific Degrees and Titles for the admission of the doctoral student Alexander Emilov Alexandrov to the defence for the acquisition of the scientific and educational degree "DOCTOR".

Question for the doctoral student: What could be the pathological role of caveolin-1 in EVs secreted by cancer cells?

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