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No.: 3-2-18/1

RESEARCH REPORT

Date: 12. July 2018

Subject of the research: *In vitro* determining the neuromodulatory potential of the Kamozin EXTRA

Client: "CarnoMed" D.O.O., Novi Sad

Scope of study: Experimental study

Data on the sample tested: Test sample supplement submitted by the client

Attachments:

1. Research report
2. Research results
3. Material and methods used in the research

Deliver to:

1. Client
2. Archives

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(Signatures and Stamp visible)

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Enclosure 1:
Research report
No.: 3-2-18 / 1
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Based on the obtained results of the study carried out *in vitro* conditions, on the continuous cell line MNA C1300 clone NA, sub-clone N2A, originating from mouse neuroblastoma, we can conclude that application of the growing concentrations (2mM, 5mM, 10MM) of the Kamozin EXTRA (Carnomed) capsule solution, increases the **proliferative activity** of the examined cell line.

Qualitative analysis of the cytoplasmic immuno-positivity of neuroblastoma on the intermedia neuron specific filament - nestin, an increased expression of the mentioned marker is observed in the application of increasing concentrations (2mM, 5mM, 10MM) of Karnozin EXTRA (Carnomed) capsule. This finding points to the **positive trophic potential of Karnozin EXTRA (Carnomed) preparation onto neuroblastoma cells *in vitro* conditions**.

Test performed:
Assistant Milan Popović, PhD

Results approved by:
Assistant Professor Ivan Čapo, PhD
Head of the Department for Scientific Research Development

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Enclosure 2:

Research results

Immuno-fluorescence staining with Ki67 antibodies on the continuous cell line MNA C1300 clone NA, subclon N2A, originating from mouse neuroblastoma, in the control groups, E1, E2 and E3, the proliferative index was calculated. The mean values of the Ki67 proliferative index for the control group are 86.26%, for group E1 are 92.67%, for group E2 are 94.29% and for group E3 95.73% (Chart 1).

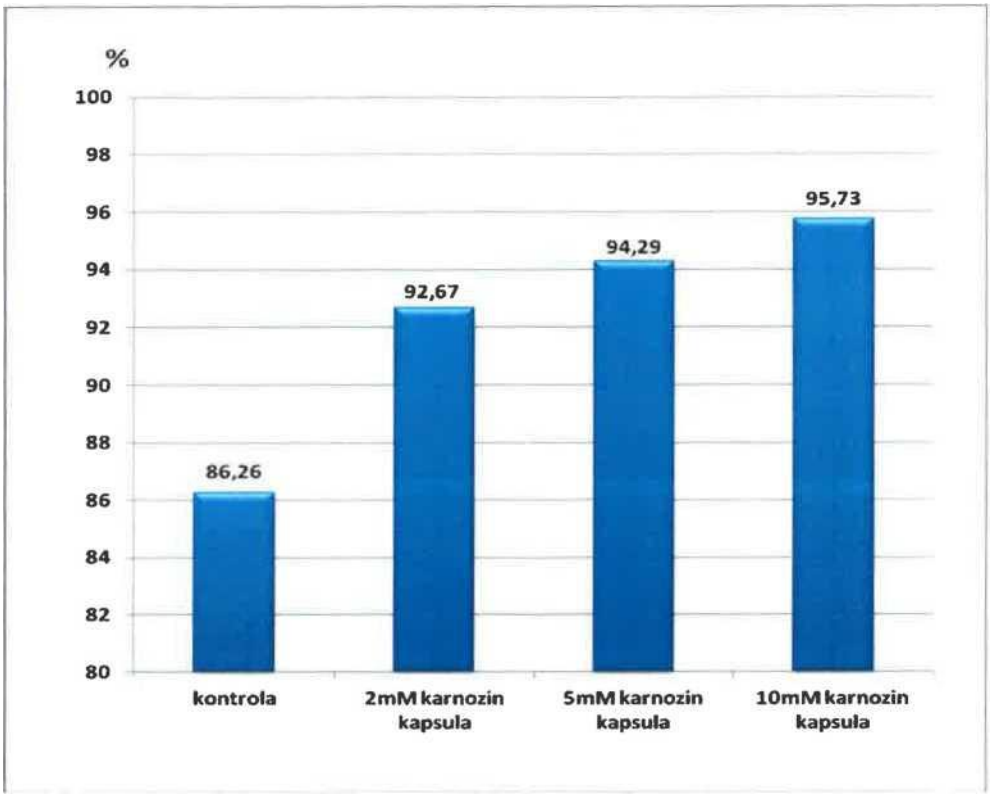

Chart 1. Mean values of Ki67 proliferative index per group

By qualitative and quantitative analysis of immuno-fluorescence images stained on Ki67 antibody, we can notice that with increasing concentration of the Karnozin EXTRA capsule, the Ki67 proliferative index, i.e. that at the highest concentration of 10mM there is a large number of cells that are in the proliferative phase (Figure 1).

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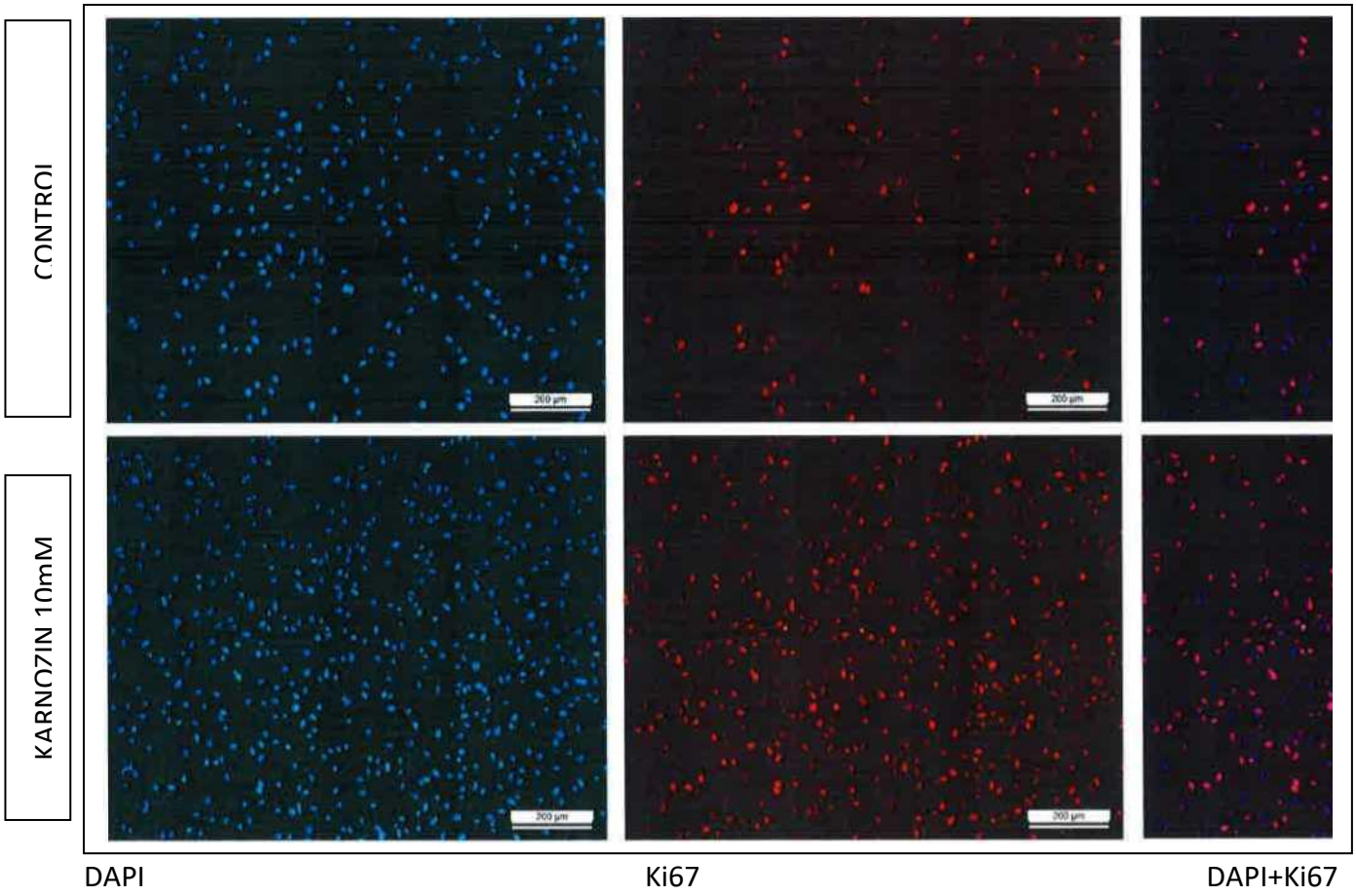

Figure 1. Continuous cell line MNA C1300 clone NA, sub-clone N2A, originating from mouse neuroblastoma-control and E3 group; Ki67 ICC / IF; 100x

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By qualitative analysis of the continuous MNA C1300 continuous cell line stained onto nestin cell line, the stronger cytoplasmic immuno-positivity of intermedial nestin filament in the E3 group could be noticed, in comparison to the control group (Figure 2).

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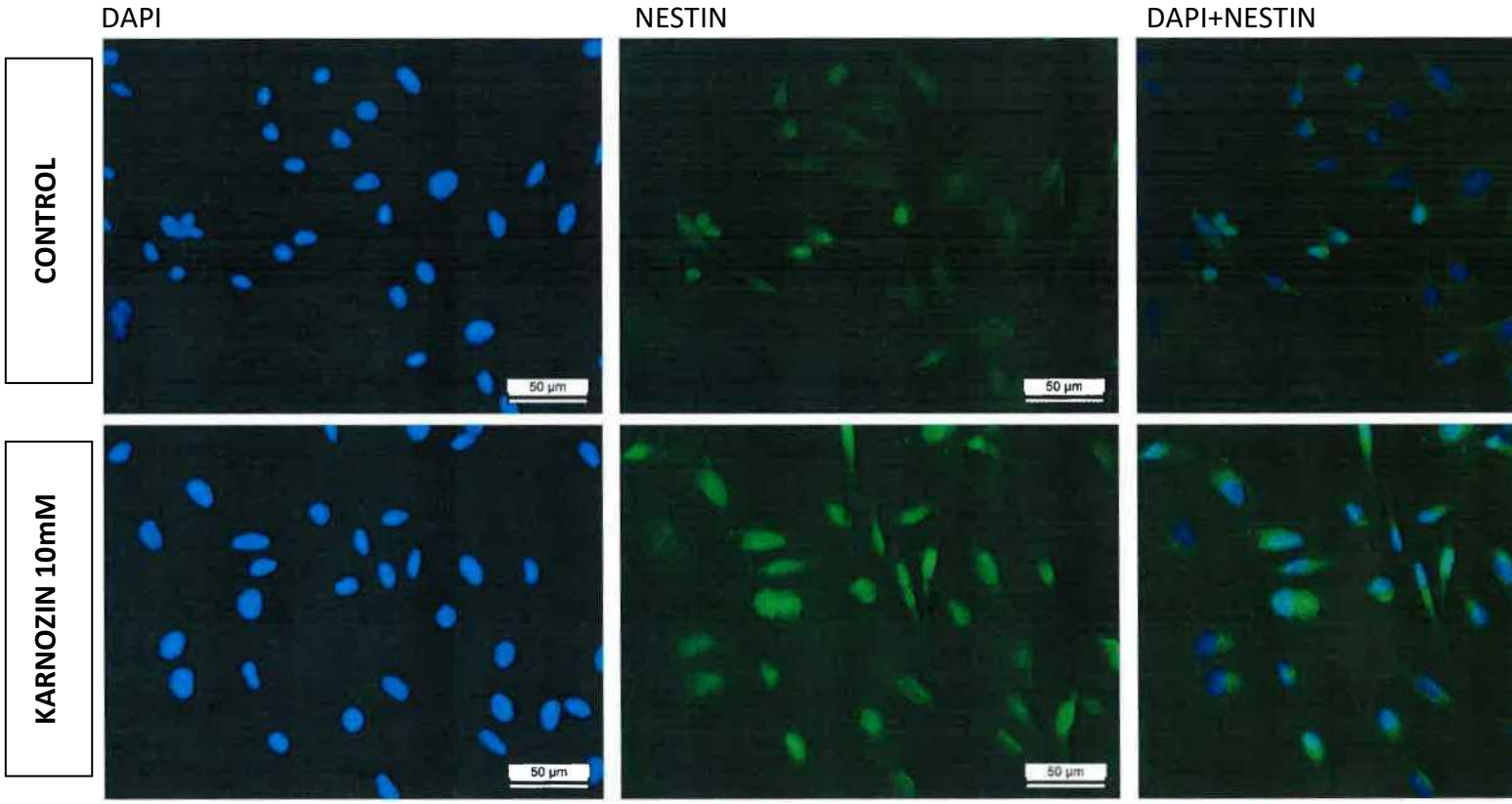

Figure 2. Continuous cell line MNA C1300 clone NA, sub-clone N2A, originating from mouse neuroblastoma-control and E3 groups; Ki67 IIC/IF; 400x

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Enclosure 3:
Materials and methods used in the research

The study was conducted on a continuous cell line MNA CI300 clone NA, sub-clone N2A, originating from mouse neuroblastoma. The experiment consisted of 4 groups: one control and three experimental. Each group was made in triplicate. In all cell groups, the cells were grown glued to the medium DMEM with 4.5 g / l glucose (DMEM HA, Capricorn Scientific), with addition of 10% fetal beef serum (FBS, Capricorn Scientific) and 2 mM glutamine.

The contents of the Karnozin EXTRA (Carnomed) capsule is prepared according to the following protocol: the content was dissolved in 1500 µl PBS, stirred and centrifuged at 3000 rpm at 22° C for 5 minutes. Aliquot of 11.94 µl of supernatant (2 mM) or 29.85 µl (5 mM) or 59.74 µl (10 mM) is added and filled up to 200 µl with PBS.

Samples of the cell line were seeded in 12 Petri cups, 35 mm in diameter, with a cap for subsequent immuno-fluorescence staining at concentration of 200,000 cells in 2 ml of DMEM medium (with 10% FBS and 2 mM glutamine). To the control group cells, 200 µl of phosphate buffer (PBS) was added into the medium. Cells from the first experimental group (group E1) were added 200 µl of capsule solution at a concentration of 2mM, cells in the second experimental group (group E2) were added 200 µl of the capsule solution at a concentration of 5mM, while in the third experimental group (group E3) was added 200µl of the capsule solution at a concentration of 10mM. The cell line was maintained for 24 hours in an incubator at 37^0 C., in an atmosphere of 100% humidity and 5% CO_2, afterwards the cap was stained with immuno-fluorescence stained antibodies of Ki67 (Thermo Scientific, RB-9043-P0) and nestin (Abcam, ab 176571).

Immunoflorescence technique for cell culture (ICC/IF)

After 24 hours, Petri cups with planted cells were removed from the incubator. The cell growth medium is drained and the cells are washed three times in PBS. They were then placed to 4% para-formaldehyde dissolved in PBS for 10 minutes. After placement and washing with PBS,

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cell permeability was performed with 0.3% Triton X-100 (Alfa Aesar, A16046) for 5 minutes.

The coats were then transferred to a humid chamber and treated with 10% normal goat serum dissolved in 1% BSA to block the non-specific staining for 30 minutes at 25 C. Then onto capsules are applied the primary antibodies of Ki67 (Thermo Scientific, RB-9043-P0) dilution 1:300 and nestin (Abcam, ab 176571) dilution 1:100. Primary antibodies were incubated at 25^0 C for 60 minutes. Secondary antibodies Alexa Fluor 555 (Abcam, Ab 150078) and Alexa Fluor 488 (Abcam, ab 150077) were applied in a dark chamber for a period of 30 minutes at 25° C. The covers were mounted onto the subject glass, using the DAPI medium (Abcam, ab 104139).

Microfotography and statistics processing

Microfilms of all four groups were analyzed in detail on the Leica DMLB 100T immuno-fluorescent microscope and photographed by the Leica MC 190 HD brand camera. On each cover, randomly selected 10 field views are enlarged at 100x and 400x magnifications. Coating microscopic photographs treated with Ki67 antibody were processed qualitatively and quantitatively while the micro-photography of the capsule treated with nestin antibody, were treated only qualitatively. The results obtained with immuno-fluorescence staining are shown graphically with corresponding descriptive statistics.

Ki67 proliferative index

Immuno-fluorescence micro-photography processing is performed in *Fiji* computer program, in with support of *Plug-in Cell Counter* counted DAP1 and Ki67 positive cell nuclei. The Ki67 proliferative index was calculated using the formula:

$$\text{Ki67 proliferative index} = \frac{\text{Ki67 positive nuclei}}{\text{DAPI positive nuclei}} \times 100\ (\%)$$