INSTITUTE OF BIOCHEMISTRY OF THE ROMANIAN ACADEMY

PhD THESIS SUMMARY

Tumor antigens with potential use for diagnosis and prognosis of malignant melanoma

Nanoparticles for targeted release of biologically active compounds

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Table of contents (PhD Thesis in extenso)

FIRST PART - TUMOR ANTIGENS WITH SIGNIFICANCE FOR THE DIAGNOSIS AND PROGNOSIS OF MALIGNANT MELANOMA

Melanoma is a very agressive type of skin cancer with rising incidence caused by the uncontrolled proliferation of the melanocytes in the basal layer of the epidermis (Cutaneous melanoma) or other anatomical sites such as gastrointestinal, respiratory and genitourinary mucosae (mucosal melanoma), iris (uveal melanoma), cochlea (primary melanoma of the cochlea). Cutaneous melanoma is the most common type with associated risk factors such as: sun burns, UV exposure from tanning beds and the presence of dysplastic naevi.

Cutaneous melanoma accounts for 1% of total skin cancer cases but is the cause of 80% of deaths due to increased dissemination potential and intra and inter tumoral citological heterogenity. These features lead to chimio or radio therapy resistance, challenges in establishing the right diagnosis and variable response rates to targeted therapy. Cutaneous melanoma can mimmic common benign non melanocytic lesions such as lentigines and seborrheic keratoses, or can be hard to distinguish from displastic or Spitz naevi and in some cases it is challenging to diagnose melanoma metastases with unknown primary tumour. (1). One third of the extremely agressive nodular melanoma cases are unpigmented and difficult to diagnose following the ABCDE rule (asymmetry, border, color, diameter and evolution) usefull for other melnoma subtypes. Besides the heterogeneous macroscopic aspect melanomas are also highly variable at the molecular level and present frequent loss of specifically expressed melanoma antigens (tirosynase-Tyr, gp100/pmel17, Melan-A/MART-1, gp75/TRP1 and DCT/TRP-2). Although the majority of melanoma cases can be diagnosed based on the hematoxylin eosin staining of histological specimens, or when necessary, after detection of melanoma antigens routinely used in the anatomo phatological laboratories (Tyr, HMB-45, Melan-A), there are cases with complete loss of differentiation antigens during tumor progression (2). This is why, adding new specific antigens to the melanoma detection antibody pannels can be beneficial for the evaluation of certain difficult to diagnose cases.

Besides challenges in diagnosis, melanoma patients face a low survival rate at 5 years if the melanoma has already spread to limph nodes and distal organs (lung, brain, liver) compared to a 90% survival rate in case of localized disease (3). Because of the forementioned aspects, accurate staging and prognosis following diagnosis is mandatory for establishment of the best treatment regimen.

In case of thin melanomas (thickness ≤ 1mm) surgical excision leads to healing and lack of recurrences for most patients, still the survival rate for this group is over 90% but not 100%. For this reason there is increased need for classifing thin melanoma pacients in low risk or high risk groups the latter benefitting from aditional treatment options like lymph node dissection or adjuvant therapy with interferon. The newest recommendations for clinical management of thin melanomas include sentinel lymph node dissection expecially if the lesions are associated with factors of unfavorable prognosis like vertical growth phase, regression, ulceration and a high number of mitoses. In case of early stage melanoma the positive sentinel limph nodes can be overlooked because tumour cells are present as micrometastases which are difficult to diagnose and able to cause disease reccurence after excision of primary tumour and limph node dissection. For this reason, numerous studies are trying to identify molecular factors associated with unfavourable prognosis in melanoma. Expression of proteins involved in cellular adhesion and extracellular matrix remodeling like N-cadherin, osteopontin and SPARC (osteonectin) has been significantly associated with metastasis development (4). Elevated expression of MCAM/MUC18 specific to melanocytes, LI-CAM specific to neurons and CEACAM-1 expressed in glandular tissues is associated with reduced survival and indicates an abnormal interaction between tumor cells and surrounding stroma. Increased expression of matrix metallo proteinase 2 (MMP-2) and tisssue plasminogen activator (tPA) is also associated with unfavourable disease outcome (5). Proteins involved in cellular proliferation, Ki67 and p16/NK4A- an inhibitor of cyclin dependent kinases which regulates cell cycle by slowing down G1-S transition, are potential useful markers for disease prognosis and indicate reduced overall survival. For thin melanomas but also for the ones thicker than 1 mm, Ki-67 expression directly correlates whith prognosis and it is superior to mitotic index (6). Investigation of molecular factors significant for melanoma progression led to the development of a prognosis test which analysis the expression of 28 genes in order to establish the potential of metastatic spread of the disease. This test is currenly used on a small scale for clinical assesment of melanoma patients

and indicates if a patient belongs to a high risk group with 69% risk of developing metastatic disease or to a low risk group with 3% risk of disease progression during the next 5 years. Decision DX toghether with sentinel limph node evaluation can identify 70-80% from the patients with negative GSL and a high risk for disease progression, but the test is expensive and demnands a certain quantity of tissue which is not always available. Although currently the anatomo pathology laboratories do not use molecular markers for evaluating patients prognosis, the existance of such markers could be extremely useful for the clinical management of patients and lowering the risk of reccurence. There is a need for continuos investigation and updating of the multiple molecular pathways that activate in cutaneous melanoma that lead to the identification of new molecules relevant for diagnosis and prognosis of this malignancy.

Results and Discussions

1. The obtainment and characterization of anti h-DCT polyclonal antibody

The levels of mRNA and protein for melanoma antigens are often decreased as a result of the activation of mechanisms which sustain tumor progression, and DCT is such an example, when its expression drops during metastatic progression from 84% in primary tumors to 58% in metastases. The immunodetection of melanoma antigens in histopathological specimens by IHC/IHF can be improved using policlonal antibodies which, unlike the monoclonals, recognize more than one epitope, significantly increasing tumor cell detection.

The sequence of luminal domain of DCT (aa 27-439) has been analyzed for the prediction of the antigenic sites (dr.Adina Milac) and the results indicated with high confidence the presence of 19 potential antigenic sites. The first 130 aminoacid residues from N-terminus which do not contain glycosylation sites demonstrated the number of peptides with the highest antigenic score. However, possible additional epitopes are indicated in other areas of the DCT polypeptide where potentially occupied glycosylation sites are present. We, therefore, considered that using the aa27-aa439 sequence, as immunizing polypeptide would represent a benefit in raising a polyclonal serum able to recognize different epitopes on DCT structure, in melanocytic tissues.

1.1 The characterization of anti-hDCT antibodies (anti-hDCT Abs)

The anti-hDCT Abs were further analyzed by different methods and techniques in order to determine their specificity and sensitivity.

Specificity of anti-hDCT Abs in western blotting– the anti-hDCT Abs detects the two DCT glycoforms which appear as a doublet of 62 and 83 kDa in melanocytic cell lines (B16F10, B16F1, MJS, SK28) but not in non-melanocytic ones (HEK, HeLa) or in cell lines which do not express endogenous DCT (A375) **(Fig.1A)**. To further confirm the antibody specificity, we temporarily decreased the expression of DCT by small interferrence RNA (si-RNA) (+) in MJS and SK28 cell lines in which DCT expression was validated (A) and compared it with control samples (-). As **Fig. 1B** shows a significant decrease of the specific signal for DCT is observed. Calnexin was used a loading control.

Specificity of anti-hDCT Abs in immunoprecipitation- Anti-hDCT Abs specifically immunoprecipitate from MJS cell lysates labeled with ^{35}S the two DCT glycoforms, the 62kDa representing the DCT precursor and the 80kDa representing the mature DCT, which were synthetized within 30 min and 180 min of labeling respectively. The lysate of HEK cells which does not express DCT was used as a negativ control **(Fig. 2).**

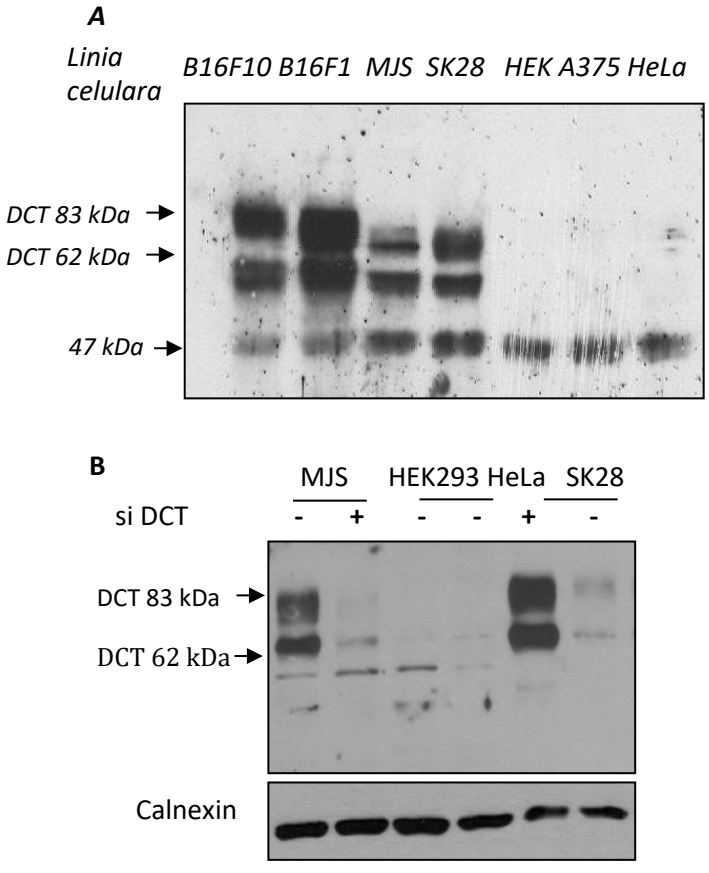

Figure 1. Analysis of the anti-hDCT Abs specificity by WB in melanocytic and nonmelanocytic cell lines (A) and by transient downregulation of DCT gene expression (B).

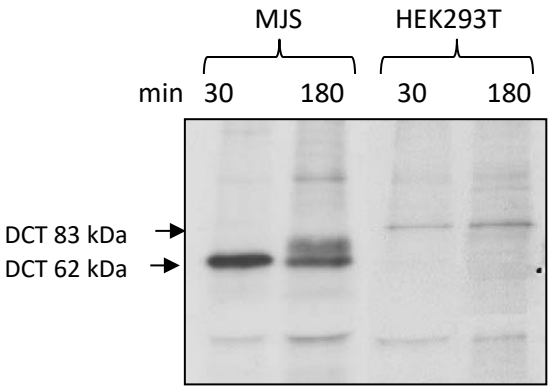

Figure 2 Analysis of anti-hDCT Abs specificity by metabolic labeling and immunoprecipitation

Specificity of anti-hDCT Abs in immunohistofluorescence and immunohistochemistry

The expression of DCT and Tyr are simultaneously detected with anti-hDCT (red) and anti-Tyr (green) in common cells **(Fig.3a, yellow)** or distinct cells (red-**Fig. 3b** or green, **Fig. 3c)**. DCT is specifically detected in nevic cells from benign lesions (**Fig. 3a,b**) and in tumor cells **(Fig. 3c,d,e,f)** which appear in intraepidermal nests (**Fig. 3 e**) or in nodular melanoma cells **(Fig. 3 ,f).** DCT is not detected in colorectal, gastric or squamous cells carcinoma **(Fig. 3 g,h,i).** The IHC images **(Fig. 3 e,f)** have been obtained by Dr. Sabina Zurac, Anatomopathology Division, Colentina Hospital.

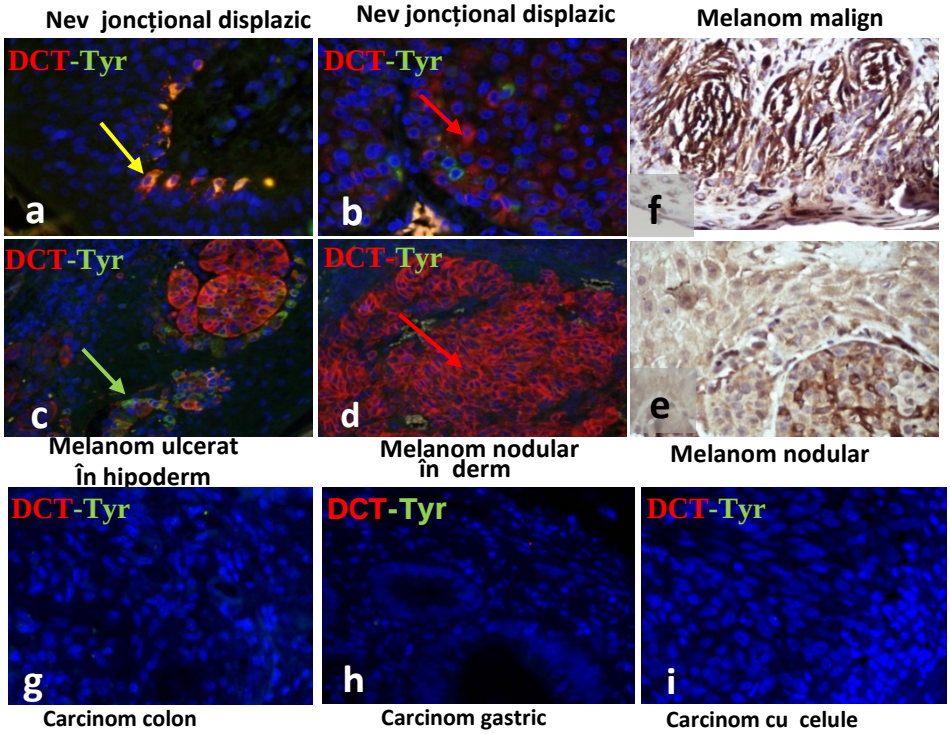

Figure 3. The analysis of histopathological specimens representing benign and malignant melanocytic and nonmelanocytic lesions with anti-hDCT Abs in immunohistofluorescence (a,b,c,d,g,h,i) and in immunohistochemistry (e,f).

2. The comparative analysis of DCT and Tyr expressions in melanoma cell lines and in histopatholgical specimens representing human melanocytic lesions

2.1 DCT is a fully processed antigen in human melanoma cell lines whereas Tyr is either not expressed or incompletely processed

DCT and Tyr are proteins with homologous structures, both being involved in melanin synthesis. The melanogenic proteins are also expressed by transformed melanocytes (melanoma cells) but their level of expression and processing are differently altered in tumors, this event having a negative impact on their detection in tumor cell populations. The reports of other groups indicated that DCT expression is increased whereas the one of Tyr is decreasing in murine melanomas in advanced stages of malignity and in the ones unpigmented as well (14). The analysis of DCT and Tyr was done here comparatively at the mRNA and protein levels, in human pigmented metastatic cells, MNT-1 cell line, in human unpigmented primary melanoma, MJS cell line and in human unpigmented metastatic melanoma, SK28 cell line **(Fig. 4**). At mRNA level the SK28 cells express both markers whereas the MNT-1 and MJS cells have lower DCT levels. Tyr is intensely expressed by pigmented MNT-1 cells but is absent in MJS cells which is positive for DCT **(Fig. 4A).** The A375 cell line, representing human metastatic amelanotic melanoma, being already known for lack of both antigens DCT and Tyr at mRNA levels, has been used a negative control for melanocytic lineage. The SaOs2 is an osteosarcoma cell line and has been used as a negative control for nonmelanocytic lineage. Both cell lines, A375 and SaOs2 have confirmed the specificity of the primers used for DCT and Tyr. At protein level DCT is detected in three lines of melanoma as the well known doublet pattern of 82 kDa and 62 kDa representing the two glycoforms complex and precursor respectively. **(Fig. 4B).** After treatment with EndoH the band of 82kDa is moving slightly is a lower position whereas the 62 kDa band which contains only oligomannosidic glycans, totally sensitive to EndoH digestion is running in the same position with fully deglycosylated DCT polypeptide with PNGase F. The Tyr band is visible in MNT-1 and undetectable in MJS, as expected **(Fig. 4C).** In MNT-1 Tyr appears as major fraction, partially sensitive to EndoH, representing mature Tyr with hybrid glycans and a smaller fraction of Tyr-precursor totally sensitive at EndoH. The SK28 cells express only imature Tyr, with oligomannosidic glycans which after EndoH digestion co-migrate with Ty-polypeptide **(Fig. 4C).**

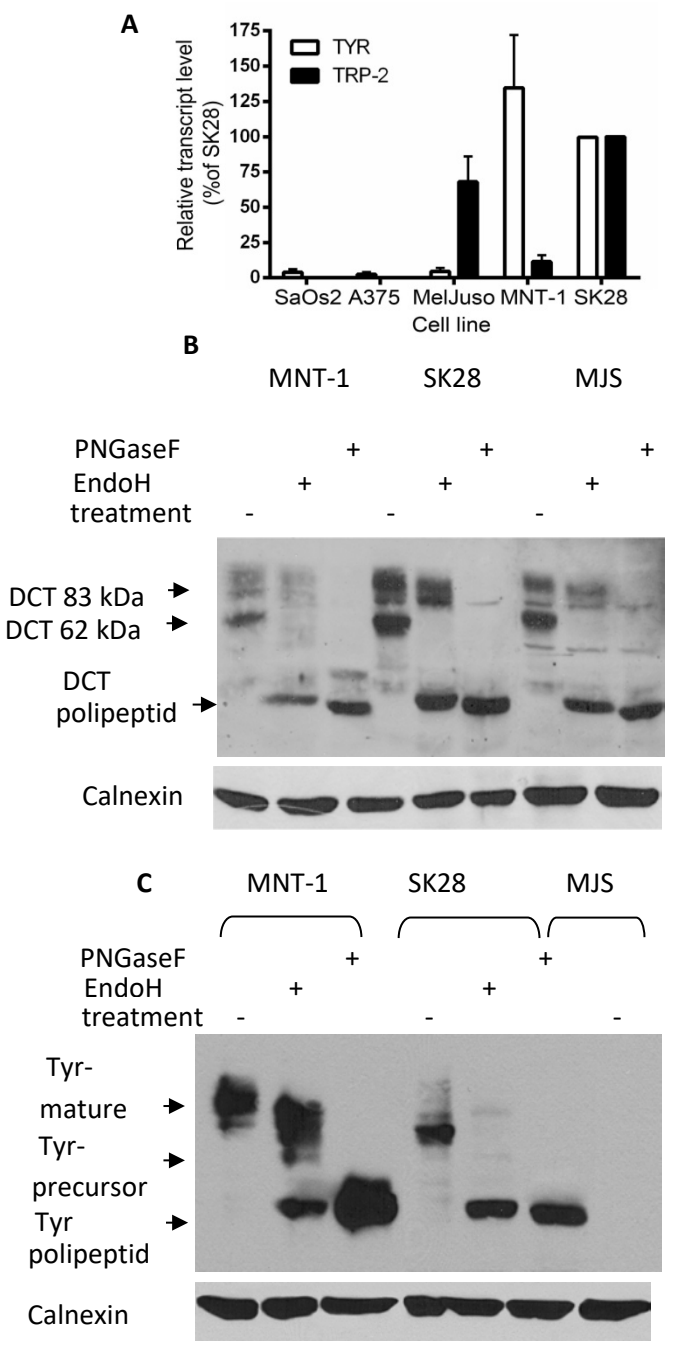

Figure 4 DCT and Tyr are antigens with distinct levels of expression and processing in human melanoma cell lines Figure was reproduced with permission Wolters Kluwer Health/ Lippincott Williams & Wilkinsafter Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

2.2 Disjunction of DCT and Tyr expressions in the process of melanocyte neoplastic transformation generates multiple antigenic profiles

The results obtained studying the cell lines indicated that distinct DCT and Tyr expressions may also occur in melanocytic tumors and represented the rationale for further analysis of these two antigens in both benign and malignant melanocytic lesions.

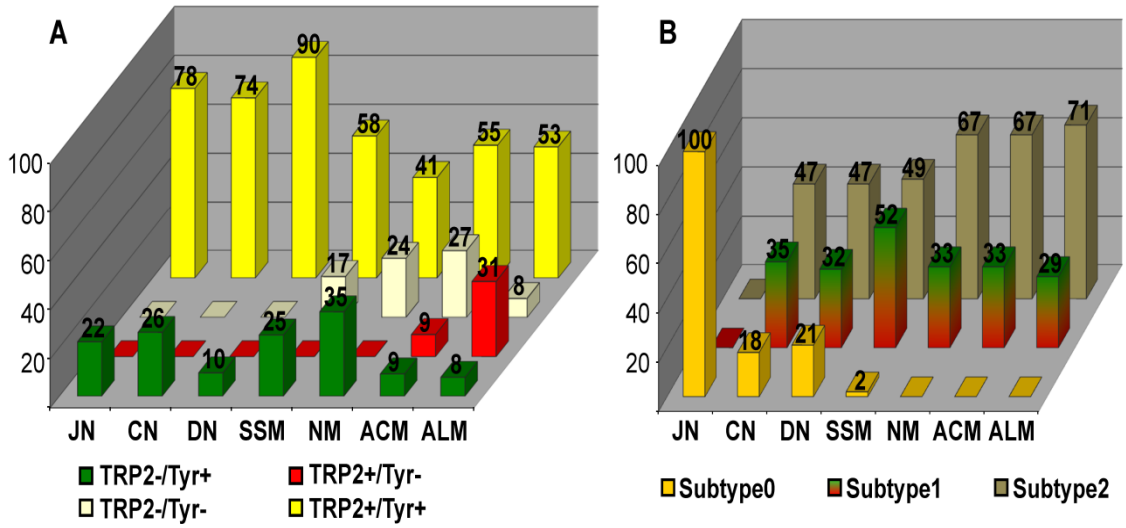

Figure 5 The schematic representation of DCT and Tyr expression analyzed by immunohistofluorescence in human histological specimens representing benign and malignant melanocytic lesions. Classification of benign and malignant melanocytic lesions in relation to the expression of DCT and Tyr. The histological specimens indicated on the x-axis are analyzed for DCT and Tyr expression and the contribution of each type expressed as percentage from the total number is indicated on each column (A). Distribution of subtype 0, 1, and 2 within DCT+/Tyr+ specimens. The histological specimens that express the subtype 0, 1, and 2 architecture are indicated on the x-axis. The contribution of each subtype calculated as percentage from the total number of DCT+/Tyr+ specimens is indicated on the corresponding column (B). ACM, achromic melanoma; ALM, Acral lentiginous melanoma; CN, compound nevus; DN, dysplastic nevus; JN, junctional nevus; NM, nodular melanoma; SSM, superficial spreading melanoma. Figure was reproduced with permission Wolters Kluwer Health/ Lippincott Williams & Wilkinst after Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

To confirm this theory and establish whether this molecular event is correlated with neoplastic transformation and malignant progression we analyzed the expression of DCT and Tyr in 166 histological specimens representing benign and malignant melanocytic lesions: 9 junctional nevi (JN), 23 compound nevi (CN), 21 dysplastic nevi (DN), 60 superficial spreading melanomas (SSM), 29 nodular melanomas (NM), 11 achromic melanomas (ACM) şi 13 acral-lentiginous melanoms (ALM). Using IHF microscopy, the simultaneous detection of DCT and Tyr in identical cell populations of tumor components in different specimens is possible, practically translating the experimental approach used on cell lines to human histological samples. The immunostained, analyzed and scored samples were grouped into four types: positive for either Tyr or DCT (DCT–/Tyr+; DCT+/Tyr–), double negative, or double positives for both Tyr and DCT (DCT–/Tyr–; DCT+/Tyr+). The percentage distribution within each group is schematically presented in **Figure 5A**. The exclusive expression of Tyr, however in different percentages, is maintained in all types of lesions which confirms that Tyr is the standard biomarker in the melanoma evaluation, whereas the samples which express DCT are specimens of ALM (31%) and ACM (9%). The presence of double negative specimens (DCT-/Tyr-) is nule in the group of the benign lesions (JNs, CNs) and premalignant (DNs) and is increased in the malignant ones (SSMs, NMs, ACMs). DCT and Tyr are the melanozomal enzymes essential for melanin production in skin melanocytes which are always positive for both antigens. In the neoplastic transformation of the melanocytes and melanoma malignant progression occurs the decrease or even the lack of the expression of the two antigens (15), DCT and Tyr being either co-expressed or distinctly expressed in the analyzed specimens. The presence of DCT in most of the tumors is in agreement with other studies which reports that DCT is expressed in 67% of the analyzed nevi, in 83% of the primary melanomas and in 100% of the metastatic melanomas (16). There is a clear correlation between the process of neoplastic transformation of the melanocytes in the malignant progression and the molecular process of the dissociation of expressions of DCT and Tyr in distinct cell populations (**Fig. 5A**). Co-expression of DCT/Tyr is characteristic only for junctional nevi (JN) which represent the most benign melanocytic tumor, appears in a lower percentage in other benign nevi as the compound ones (CNs-18%) or premalignant tumors as dysplastic nevi (DNs-21%) and is absent in malignant lesions (SSMs, NMs, ACMs). Oppositely, the double negative specimens are not detected in nevi and their percentage is increased in malignant melanomas. This is in agreement with the theory that there is a severe decrease of the

melanoma antigen expression during the increase of the malignity, which contributes to the tumor evasion from the control of the immune system.

2.3 The molecular architecture of DCT+/Tyr+ specimens - The DCT phenotype

In each type of lesion more than 50% of the tested specimens express both antigens DCT and Tyr (DCT+/Tyr+). Following the distribution of the two antigens in the cells of the tissue layers we identified three situations, conventionally named subtypes 0, 1 and 2 **(Fig.5B,Fig.6).** ***Subtype 0*** , defined by nevus or tumor cells that coexpressed DCTand Tyr, is characteristic of JNs (JN0 represents 100% of all double positive JNs), to a less extent to CNs, DNs, and is almost absent in SSMs, NMs, ACMs, and ALMs. A representative image of the subtype 0 presented in **Fig. 6a-c**, shows a JN0 specimen in which all nevus cells from distinct parts of the sample contain both Tyr **(Fig. 6a, c)** and DCT **(Fig. 6b,c).** ***Subtype 1 or DCT-phenotype*** is characterized by the appearance of numerous individual or clustered cells that are positive for DCT or Tyr. The segregation of DCT+/Tyr– and DCT–/Tyr+ mixed cell populations becomes increasingly more marked as the tumor cells are located within the dermis, and this process culminates with the complete dissociation of DCT and Tyr expressions in cells occupying large ID areas. The DCT phenotype is absent in JNs and expressed in approximately similar percentages in CNs, DNs, NMs, ACMs, ALMs, and SSMs **(Fig. 5B).** A representative image of this subtype in a DN1 specimen is presented in **Fig. 6d–i.** Individual or grouped cells expressing Tyr are located in the subepidermal area (SE) **(Fig. 6,d**) and they may or may not express DCT **(Fig. 6e).** In tissue depths, Tyr staining becomes completely negative (Fig. 6g) and tumor cells retain only DCT expression **(Fig. 6h).** The merged images and the corresponding insets clearly show that the dissociation process of DCT and Tyr expressions starts in the upper layers of tumor components **(Fig. 6f)**, where cells that still preserve the expression of both DCT and Tyr appear as yellow next to green or red cells, which express only Tyr or DCT, respectively. The perpetuation of DCT+/Tyr– clones continues in the middle or deep dermis which completely lacks green fluorescence **(Fig. 6i).** Similar patterns defining the subtype 1 were also detected in CNs or in SSMs and are further presented in **Fig. 7** and **Fig.8**. The subtype 1 is detected in NMs in a similar range of percentage as in CNs and DNs **(Fig. 5)**, showing, however, a pattern of DCT/Tyr different from that described in CN1, DN1, or SSM1. In CN 1, DN 1, or SSM 1 samples, the segregation between DCT and Tyr expression is gradual, whereas in NM1, this process is more

abrupt. In the SE area of the NM1 specimen, no cells coexpressing both antigens could be observed, but cells expressing either DCT (red) or Tyr (green) were already grouped into small nests or clusters **(Fig. 6j).** The ID component **(Fig. 6k)** or deep dermis **(Fig. 6l)** of NM1 specimens also contains large areas with DCT+/Tyr– cells similar to that observed in the lower dermis of CN1 or SSM1. A particular situation of subtype 1 is encountered in several ALM and ACM specimens **(Fig. 6m–o)** in which Tyr expression is completely negative in the entire specimen, whereas DCT is intensely expressed in tumor nodules already formed in the SE area **(Fig. 6m)** as well as in the ID component **(Fig. 6n)** or in the deep dermis in cells positioned along the tumor vasculature **(Fig. 6o).** ***Subtype 2*** is defined by the presence of cells that express DCT and Tyr either together or dissociated along different tissue layers of the investigated specimen. In specimens scored as subtype 2, there is not a complete lack of DCT or Tyr expression in the lower dermis **(Fig. 6p, r and s**

2.4. The clinical significance of the DCT -phenotype

In an attempt to find the clinical significance for the DCTphenotype defined above, we associated the data obtained from DCT/Tyr analysis by IHF with the data from anatomopathological reports. We have identified 2 benign lesions represented by compound nevi and 2 malignant lesions of SSM and following their analysis we observed that they support the theory according to which DCT phenotype could be associated with some clinical parameters of unfavourable prognostic and these data are presented in **Fig. 7** and **Fig.8.**

The DCT –phenotype is associated with neurotization process in benign melanocytic lesions

In **Fig. 7** are presented two specimens, one representative for DCT phenotype and another with a different molecularity, conventionally referred as CN1-A **(Fig.7,a-f)** and CN-B **(Fig. 7, g-l)** respectively. The CN1-A specimen belongs to DCT-phenotype, characterized by the dissociation observed in the SE layer **(Fig.7,a-c)** and continued by perpetuation of nevic cells DCT+/Tyr- in middle dermis **(Fig.7, d-f).** The CN-B specimen belongs to subtype 2, with cells co-expressing DCT and Tyr groupped into subepidermal nests **(Fig. 7,g-i).** Intradermally, the immunostaining for DCT and Tyr is weak but detectable **(Fig. 7, j-i).** The anatomopathological reports for the described specimens in **Fig. 7** indicate that specimen scored as DCT-phenotype

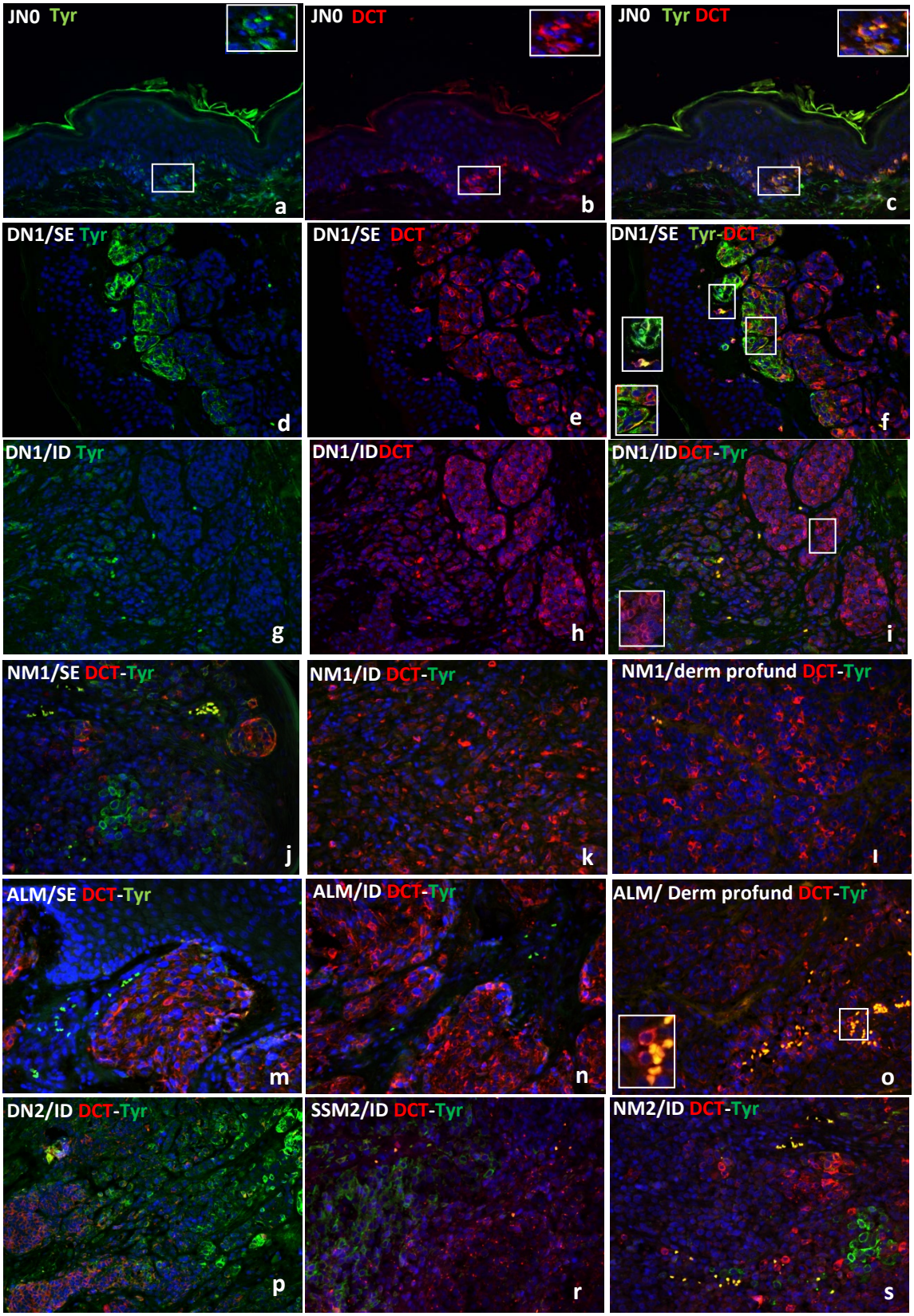

Figure 6 The molecular achitecture of subtype 0,1 and 2 in DCT+/Tyr + specimens. Samples were labeled for DCT (anti-hDCT), Tyr (T311) and nuclei (DAPI/blue) and analyzed by IHF. Representative specimens for each subtype (0, 1 și 2) and tumor components (SE, subepidermal; ID, intradermal; deep derm) are presented. Original magnification 40X. Figure reproduced with permission Wolters Kluwer Health/ Lippincott Williams & Wilkinst after Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

(CN1-A) shows and increased neurotization in tissue depth, unlike the specimen CN-B which shows moderate neurotization.

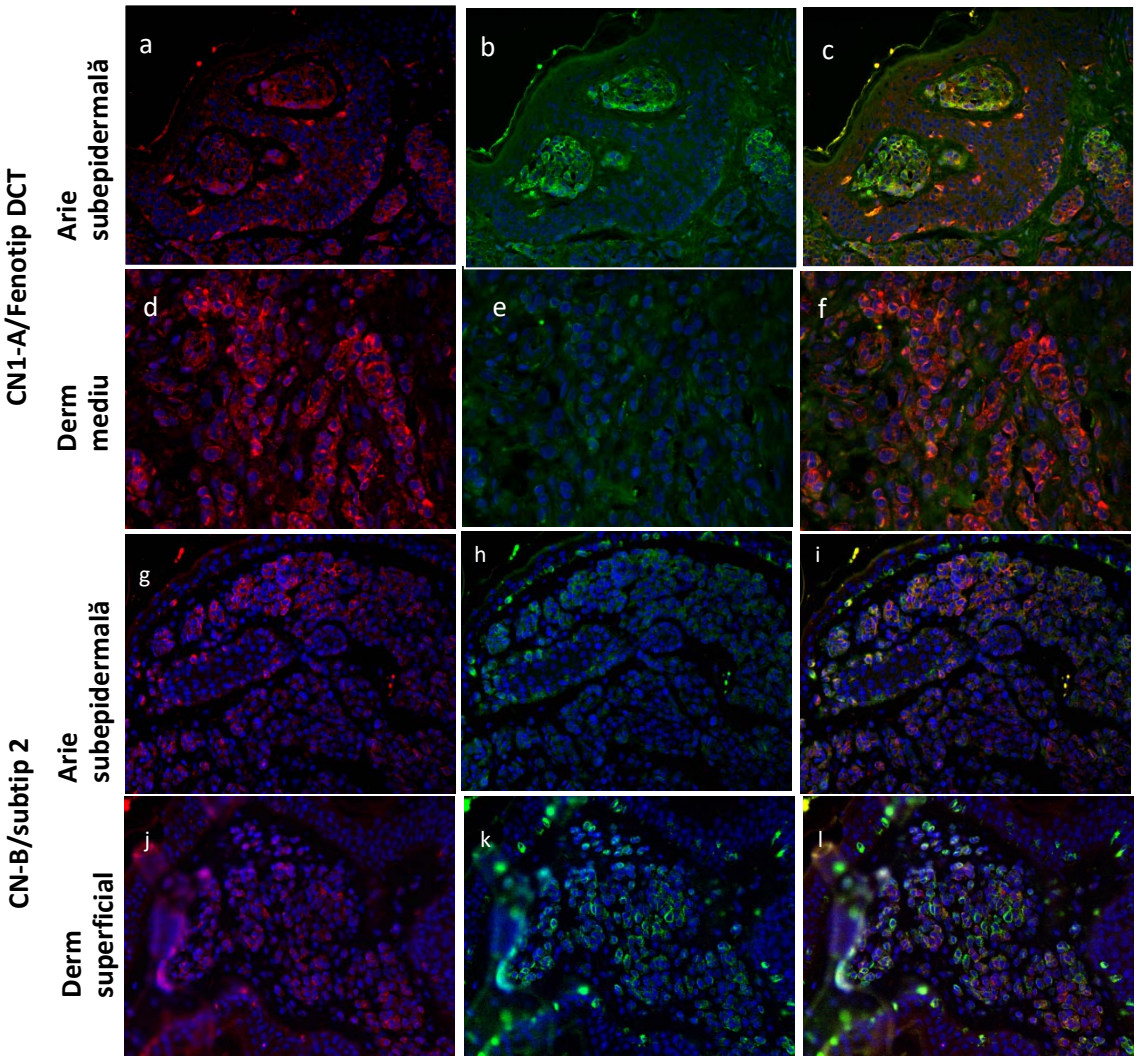

Figure 7. The molecular analysis of two specimens of benign melanocytic lesions The samples were labeled for DCT (anti-hDCT), Tyr (T311) and nuclei (DAPI/blue)and analyzed by IHF. The DCT fenotype is characteristic of the specimen with increased neurotization (CN1-A). The original magnification is 40X. Figură adaptată cu permisiunea Wolters Kluwer Health/ Lippincott Williams & Wilkinst după Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

The DCT –phenotype is associated with ulceration and mitoses in malignant mmelanocytic lesions

In **Fig. 8** are presented two SSM specimens referred here as SSM1-A **(Fig. 8a,f)** and SSM1-B **(Fig. 8g,l).** The specimen SSM1-A shows the pattern of DCT-phenotype with the dissociated expression in the melanoma cells of DCT and Tyr which starts in IE areas **(Fig. 8a,c)** and the protrusion of tumor cells DCT+/tyr- in dermis **(Fig. 8d-f).** The SSM-B specimen belongs to subtype DCT-/Tyr+ with large intraepidermal areas occupied by DCT-/Tyr+ **(Fig. 8g-i)** which invade the papilar dermis **(Fig.8j,l).** The specimens scored as DCT-phenotype, SSM1-A **(Fig. 8g-i)** distingueshes from the other one SSM-B by ulceration and number of mitosis. Inportantly to note is that in both specimens , SSM1-A and SSM-B, the Clark and Breslow indexes are identical (II) and 0, 45 and 0,45 respectively and what makes the differences between them are ulceration, the number of mitoses and the molecular architecture of DCT/Tyr. Previous reports about murine melanoma cell lines showed that DCT expression is independently regulated from the other TRPs (17). The data of this study about DCT and Tyr confirmed that expression and processing of the two antigens is distinct in human melanoma cell lines as well. Therefore, the result that cell populations in histopathological sections express separately DCT and Tyr was not an unexpected finding. More unexpected is the fact that in the DCT-phenotype, Tyr+cells always remain in the upper part of the tumor components, whereas DCT+ clones are always in the deep dermis. The different DCT/Tyr protein expression in tumor components complements the study of Hashimoto et al. (18) which showed that in acquired melanocytic nevi, Tyr protein expression is restricted to the basal/upper dermis, whereas the mRNA for Tyr and DCT are expressed in basal, upper, middle, and lower dermis layers. In agreement with these data is the study of Vries et al. (19) which report a significant decrease in Tyr with melanoma progression.

DCT but not Tyr is expressed in 31% ALMs all having the characteristics of advanced malignity and bad prognostic. Moreover, the ALM category is one with the highest percentage of double negative specimens. The total loss of the Tyr expression and retention of DCT may represent a particularity of some patients with ALMs. This molecularity would correlate with some characteistics of the ALM type which distinguish from other melanoma types in terms of bad prognostics, increased aggressiveness and a more advanced stage than in the one indicated by clinical parameters (20,21). In addition to ALMs, the achromic category also showed specimens

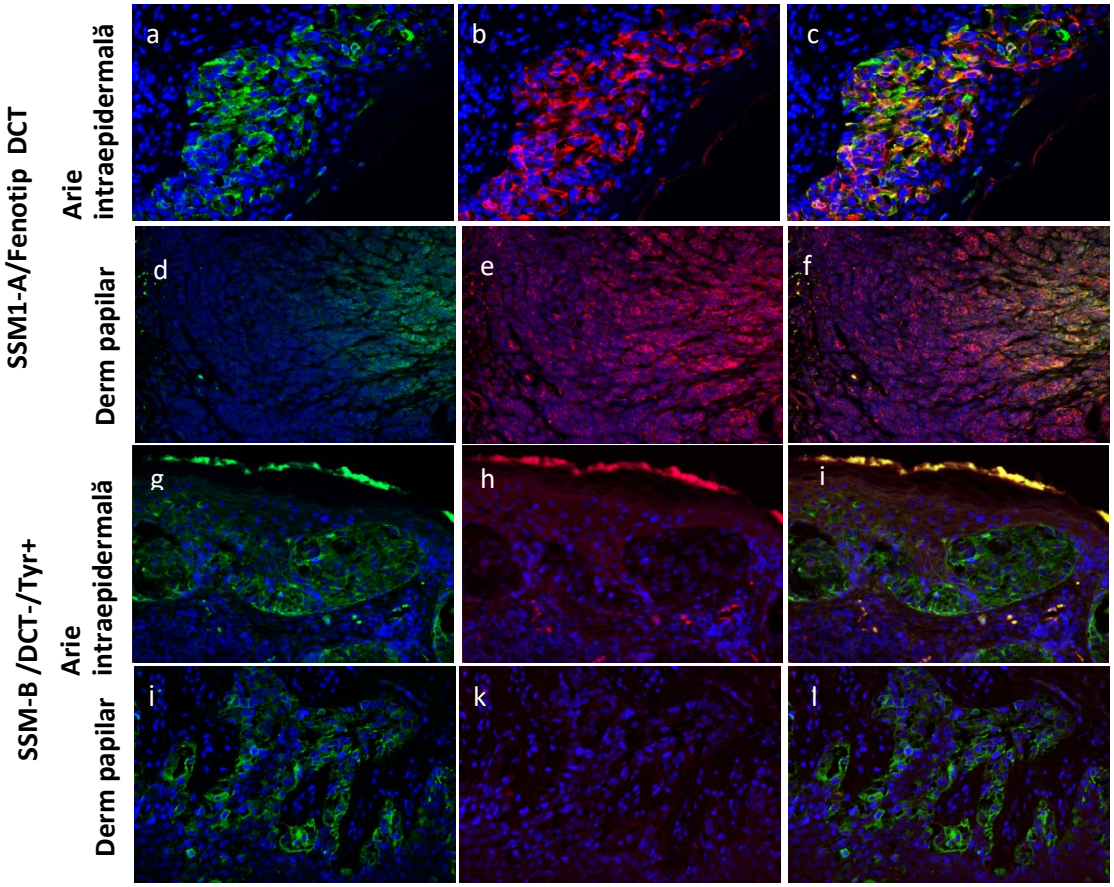

Figure 8 The molecular analysis of two specimens in malignant melanocytic lesions The samples were labeled for DCT (anti-hDCT), Tyr (T311) and nuclei (DAPI/blue)and analyzed by IHF. The DCT fenotype is characteristic to the specimen with ulceration (SSM1-A) (Table 4). The original magnification is 20X in SSM-B and 40x in SSM1-A. Figure was reproduced with permission Wolters Kluwer Health/ Lippincott Williams & Wilkinst după Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

expressing only DCT and negative for Tyr. In most ACMs, Tyr is detected, probably as an unstable precursor, similar to Tyr in amelanotic SK28 cells **(Fig. 4C)** (22) or in other amelanotic cell lines (23), and this may be an explanation for the relatively high number of AC specimens counted here as Tyr-positives. For these reasons DCT would represent a more adequate marker than Tyr in this type of lesions. This conclusion is supported by observations made by Orlow et al. (24,25) that in amelanotic tumors, Tyr is often deficient, whereas DCT is most often persistently expressed, and with the more recent reports of Choi et al. (26) and Smedley et al. (27) which recommend DCTas a diagnosis marker for canine amelanotic melanomas. The ACMs samples positive for DCT and negative for Tyr similar to the ALM ones have an advanced

malignant profile, suggesting that DCT expression, already known to play a role in tumor stress resistance, could also be involved in the peculiar and aggressive evolution of some ACMs (28).

Regarding the significance of DCT phenotype, in case of specimens analyzed in **Fig. 7**, large areas of intradermal cells DCT+/Tyr- are detected in compound nevi characterized by increased neurotization. The neuroid changes in a benign melanocytic proliferation are recorded at the base of a dermal nevus. The tumor structures consist of type C nevus cells (elongated cells with a spindle-shaped nucleus, resembling fibroblasts or Schwann cells) arranged in patterns reminiscent of nerve fibers or neural organs such as Wagner–Meissner corpuscles (29). The already-documented antiapoptotic activity of DCT (30), shown here to be intensely expressed in the highly neurotized areas, may contribute toward the wellacknowledged enhanced stability and low proliferation rate of these nevus cells (31,32).

In the superficial malignant tumor specimen presented in **Fig. 8**, the DCT-phenotype distinguishes itself from its counterpart with identical pathological characteristics only by ulceration. Although ulceration and tumor thickness are two well-correlated parameters (33,34), in this case, one patient has ulceration and the other does not; however, both have tumors with identical Breslow and Clark indexes. The two patients did not return for follow-up after surgery; thus, we do not have any information on their postoperative status. A recent communication indicates that although the majority of thin melanomas will never lead to harm, there is a small category of thin melanomas with ulceration that can represent a more aggressive type (35). Whether the segment of patients with thin ulcerated melanomas developing unexpectedly aggressive tumors has as an additional characteristic the pattern of DCT-phenotype is a possibility supported by the data presented in the final section of this study.

2.5 Intradermal DCT-clones in thin melanomas acquire the expression and subcellular distribution of the poor prognostic markers

The next step of this study aimed to characterize in more detail the malignant DCT+ clones, especially those observed to result from DCTselection and that populate the innermost dermal components. We selected the SSM1-A specimen **(Fig. 8)** and the DCT+/Tyr– ALM specimen **(Fig. 6),** representing a tumor in a highly advanced stage, and therefore expected to contain the progression/poor prognostic markers and considered in this case the ‘positive control’ for their

expression and subcellular distribution. Moreover, this ALM specimen is negative for TRP-1 **(Fig. 9a, a1)** and MART-1 **(Fig. 9b, b1**) and expresses gp100 poorly **(Fig. 9 c, c1).**

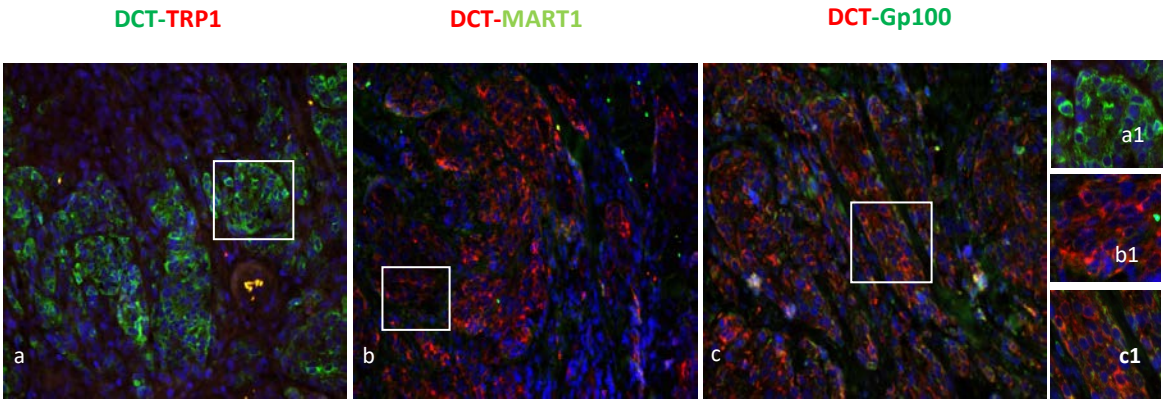

Figure 9 The analysis of DCT expression comparative with other melanosomal antigens TRP-1, MART-1 and gp-100 , in an ALM lesion with advanced malignit Figure was reproduced with permission from Wolters Kluwer Health/ Lippincott Williams & Wilkinst after Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

Hif-1α is the main regulator of cellular responses to hypoxic conditions and of over 60 genes involved in tumorigenesis, antiapoptosis, and in the malignant progression of several cancers. In the SSM1 specimen, the isolated melanocytes residing at the D–E layer expressing DCT, as expected, do not stain for Hif-1α **(Fig. 10a, a1).** The DCT+ cells invading the dermis in SSM1 **(Fig. 10b, b1)** are coexpressing cytoplasmic Hif1α, similar to the ones in the hypodermis of the ALM specimen **(Fig. 10c, c1).** This indicates the selection of cells capable to to survive in severe environmental stress conditions in which Hif-1α is stabilized into cytoplasm as result of its impaired degradation, as reported in breast cancer (36) or colorectal carcinoma (37).

Caveolin-1 (Cav-1) is a protein involved in multiple processes including oncogenesis and tumorigenesis. In SSM1, DCT+ cells in the IE layer coexpress Cav-1 in cytoplasmic/nuclear granules or decorating segments of PM **(Fig. 10d, d1).** In the ID component, Cav-1 is still expressed, however, in cells negative for DCT **(Fig. 10e, e1),** whereas in the ALM specimen, all cells are negative for Cav-1 staining **(Fig. 10f, f1).** In melanoma cell lines, Cav-1 overexpression is correlated with the attenuation of the migratory properties and metastatic potential of tumor cells (38). The intradermal DCT-clones, negative for Cav-1, similar to the ones in the ALM

specimen, may acquire migratory capacities, unlike their counterparts from the intra epidermal areas that coexpress Cav-1.

Cyclin D1 (Cycl D1) and *Cyclin E (Cycl E)* together with their regulatory-dependent kinases and inhibitors that act to drive cells through the G1 and S phase, are consistently overexpressed in metastatic melanomas compared with nevus tissue. In the SSM1 specimen in the IE component, numerous DCT+ cells are also intensely positive for nuclear Cycl D1 **(Fig. 10g, g1)**, whereas in the ID area, DCT+ cells are poorly stained for nuclear Cycl D1, and cells that are still strongly positive for nuclear Cycl D1, no longer express DCT **(Fig. 10h, h1).** In the ALM specimen here **(Fig. 10i, i1),** nuclear Cycl D1 is occasionally expressed in DCT+ cells, whereas many more cells positive for DCTare also expressing cytoplasmic Cycl D1. No Cycl E staining was detected in tumor cells including DCT+ cells located in IE areas of the SSM1 specimen **(Fig. 10j, j1).** However, this marker is intensely expressed in the cytoplasm of DCT+ cells in ID nodules in SSM1 **(Fig. 10k, k1)** as in DCT+ clones found in the ALM specimen **(Fig. 10l, l1),** which may thus confine advanced metastatic characteristics to intradermal DCT+ clones in SSM1. The intradermal DCT+ clones no longer express nuclear Cycl D1, which is in agreement with the data reported by Ramirez et al. (39) showing that Cycl D1 expression decreases in lower tumor components and also indicating that the proliferation capacities of DCT+ cells are lower in the dermal component. We also show here that DCT+ clones in both the ALM specimen and in the intradermal component of the SSM specimen express cytoplasmic Cycl D1 and Cycl E. Cycl D1 is reported to bethe particularly amplified in acral melanoma (40) and its abnormal sequestration in the cytoplasm of mammalian tumor cell lines (41) prevents apoptosis similar to that reported for neuronal cells (42). Cycl E low molecular forms (LMW-E) particularly produced in tumor but not in normal cells (43) are linked to the increased angiogenic and metastatic potential of human melanoma cells in vivo (44) and their specific cytoplasmic accumulation contributes toward the cellular tumorigenicity (45).

Bcl-2 is primarily known as a protein having the capacity to prolong the survival of hematopoietic and neuronal cells by blocking apoptosis. In the SSM1 specimen, groups of cells at the D–E layer or in the IE component are positive for either DCTor Bcl-2 **(Fig. 10m, m1).** In the SSM1 dermis within a tumor cell cluster, DCT+ cells expressing nuclear Bcl-2 **(Fig. 10n, n1)** similar to the DCT+ cells in the ALM specimen could be detected **(Fig. 10o, o1)**. Finally, DCT+

intradermal clones acquire Bcl-2 expression. A possible increase in Bcl-2 levels in the DCT+/Bcl-2+ cell phenotype may alter tumor characteristics, enabling progression to highly malignant phenotypes as has been reported by other studies in melanoma or tumor-associated endothelial cells overexpressing Bcl-2 (46,47).

Based on the data of the present study and on what is already known about DCT , our theory , schematically presented here in **Fig. 11,**is that disjunction between DCT and Tyr expressions is an early molecular event in neoplastic transformation of melanocytes. DCT and Tyr are co-expressed in normal skin melanocytes **(Fig. 11, yellow/orange).** In junctional nevi, DCT/Tyr are still coexpressed in all nevus cells (subtype 0). A particular tumor architecture in which DCT–/Tyr+ cells are always retained in the upper part of the tumor component,whereas DCT+/Tyr– cells invade the innermost dermis is defined as subtype 1/'**DCT-phenotype'** and is found in compound (CN), dysplastic nevi (DN) and malignant phenotypes (SSM, NM, ALM, ACM). The ***abrupt*** disjunction process of DCT/Tyr expression seems to be representative for nodular melanomas and specimens with a deep vertical growth phase (ALMs or ACMs with Breslow 12, 17, 22mm). In compounds nevi this phenotype is associated with neurotization. In superficial melanomas, unlike DCT-cells in the junctional component or the subepidermal (SE) layer, intradermal DCT-clones acquire the expression and subcellular distribution of molecular markers associated in other neoplasms, including melanoma, with migration (Cav-1-), survival under stressful conditions (cytoplasmic Hif-1α), activated antiapoptotic mechanisms (Bcl-2+, cytoplasmic Cycl D1), and increased angiogenic and metastatic potential (cytoplasmic Cycl E+).

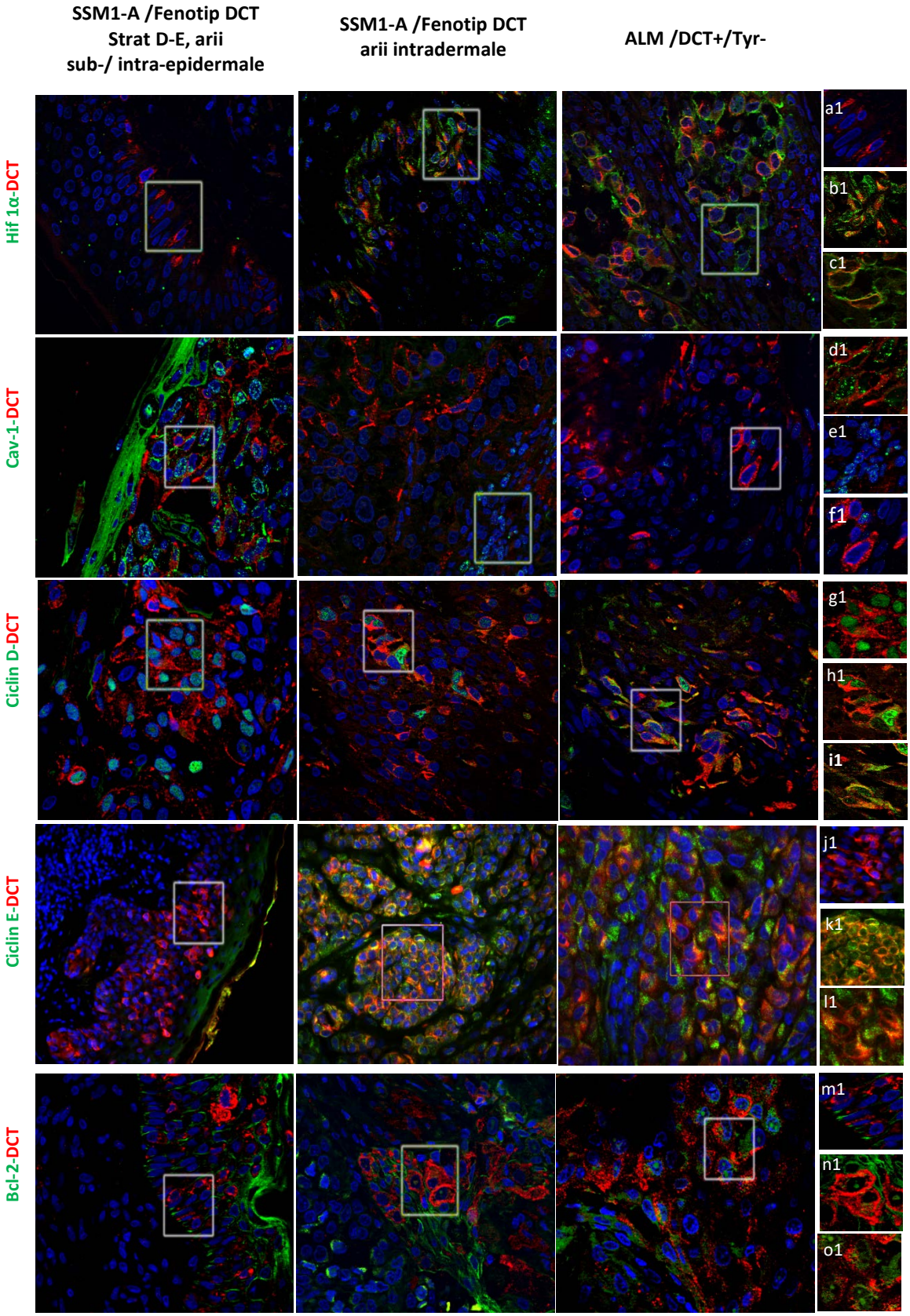

Figure 10. The characterization of DCT phenotype with biomarkers of bad prognostic and advanced malignity Figure reproduced with permission from Wolters Kluwer Health/ Lippincott Williams & Wilkinst after Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24 ,No3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

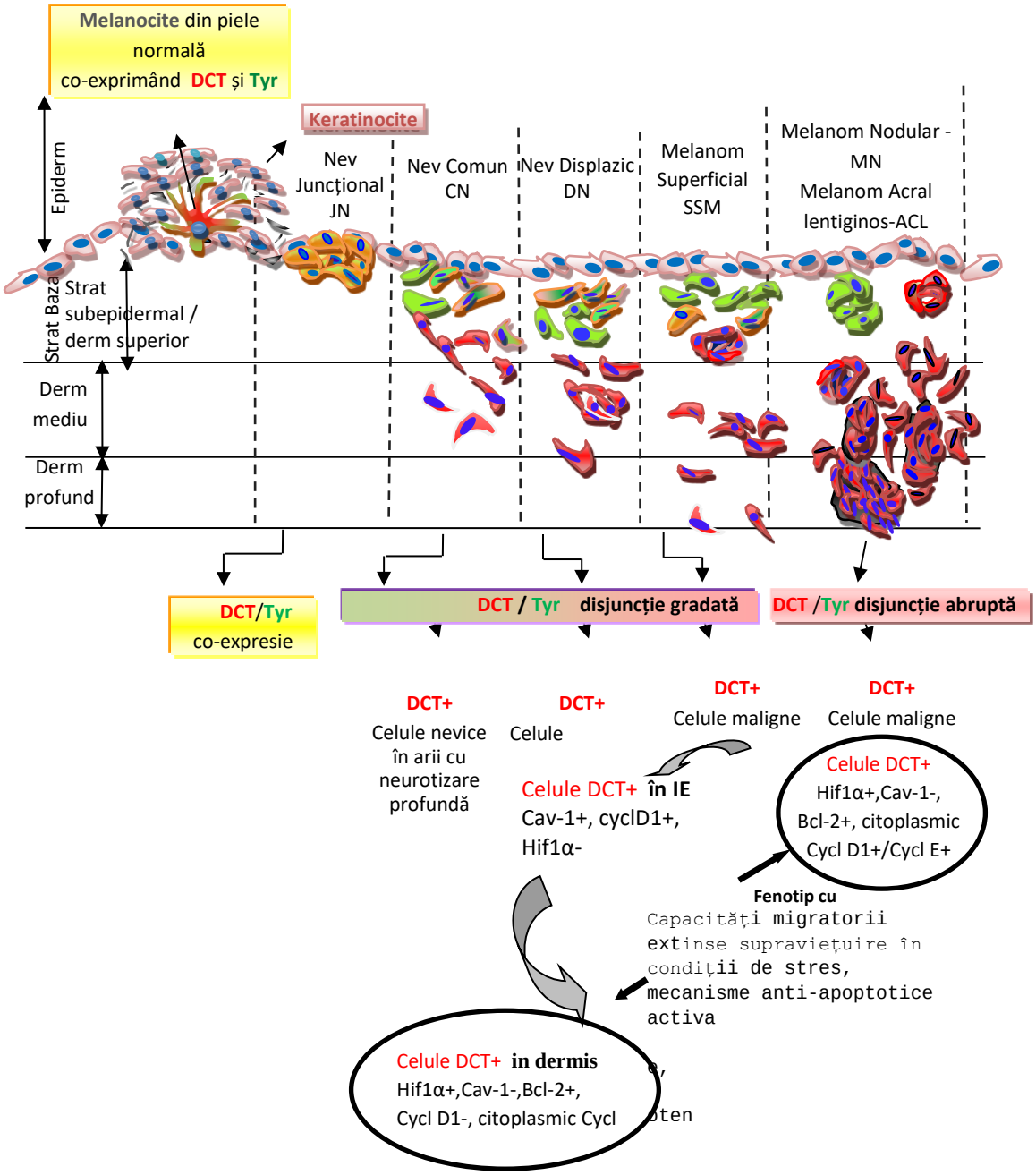

Figure 11 The schematic representation of the dissociation process of DCT and Tyr in melanocytic lesions and the molecular anatomy of the DCT-cellular phenotype in different melanocytic lesions Figure reproduced with permission from from Wolters Kluwer Health/ Lippincott Williams & Wilkinst after Filimon et. al Value of Dopachrome tautomerase detection in the assesment of melanocytic tumours, Melanoma Research 2014, Vol 24, No 3.http://journals.lww.com/melanomaresearch/fulltext/2014/06000/Value_of_dopachrome_tautomerase_detection_in_the.5.aspx

2.6 Conclusions and Perspectives

- DCT unlike Tyr is expressed and completely processed along the secretorii pathway in both pigmented and acromic melanoma cells

- Most of the investigated tumors are positie for both antigens DCT and Tyr which demonstrate that DCT is a stabile, well-expressed antigen

- The disjuct expressions of DCT and Tyr in distinct cell populations support the utility of addition of DCT on the list of biomarkers for the increase of the sensibility of the detection of tumor cells in which Tyr and other biomarkers are poorly expressed or absent.

- The disociation of DCT and Tyr expressions in melanocytic lesions is correlated with the neoplastic transformation of melanocyties and with malignant progression and can be considered an molecular event characteristic for initiation and evolution of skin melanoma

- It has been identified a molecular specific architecture, named by us „DCT-phenotype" which is correlated with specific pathological parameters in benign and malignant lesions as neurotization and ulceration respectively. In some thin melanomas the DCT+ intradermal populations express biomarkers of metastatic progression and bad prognostic, bringing into attention a possible phenotype characterized by aggressiveness and therapeutic resistance.

-DCT-phenotype can be considered as a „die-hard" one which in benign tumors does not represent a vital threat. However, the DCT cells present in deep dermis of the early maligant lesions acquire the expresion and subcellular distribution of of the molecular markers reported in other studies for being associated in other neoplasms with: extended migratory capacity (Cav-1-), survival in stressful conditions (cytoplasmic Hif-1α), activated antiapoptotic mechanisms (CyclinD1-, cytoplasmic Bcl1+),potential angiogenic and metastatic (cytoplasmic Cyclin E). The intradermal DCT phenotype represents a parameter which should be evaluated in a multifactorial analysis for the prognostic of melanocytic lesions.

- The anti-hDCT antibody , obtained in the Institute of Biochemistry, Bucharest is suitable for DCT identification in histopathological specimens and for other molecular studies about DCT analysis in melanoma cell lines.

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PART II NANOPARTICLES FOR TARGETED DELIVERY OF BIOLOGICALLY ACTIVE COMPOUNDS

INTRODUCTION

Nanotechnology is a science that comprises the sinthesys, characterisation and use of materials with dimensions of maximum 100 nm in at least one dimension. Dendrimers are polimeric nanoparticles of 1-15 nm, with highly branched 3D structure and a globular shape in which all the chemical bounds start radially from a centrale core that can be a small molecule or a liniar polymer. The ter dendrimer comes from greek language and underlines the unique shape of this structures: dendron means tree and meros means part. The structure of dendrimers is organized in three different areas: a central multipurpose area, generations of repetitive units (marked with G) attached to the central zone and a external surface with terminal functional groups. Dendrimers have a high degree of molecular uniformity and properties shared between molecular chemistry due to gradual sinthesys in controlled conditions and polimer chemistry because they are polimeric structures consisting of monomers. Unlike liniar polymers, dendrimers have a series of superior physico-chemical properties that makes them more suitable for the release of pharmacological compounds: compact and globular structure comparing with the unstructured conformation of liniar polymers, low compressibility and high solubility in water and non-polar substances (1). Thus dendrimers are able to improve some shortcomings of the therapeutical agents such as low water solubility and rapid degradation once inside biologic systems (2). Dendrimers also display retention effect due to internal cavities formed in their structure which allows them to incpsulate pharmacological agents, cosmetic ingredients, metalic ions whith a role in catalisys. Because of these properties dendrimers are able to bind a series of biomolecules through several mechanisms: complexation, conjugation, encapsulation. Initially synthesized by Vogtle F in 1978 through divergent synthesis methods the dendrimers have transformed in an attractive target for nanotechnology industry because they can be functionalized through conjugation with various molecules resulting in multivalent systems. These systems allow the conjugation of molecules that determine the targeted delivery but also pharmacological compounds to the same dendrimeric nanoparticle which can significantly lower the side effects of administred therapies on healthy tissues and cells. Dendrimers already have a large spectrum of applications in medical sciences but also in other fields. In the medical field

dendrimers are used per se because of their antibacterial and antiviral properties or as scaffold molecules for various pharmacological agents and other chemical compounds used for diagnosis (3). In cancer therapy dendrimers already have numerous potential applications as: tumor imaging, photodinamic therapy, neutron capture therapy used as an alternative method to the conventional radiation therapy in case of glioma, gene therapy and targeted release of pharmacological compounds.

The use of nanotechnologies in melanoma aimes to lower or completely abolish treatment resistance, increase therapy efficiency, lower the side effects and improve diagnosis. Therapeutical formulation with liposomes, dendrimers, human albumin, polymerosomes and carbon based nanoparticles have been conceived for chemotherapeutic compounds release in melanoma treatment (4), targeted therapy, immunotherapy, photodinamic therapy, inhibition of the cellular detoxification system and targeting of the apoptotic processes mediated by mitochondria. Despite the fact that the use of nanotechnology for new therapeutical agents has led to clinical testing or even commercialisation of numerous drugs for some cancer types (5), in melanoma the studies have not passed the preclinical stage. A series of dendrimers formulations with various chemotherapeutic compounds have been tested in vitro and proved to be superior to the sole use of the antitumoral drugs. Conjugation of temozolomide (TMZ) with PAMAM dendrimers aimes to improve therapeutical efficiency by increasing the plasmatic half time of the compound. In vitro studies have shown that internalisation of nanostructures TMZ-PAMAM-PEG-GE11-HA was more efficient in metastatic melanoma cells A375 comparing with human epithelial fibroblasts (6). The synthesis of tecto-dendrimers as sandwiches composed of 5-th generation PAMAM dendrimers covalently bound to a shell of 2,5 generation dendrimers in which methotrexate (MTX) and zolendronic acid (ZO) are incorporated, aimes to facilitate the use of these compounds in the therapy of malignant melanoma. Usually MTX is not efficient in the treatment of malignant melanoma beacuse of its uptake and elimination from the cells as the MTX-FRα complex (folate receptor) by melanosomes and ZO is a compound from biphosphonates class mainly retained in the bones which makes it useless in the treatment of melanoma. Loading of tecto-dendrimers with 6 molecules of MTX caused increased toxicity for SkMel-28 metastatic melanoma cells compared with HaCat keratinocytes. Thus MTX conjugation with dendrimers can modify the internalisation of the drug from a mechanism based on the interaction with the folate receptor to a mechanism based on macropinocytosis that can

block MTX exocitosys from melanoma cells. Loading of the dendrimers with 31 molecules of ZO caused a toxic effect on melanoma cells (85%) but also on keratinocytes (50%). Conjugation of doxorubicin (DOX) with 4th generation PAMAM dendrimers and its pulmonary administration improves the antitumoral effect and limits the cardiotoxic effects in a model of murine melanoma with pulmonary metastasis (7). Pulmonary administration of conjugated or free DOX is more efficient comparing to intravenous administration and conjugation with dendrimers lowers the pulmonary metastatic burden and the distribution of DOX in the cardiac tissue. The formentioned examples illustrate the benefits of conjugation of chemotherapy agents with dendrimers: improvement of therapeutical efficiency through reduction of half time, lowering the toxicity towards healthy tissues, introduction of new pharmacological agents in melanoma therapy which are otherwise totally ineffective if not conjugated with dendrimers. Lowering of the systemic (generalized) toxicity through preferential accumulation inside tumors is potentiated by certain physico-chemical properties specific to nanoparticles that lead to preferential internalisation in certain cell types; attachement of specific ligands for certain receptors overexpressed in tumours; the ERP effect (enhanced permeability and retention) by which molecules with certain dimensions (liposomes, dendrimers, other types of nanoparticles and chemical compounds) accumulate in tumoral tissues more than in normal tissues. ERP is due to intratumoral vasculature composed of endothelial cells faultily aligned with spaces between them and devoided of the smooth muslcular layer, with a larger lumen and a modified expression of angiotensin II receptors as opposed to the vasculature found in normal tisssues. For these reasons sufficiently large particles cand enter tumor blood flow and not the normal tissues. The purpose of the experiments further described is the evaluation for the first time of 5 th generation glycodendrimeric structures based on poly (propilene imine) in cultures of human primary and metastatic melanoma cell lines in order to analyse the possibility of using these nanoparticles for targeted release of biologically active compounds.

1. The Effect of the Maltose Coupling on G5-PPI Dendrimer Cytotoxicity

The nanoparticles used in this study were the 5th generation of poly(propylene imine) dendrimers and to them were added maltose shells in the laboratory of Dr Dietmar Appelhans in Leibniz-Institute für Polymerforschung Dresda, Germania.these compounds, named further conventianally G5-PPIs were supplied to our Institute within a collaborative project. G5-PPIs

were nanoparticles synthetized using divergent method starting from a diamnobutane nucleus to which was sequencially added a number of 64 amino primary groups. This type of dendrimers have a cationic charge due to multiple amino surface groups and can destabilize the cell membranes inducing cell lysis. In the same time the surface amino groups can be linkage points for various functional groups (fluorescent molecules, mall interferrence RNA, etc) which can generate diverse compounds with various biomedical applications, therefore the maintainance of amino terminal groups together with a low toxicity is desired.

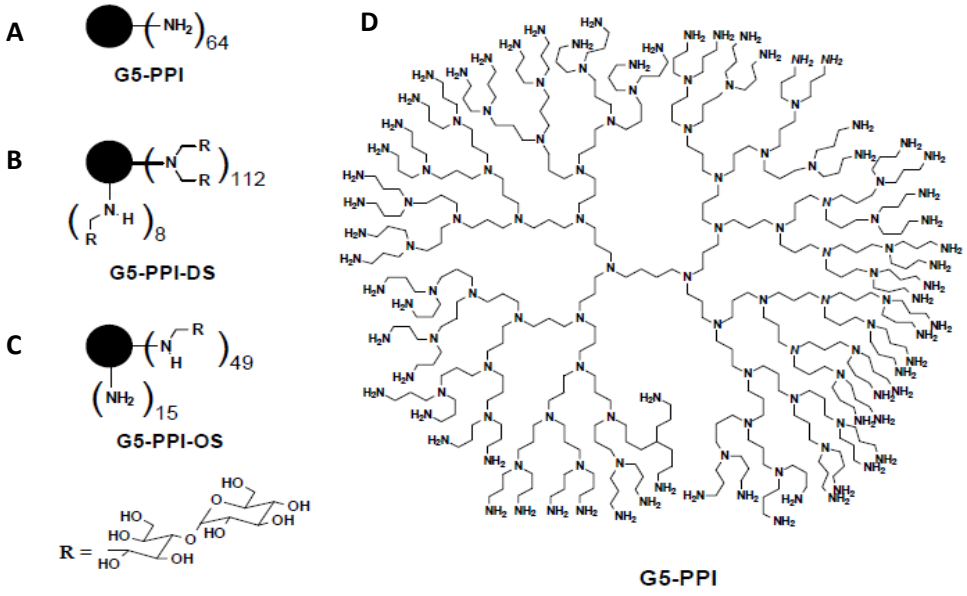
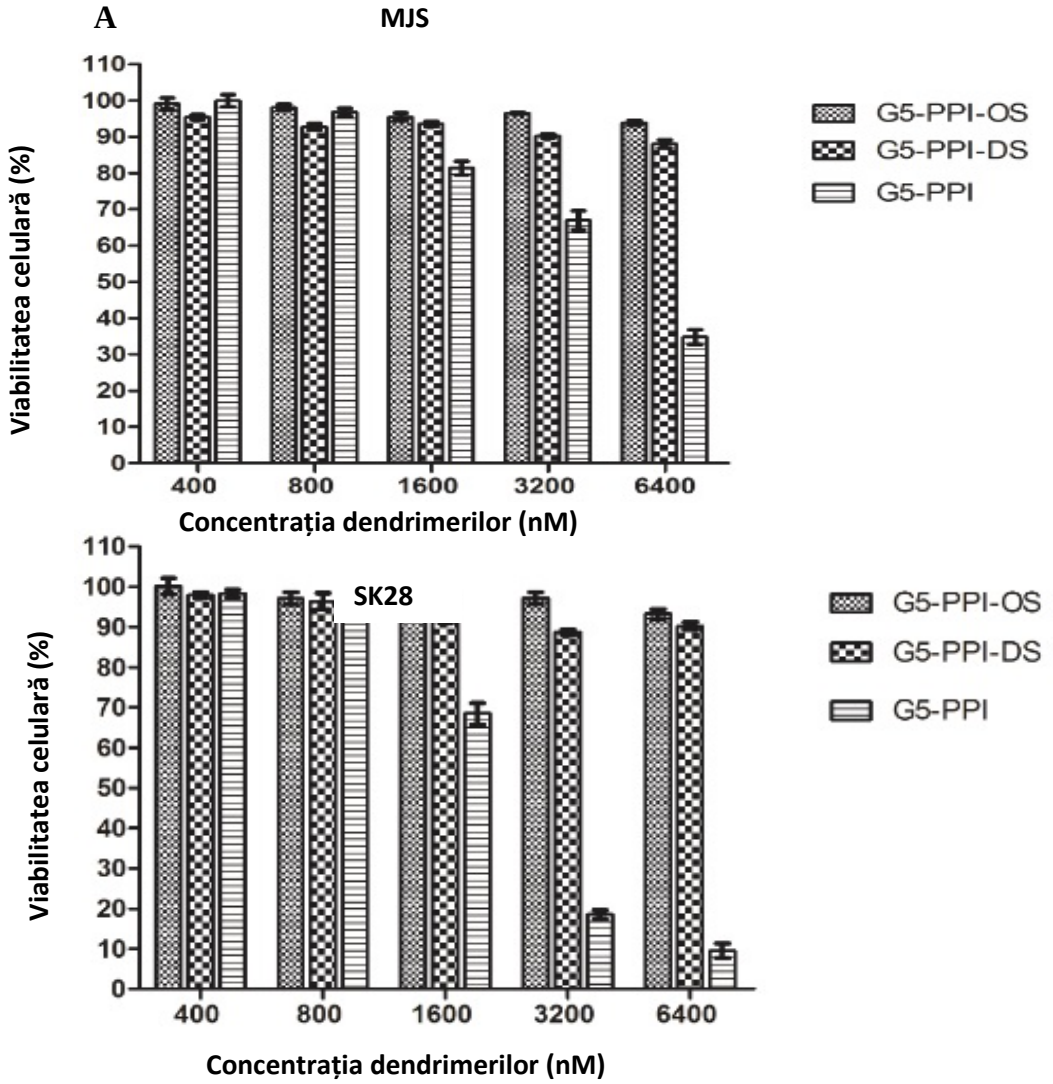


Figure 1. The schematic structure of poly(propilen imine) dendrimers G5-PPI (A), of neutral dendrimers with maltose shell G5-PPI-DS (B), cationic maltose shell dendrimers G5-PPI-OS (C) and chemical structure of G5-PPI dendrimers (D). Figure addapted with permission Bentham Science după Filimon et al., Internalization and intracellular trafficking of poly(propylene imine) glycodendrimers with maltose shell in melanoma cells, Curr Med Chem 2012, 19(29): 4955-68.

For imagistic analysis in the dendrimer structure were attached molecules of FITC or rhodamin B which allow the visualization of these by fluorescence microscopy as green elements (FITC) or red (rhodamin B). The surface decoration with maltose in different amounts may determine the decrease of cationic groups and subsequently of their citotoxicity which represent a positive effect and would extend their use as platforms for the release of targeted active compounds.



Figur2 The effect of maltose conjugation on citotoxicity of G5-PPI dendrimers. The melanoma cells MJS and SK28 have been exposed for 1h at increased concentrations of
Dendrimers without maltose shell (G5-PPI), cationic (G5-PPI-OS) or
neutral (G5-PPI-DS) dendrimers. Cell viability was calculated according to protocol described in Material and Methods. The data represent average of 3 independent experiments (n=3) done in triplicate. Figure addapted with permission Bentham Science după Filimon et al., Internalization and intracellular trafficking of poly(propylene imine) glycodendrimers with maltose shell in melanoma cells, Curr Med Chem 2012, 19(29): 4955-68.

Starting from G5-PPI (Fig.1 A,D) cationic dendrimers with maltose open shell (G5-PPI-OS, Fig.1C) and neutral dendrimers with maltose dense shell (G5-PPI-DS, Fig.1B) have been synthetized and evaluated as potential systems with applicability in melanoma diagnostic and therapy. The experiments used mainly two amelanotic melanoma cell lines , MJS and SK28 (8),

representing two distinct phenotypes VGP (early or primary) and metastatic respectively. The cytotoxicity analysis of G5-PPI-OS and G5-PPI-DS comparatively with G5-PPI showed that at concentrations between 400 and 800 nM none of the tested dendrimers have toxic effects on MJS primary melanom cell line (Fig. 2A) or metastatic, SK28 (Fig. 2B). The increase above 800nM of G5-PPI, G5-PPI-OS și G5-PPI-DS,results in viability decrease whereas the viability of the cells exposed at identical concentrations of G5-PPI-OS și G5-PPI-DS dendrimers is more than 90% even at the highest dendrimer concentration of 6400 nM (Fig.2A,B). These results demonstrate that *cytotoxicity of G5-PPI is significantly decreasing as a result of the modification of their surface with maltose*.

2. The internalization of G5-PPI-OS and G5-PPI-DS in human melanoma cell lines

The analysis of internalization of G5-PPI-OS and G5-PPI-DS dendrimers in various cell types is necessary to establish the capacity of these structures to cross the cell membrane with the purpose of being used as active pharmacological molecules transporters in vivo. With this purpose in mind we used a number of 5 human cell lines from which 4 were melanoma cell lines (A375, MNT-1, SK28, MJS) and an embryonic cell line (HEK293T) in which was analyzed ***the percentage of cells which have internalized the dendrimers, the level of intracellular fluorescence and the efficiency of nanoparticle uptake by these cells.***

The analysis of cell percentage (Fig.3) which internalize the dendrimers indicate that G5-PPI-DS is uptaken by a higher nuber of cells comparative with G5-PPI-OS, the differences being more visible in case of A375 (74,1% versus 88,5%) and less evident in case of SK28 (79,67% versus 81,8%). The level of intracellular fluorescence was measured by flow cytometry following treatment of cells with trypan blue for 10 min at 37C. The trypan blue quench the extracellular fluorescence generated by dendrimers absorbed at plasma membrane (PM) and makes possible the measurement of intracellular fluorescence resulted from dendrimer internalization by the cells. ***The levels of intracellular fluorescence*** indicate that G5-PPI-OS dendrimers are internalized without any major differences in all 5 cell lines. MNT-1, MJS and HEK293T intenalize more G5-PPI-OS dendrimer (8, 52, 8,75, 8,35) than Sk28 (6,78), A375 (6,27) (Fig. 4A). The level of flourescence determined by internalization of neutral G5-PPI-DS dendrimers varies in all analyzed cell lines. Sk28 and MJS internalize more G5-PPI-DS (7,71 and 7,39 rspectively) versus MNT-1 (6,48), HEK293T (6,06) și A375 (4,76) which internalize the lowest

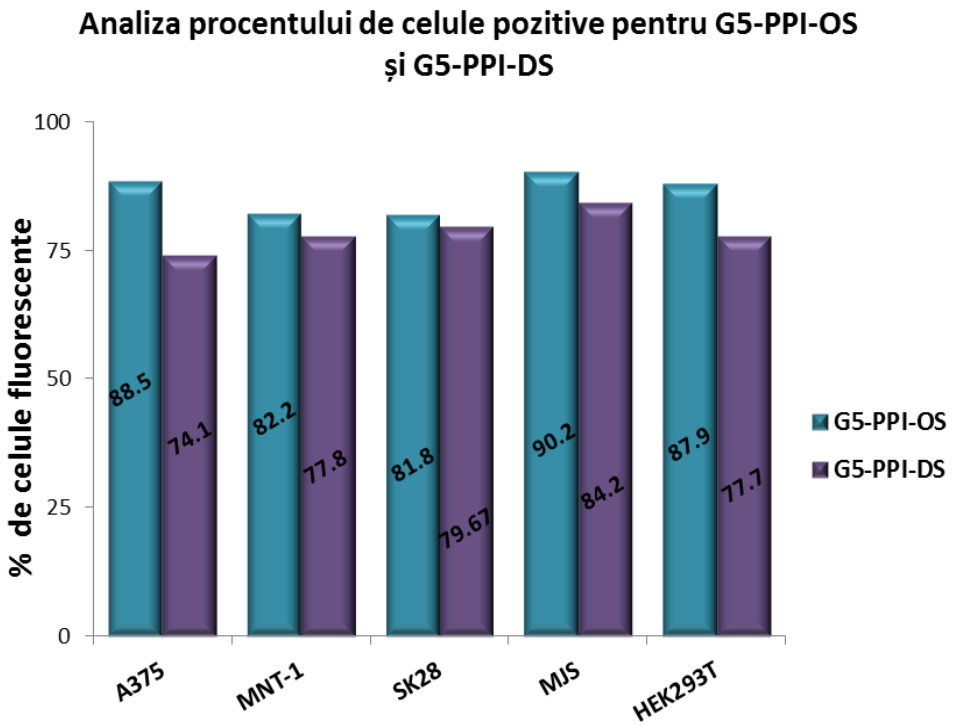


Figure 3. The internalization of G5-PPI-OS and G5-PPI-DS dendrimers in melanocytic and non-melanocytic cell lines (the percentage of cells which have internalized dendrimeric nanoparticles). The melanoma cells (A375, MNT-1, SK28, MJS) and embryonic kidney cells (HEK293T) were incubated with 800 nM G5-PPI-OS and G5-PPI-DS dendrimers conjugated with FITC for 1h and treated (+) or not-treated (-) with trypan blue (0,0025%) and analyzed by flow cytometry. The bars represent the average of two independent experiments done in duplicates.

amount of this type of dendrimer (Fig. 4C). Exccepting SK28 cells the internalization of G5-PPI-DS (Fig.4C) determine a lower level of intracellular fluorescence versus G5-PPI-OS (Fig.4A).

The yield of internalization of G5-PPI-OS is higher in MNT-1 (68,36%) and HEK293T (76,72%) than in SK28 (41,88%) and MJS (31,33%), and A375 internalizes the G5-PPI-OS dendrimers with the lowes yield (30,68%)(Fig. 4B). The MNT-1 cell line internalizes the highest amount of G5-PPI-OS (8,75) with the highest yiels (68,36%), and A375 cell line internalizes the lowes amount of this dendrimer type (5,71) with a low (30,68%). The cell lines SK28 and MJS internalize these

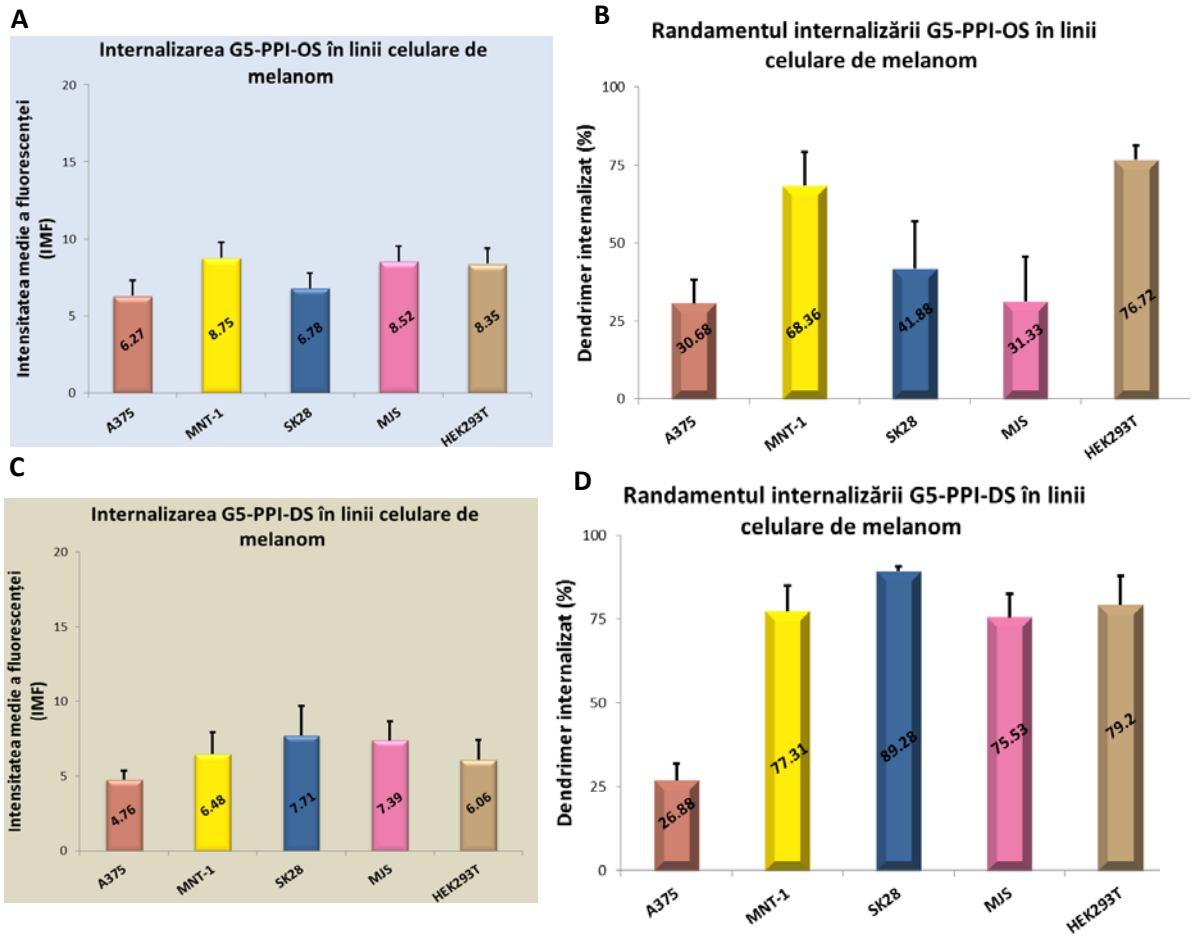


Figure 4. the internalization of G5-PPI-OS and G5-PPI-DS dendrimers in melanocytic and non-melanocytic cell lines (the level of intracellular fluorescence nivelul de fluorescență and the yield of naoparticle uptake by these cells). The internalization of G5-PPI-OS dendrimers in A375, MNT-1, SK28, MJS și HEK293T. The internalization of dendrimers in presence of trypan blue is graphically represented as absolute values of mean fluorescence intensity MFI (A,C).

nanoparticles with a yield below 50% (41,88 and 31,33% respectively). In case of MJS there is a discrepancy between the level of fluorescence of the cells (8,52) and the internalization yield (31,33%). These data can be interpretated that a significant amount of dendrimers is retained at PM which would necessitate a higher amount in order to attain the same level of internalized fluorescence as the one found in MNT-1 cells. The neutral dendrimers, comparatively with the cationic ones are more efficently internalized in MNT-1 (77,31%), SK28 (89,28%) and MJS (75,53%) , equally in HEK293T (79,2% versus 76,72%) (Fig. 4D). In A375 the G5-PPI-DS dendrimers are internalized with the lowest yield comparatively with the other cell lines (26,88%) and approximately similar with the cationic structures G5-PPI-OS (30,68%).

Although there are numerous studies which confirm that cationic structure of as a factor which favours the internalization of nanoparticles (9,10,11,12), it is very likely that the lower internalization yield of the G5-PPI-OS comparatively with the G5-PPI-DS, in 4 out of the 5 analyzed cell lines, is due to an increased retention of this structure at PM generated by the interaction between the positive charge of the NH2 groups from the dendrimeric structure and the negative charge of the PM extracellular surface. This would explain the results showing the much higher differences between the extracellular and intracellular fluorescence in case of G5-PPI-OS in MJS (55,96 versus 16,34) and SK28 (26,46 versus 69) comparatively with the differences generated by G5-PPI-DS in MJS (15,8 versus 9,19) and SK28 (8,86 versus 6,8). The cationic G5-PPI-OS can create an effect of adhesion at PM decreasing the internalization yield but not the level of the intercellular fluorescence which is higher compared with G5-PPI-DS, excepting the SK28 cell line.

In conclusion, even that in case of MJS, MNT-1 and HEK293T cell lines, the internalization of G5-PPI-DS determines a lower level of fluorescence comparatively with G5-PPI-OS the yield is higher in case of MNT-1, more than double in MJS and approximately equal in HEK293T. In SK28 G5-PPI-DS determines an higher increase of the fluorescence level and is internalized with more than double yield compared with G5-PPI-OS. These data show that G5-PPI-DS would be a good candidate for in vitro studies due to its good internalization yield in 4 out of 5 analyzed cell lines, being possibly administrated in lower amounts and minimizing thus its possible toxic effects. In melanoma cell line group unlike MNT-1 and SK28, A375 has a lower capacity to internalize G5-PPI-OS or G5-PPI-DS. These differences are in agreement with intra- and inter- tumor heterogenenicity observed in case of melanoma and generated by the specific molecular characteristics acquired by some cell populations during metastaic progression.

3. The study of G5-PPI glicodendrimer internalization in human primary and metastatic melanoma cell lines

3.1 The internalization of G5-PPI-OS and G5-PPI-DS in human melanoma cell lines treated with inhibitors of clathrin- or cholesterol-mediated endocytosis

Considering that the values of Mean Fluorescence Intensity measured in the absence of the inhibitors represented 100% internalization yield, the internalization yield of glicodendrimers in the presence of inhibitors was expressed as percentage and graphically represented in Fig. 5A.

The data anlysis shows that in MJS cells treated with CPZ the internalization of cationic G5-PPI-OS dendrimer is not affected whereas in cells treated with MβCD the yield is only 28%. The neutral G5-PPI-DS dendrimers are affected in a similar manner by the endocytosis inhibitors, CPZ and MβCD which decrease the internalization to 90% and 48% respectively. Unlike the MJS cells the treatment with CPZ reduces the G5-PPI-OS internalization in SK28 cells to 82% whereas MβCD has a less pronounced to 70%. The internalization of the neutral G5-PPI-DS in SK28 cells is not affected in cells treated with endocytic inhibitors.

These data are schematically represented considering that a 100% internalization yield in the presence of an inhibitor signifies a total independence of endocytosis of that inhibitor, whereas a 0% yield is a total dependence of that pathway (Fig. 5B). All together these data demonstrate the both cell lines use more than one endocytic pathway for both glycodendrimers. However, the integrity of the cholesterol-rich regions is very important for their endocytosis, especially in MJS where the treatment with MβCD demonstrates a 72% dependence of PPI-OS and 52% of G5-PPI-DS for the endocytic processes cholesterol-mediated (Fig. 5B). In SK28 metastatic cell line the internlization of G5-PPI-OS depends less than cholesterol (30%) and internalization of G5-PPI-DS is cholesterol-independent. The diminuation of dendrimer internalization of the cholesterol-rich domains (with or without caveolin) in metastatic SK28 cell line comparative with MJS is in agreement with studies which show that cholesterol and Caveolin-1 levels are decreasing in melanoma metastaic progression (13), and this was also found by our group in MJS and SK28 cells (unpublished data).

In SK28 cells the internalization of 48% of G5-PPI-OS dendrimers depends on cholesterol and clathrin and 52% are internalized using non-convetional pathways. An interesting result is that neutral G5-PPI dendrimers which are 38% dependent of non-conventioanl pathways in MJS are 100% internalized via these pathways in SK28 cell line. *In conclusion the internalization pathways are predominantly dependent on cholesterol in primary melanoma cell line and on non-conventional pathways (non-clathrin, non-cholesterol) in metastatic melanoma cells, whereas clathrin-mediated endocytosis is insignificant in both cell types for both glycodendrimer structures.*

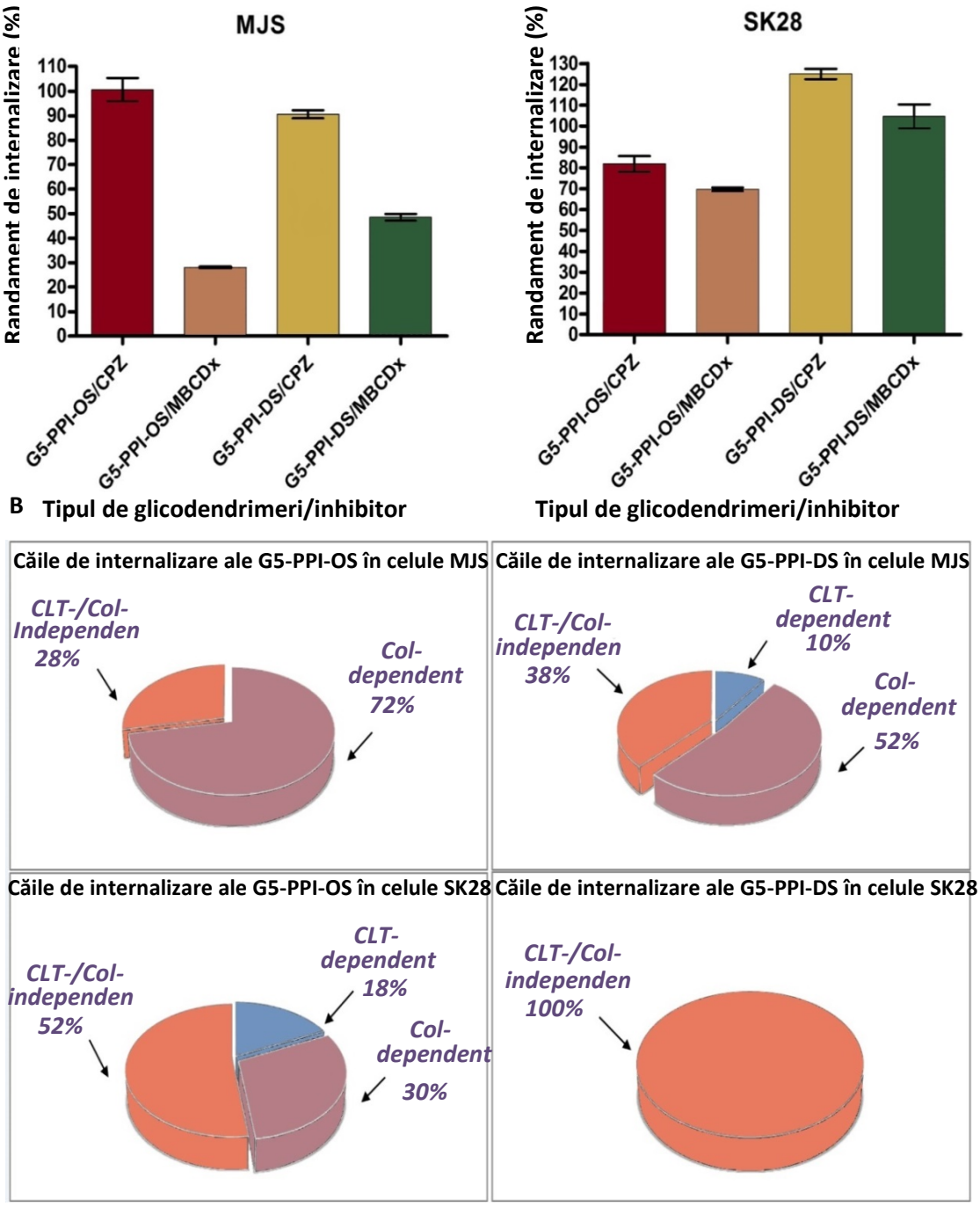


Figure 5 The effect of endocytosis inhibitors on the maltose-shell glycodendrimer internalization in melanoma cells. The cells have been pretreated with CPZ or MβCDx and incubated for 1h with G5-PPI-OS and G5-PPI-DS conjugatd with FITC simultaneously with the inhibitors. The cells incubated with glycodendrimers in the absence of the inhibitors represent the controls. All the samples were treated with with trypan blue in order to determine only the intracellular fluorescence and were analyzed by flow cytometry. The values for the internalized glycodendrimers (%) were calculated as indicated in Material and Methods. The data represent the average of 3 independent experiments performed in duplicates (A). The schematic representation of the endocytic pathways used by melanoma cell lines for the dendrimer internalization (B). Figure was addapted with the permission of Bentham Science după Filimon et al., Internalization and intracellular trafficking of poly(propylene imine) glycodendrimers with maltose shell in melanoma cells, Curr Med

3.2 The internaliztion and traffic of structures positive for G5-PPI-DS are modulated by elements of cytoskeleton in human melanoma cell lines

In order to establish whether the elements of cytoskeleton are involved in trafic of G5-PPI-DS in melanoma cell lines, two chemical compounds which modulate the integrity of cytoskeleton were used: c*ytochalsine B (CKL)* of which principal mechanism consists in inhibation of actin polymerization due to its binding to the actin ends with a rapid growth of actin F filaments and of *nocodazol (NCZ)* which interferes with microtublue polymerization. In control samples G5-PPI-DS shows in fine, punctuate structures which accumulate in nucleus proximity (Fig. 6a și d). In cells treated with CKL the G5-PPI-DS is accumulated in structures retained in MJS and SK28 cell pheriphry (Fig. 6b și e). In cells treated with NCZ G5-PPI-DS appears in vesicular structures well-defined redistributed from perinuclear zone in entire cell body (Fig. 6c și f).

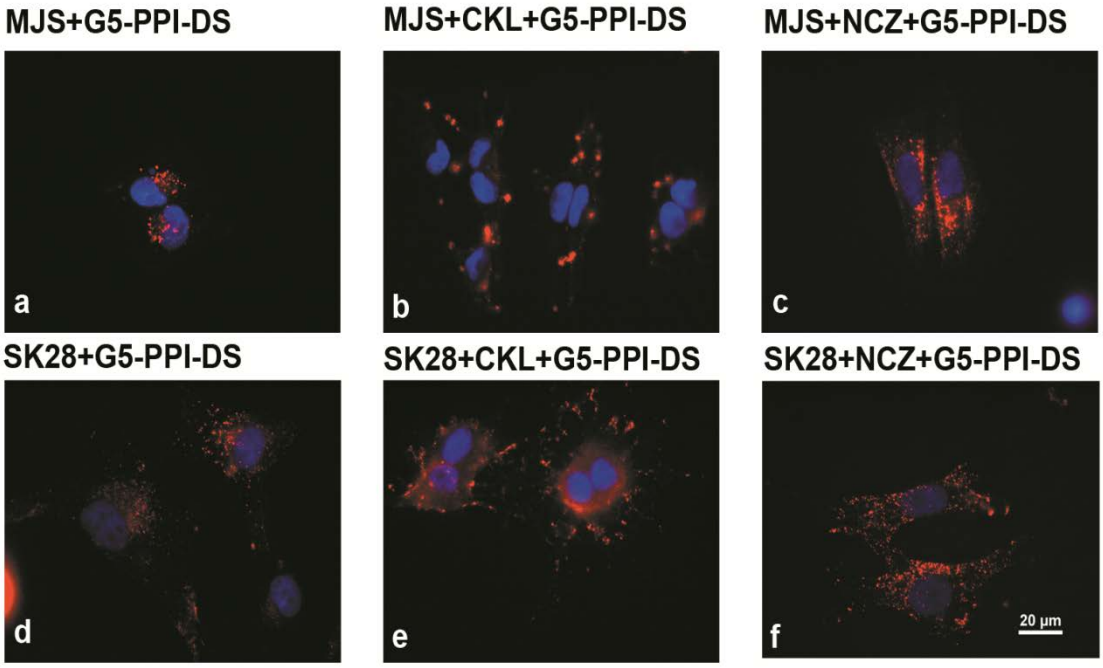


Figure 6. The effect of cytoskeleton alteration on the subcellular distribution of maltose-shell glycodendrimers in human melanoma cell lines. MJS and SK28 cells were pre-treated with CKL and NCZ and incubated with G5-PPI-DS dendrimers conjugated with Rodhamin B. The subcellular distribution of positive structures for fluorescent dendrimers in cells treated and non-treated were analyzed by fluorescent microscopy. Figure was addpted with permission of Bentham Science după Filimon et al., Internalization and intracellular trafficking of poly(propylene imine) glycodendrimers with maltose shell in melanoma cells, Curr Med Chem 2012, 19(29): 4955-68.

It can be concluded that the integrity of actin filaments is important for the internalization of G5-PPI-DS and microtubules play an important role in post-endocytic intracellular trafic. Due to the fact that G5-PPI-DS is endocytosed via clathrin-and cholesterol-independent mechanisms 100% in SK28 and 38% in MJS and the endocytic processes independent of clathrin are well-correlated with actin filament reorganization (14), it is very likely that mechanisms as macropinocytosis to be involved, at lest partially, in internalization of this type of glycodendrimers, but this will need further investigation.

3.3 The subcellular distribution of structures positive for G5-PPI-DS in SK28 human melanoma cell line

The characterization of intracellular structures positive for G5-PPI-DS in relation with cellular compartments and processes in SK28 cell line was performed using specific molecular markers for the main cell compartments or cell structures (15): calnexin for endoplasmic reticulum (ER), EEA1 for early endosomal compartment (EE early endosomes), Rab 11 for recycling endosomes (RE-recycling endosomes), Lamp-1 for lysosomes (LYS), Rab9 for late endosomes (LE-late endosomes), syntaxin 8 for transport processes independent of clathrin and membrane fusion events in protein transport from EE to LE (16) and dextran-FITC (molecular weight 10.000) as marker for macropinocytosis (17). All images obtained from the immunofluorescence experiments are presented in Fig. 7. The G5-PPI-DS associated fluorescence is detected in an area totally opposite to ER positive for calnexin, which suggets that post-endocytic traffic of these glycodendrimeric structures doen not involve this cellular compartment. Moreover, the fluorescence G5-PPI-DS does not overlapp with LAMP-1, despite the fact that vesicles positive for dendrimers are detected in the proximity of lysosomal structures labelled for Lamp-1. Very few structures positive for G5-PPI-DS and EEA1, syntaxin 8 and Rab11 are observed. G5-PPI-DS and late endosomes positive for Rab9 seem to share the same perinuclear region, demostrated by a partial co-localization. This would indicate that that at least a fraction of neutral internalized glycodendrimers is in this type of endosomes. There are also detected very few positive structures for G5-PPI-DS and FITC dextran, a marker for micropinocytosis. A possible explanation would be that SK28 internalize FITC-dextran but this

is rapidely exocytated. It can be hypothesized that both structures use a common internalization pathway (macropinocytosis) but follow distinct intracellular routes distinctly modulated.

3.4 The characterization of structures positive for G5-PPI-DS in SK28 human melanoma cell line

In order to characterize in more detail the positive structures for G5-PPI-DS we used specific pharmacological agents (wortmanin-WO, brefeldin-BRF and cloroquin-CQ) to treat cells post-dendrimer incubation. Cells which have endocytosed the G5-PPI-DS dendrimers for 1h have been washed and further incubated for another 30 min in the presence of the above mentioned pharmacological agents. Furthermore the cell morphology and intracellular distribution of the internalized structures positive G5-PPI-DS in relation with molecular markers and the specific mediated biological processes (Fig. 8) comparatively with the untreated positive controls (Fig. 7).

WO is an inhibitor of phosphatidilinozitid-3-kinases (PI3K), enzymes which catalyze the phosphorylation of the lipidic molecules of phosphatidilinositol to phosphatidilinositol-3-phosphate (PtdIns(3)P), important event in intracellular membrane traffic and fusion between plasma membrane (PM) and endosomes, late endosomes (LE) and lysosmes (LYS), endoplasmic reticulum (ER) and PM or homotypic fusion of early endosomes (EE). PtdIns(3)P together with Rab5 and EEA1 are molecules which involved in endosome trafic and fusion (18), being well known that EEA1 binds to PtdIns(3) and is their best known effector. WO induces a) the formation of enlarged endosomes and association of Rab5 at thier membranes and b) the homotypic fusion of EE (19). In most of the cases in SK28 cells treated with WO

Significant changes can be observed in the morphology of structures positive for EEA1 and G5-PPI-DS (Fig. 8) comparatively with the fine, punctuate morpholgy detected in untreated cells (Fig.7).

WO induces the formation of intense fluorescent structures and visibly larger than in controls positive for G5-PPI-DS or EEA1 which partially colocalize. The effect of WO on subcellular distribution of syntaxin 8, a protein which partially colocalize with structures positive for G5-PPI-DS (Fig.7). In untreated cells syntaxin 8 shows a punctuate pattern in a polarized perinuclear area, TGN-specific, whereas in cells treated with WO, the positive structures for this protein colapses in perinuclear aggregates without having a morphology clearly positive for G5-PPI-DS (Fig. 8). Moreover, WO induces a similar effect in structures positive for Rab9 causing a dense

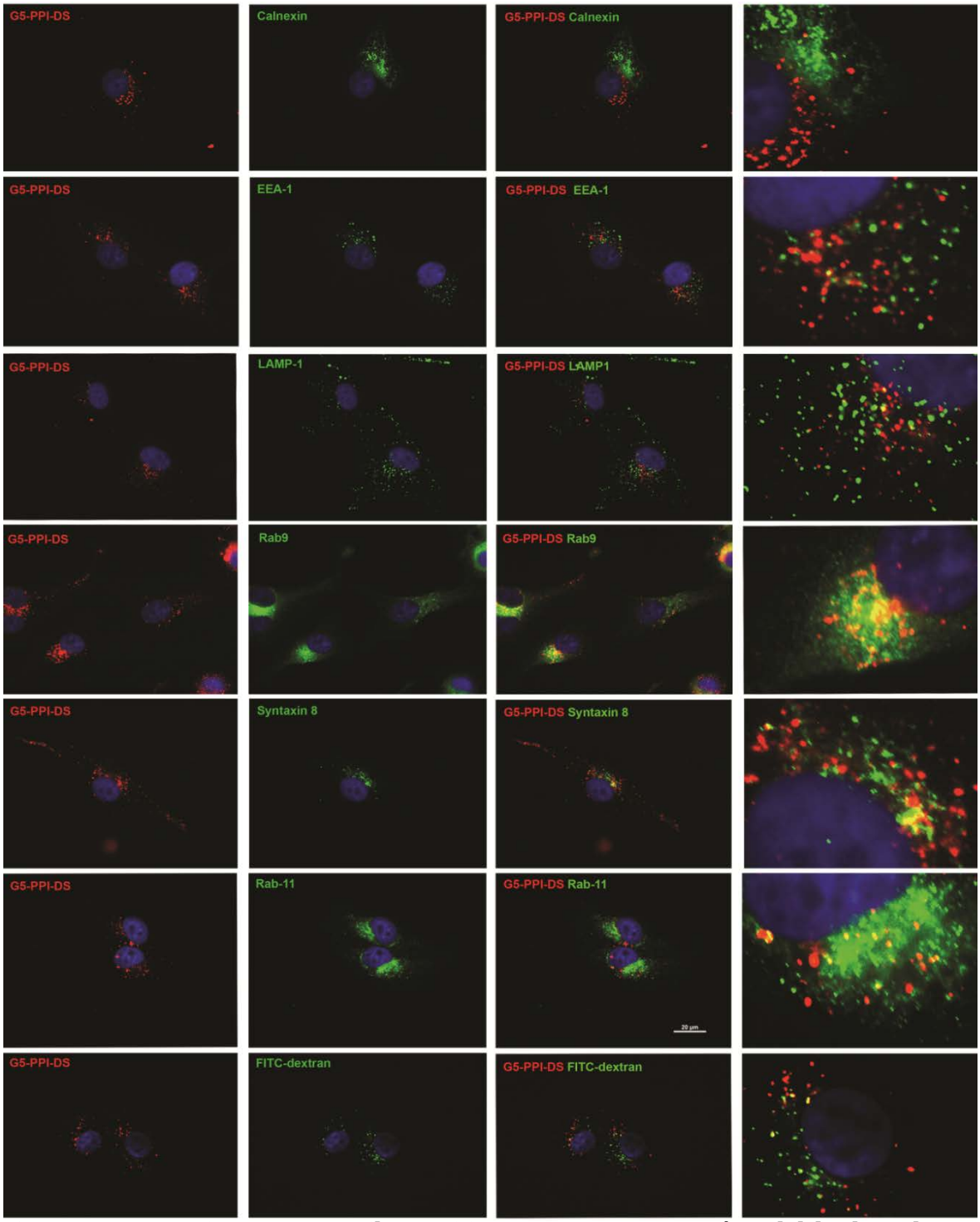


Figure 7. Caracterizarea structurilor pozitive pentru G5-PPI-DS în celulele de melanom metastatic SK28 prin imunomarcarea specifică a compartimentelor intracelulare. Celulele au fost marcate timp de 1 h cu dendrimeri G5-PPI-DS conjugați cu rodamina B și monitorizate în mediu fără dendrimeri timp de 30 de minute sau 1 h acolo unde este indicat. Toate probele au fost fixate, permeabilizate și marcate cu anticorpi pentru markerii moleculari indicați și analizate prin microscopie de fluorescență. Înafară de proteina Arf1 și Rab11 prezintă domenii structurale sensibile la acest inhibitor (78), ceea ce indică faptul că traficul veziculelor pozitive pentru Rab11 este influențat de acțiunea BRF. Figura adaptată cu permisiunea Bentham Science după Filimon et al., Internalization and intracellular trafficking of poly(propylene imine) glycodendrimers with maltose shell in melanoma cells, Curr Med Chem 2012, 19(29): 4955-68.

perinuclear distribution of Rab9 which overlapps on the fluorescence generated by G5-PPI-DS. All together these results advance the hypothesis that G5-PPI-DS is part of a cargo with the dymanics regulated by PtdInsP3 and modulated by PI3K together with Syntaxin 8, Rab9 and less EEA1.

Another pharmacologic agent to which G5-PPI-DS structures are sensitive is BRF. The most extensively documented target of BRF is Arf1 which mediates the recruitment of β-COP at transport vesicles between RE and Golgi (20). The treatment of cells with BRF determines the colaps of ER and Golgi due to the prevention of the formation of transport vesicles and membrane fusion of the two compartments. Besides Arf1, Rab11 shows structural domains sensitive to BRF as well (21), which indicates that the traffic of the vesicles positive for Rab11 is controlled by BRF.

As Fig. 8 shows, as in case of the untreated cells the structures positive for Rab11 and G5-PPI-DS do not colocalize but are redistributed from perinuclear area through entire cytoplasm. Based on these data it can be concluded that the dymanics of the coat formation for G5-PPI-DS positive structures and and of the recycling endosomes Rab11-positive is modulated by BRF.

Taking into consideration that both G5-PPI-DS pozitive structures as well as RE –Rab11-positive were similarly afftected by BRF although their colocalization is poor, we considere worthwhile to determine whether a small fraction of glycodendrimers enters a recycling route. If this theory is true means that this process is very rapid because the cells analyzed at 1h after dendrimer internalization and 30 min post-internalization (Fig.7) was not detected the presence of of dendrimers at PM. This hypotheis was confirmed by labeling cells for 1h with G5-PPI-DS and WO treatment for a prolongued time period of 1h. In addition to the perinuclear colocalization a glycodendrimer fraction accumulated at PM can be detected in all 4 images noted with G5-PPI-DS+WO/1h in Fig.8.

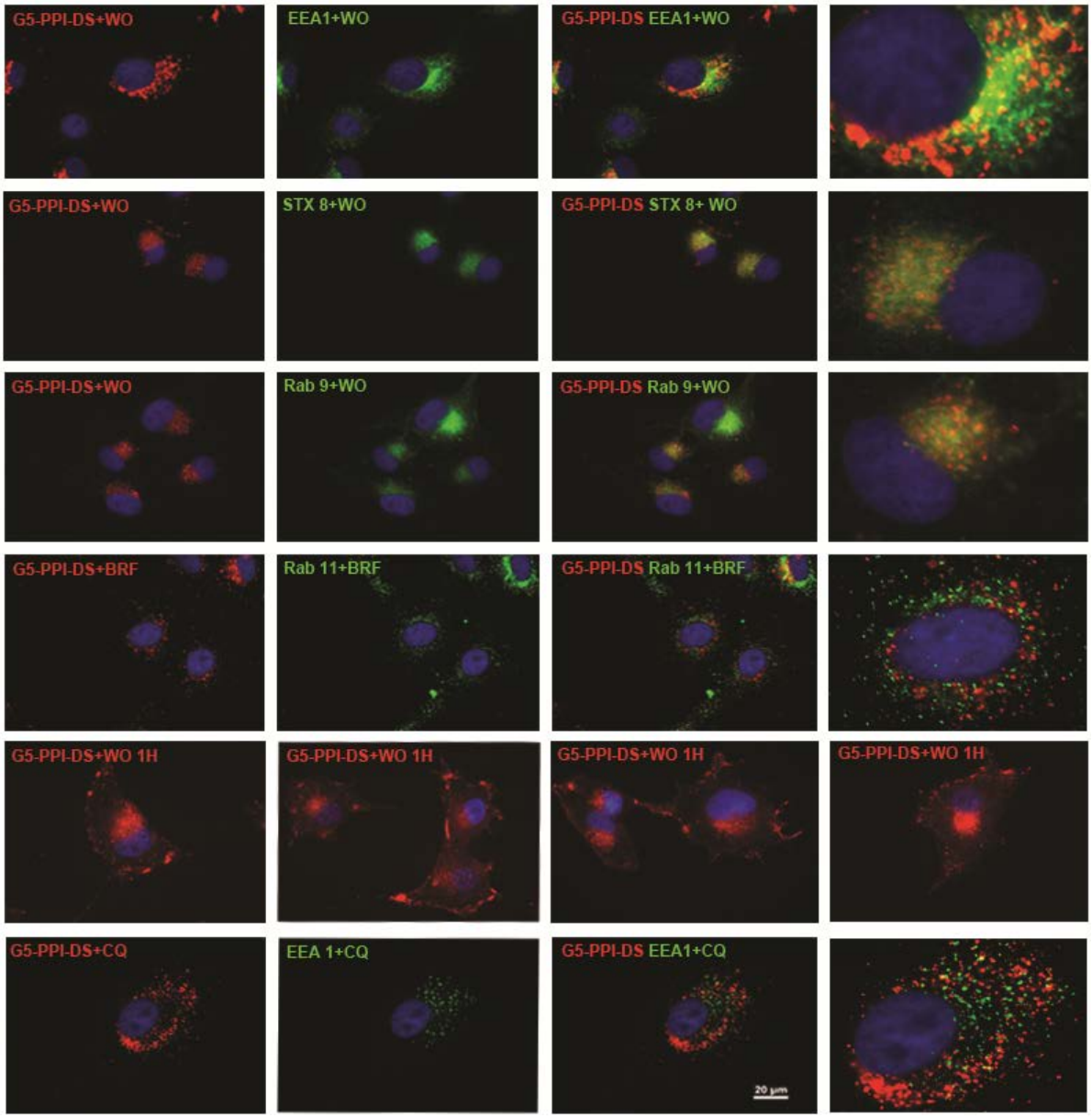


Figura 8. Characterisation of positive structures for G5-PPI-DS in SK28 metastatic melanoma cells in the presence of Wo, BRF and CQ inhibitors.

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Publications and patents list

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***Romanian Journal of Biochemistry*, 299: 36-52 (2009)**

Brevet

Polyclonal Antiserum anti-human Dopachromtautomerase

Patent Assignee Name(s) and Code(s):Institutul de Biochimie, Bucharest

Patent Number(s): 123570

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