

Latest News on Nanotechnology for
Melanoma Therapy and DiagnosisMartina Daga¹, Chiara Dianzani², Benedetta Ferrara², Valeria Nardoza¹, Roberta Cavalli², Giuseppina Barrera¹ and Stefania Pizzimenti^{1*}¹Department of Clinical and Biological Sciences, University of Turin, Italy²Department of Drug Science and Technology, University of Turin, Italy

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Abstract

Melanoma skin cancer is an aggressive tumour with an increasing incidence. In recent years, the treatment options for the advanced disease have expanded dramatically with the employment of targeted therapy and the immunotherapy. However, the high rate of non-response, the toxicity, and the induced drug resistance remain unmet clinical problems. Scientists are expecting a further advance with the application of nanotechnology in melanoma treatment and diagnosis. In this review, we present an up-date on the latest pre-clinical studies (2015-2016) on nanomedicine with potential use in the clinical management of this disease.

Introduction

Melanoma skin cancer is an aggressive tumour whose incidence has been steadily increasing over the last 50 years, now representing 3% of total tumours. In 2012, more than 232,000 new cases of melanoma were diagnosed worldwide; in the same year, mortality estimation calculated 55,500 deaths from melanoma in the world [1], with an increasing stage-specific mortality rate [2]. The treatment of localized disease (stage I and II) is surgical excision, while, in the advanced disease, pharmacotherapy is included in the treatment [3]. Chemotherapy was the first therapeutical option: Dacarbazine (DTIC), an alkylating agent, was approved in 1974 by US Food and Drug Administration (FDA) for the treatment of metastatic melanoma. Later, other chemotherapeutic drugs were used, alone or in combination, in several clinical trials, such as Temozolomide (TMZ), a combination of Cisplatin, Vinblastine and Dacarbazine (CVD), or Carboplatin and Paclitaxel (CP). However, these regimes have been compared with DTIC alone and no significant improvements in overall survival were observed, or they elicited important toxic effects, such as myelosuppression, peripheral neuropathy and fatigue [4].

A recent report has calculated that less than 5% of patients achieve a complete response with DTIC, and the 5-year survival rate is only 2-6% [5]. Recent improvements in treatment effectiveness have been obtained with Immunotherapy and Targeted therapy (Table 1).

The immune system plays a very important role in melanoma progression. Indeed, melanoma can evade immune response which can be associated with immunosuppression [6]; thus, the possibility of re-activating a specific antitumour immune response has been widely explored. IL-2 and interferon were at first used as adjuvant therapy, but, more recently, very specific modulators of the immune response, such as the Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) and Programmed Death-1 (PD-1) inhibitors have been approved. CTLA-4 is a protein receptor present on the surface of cytotoxic T-lymphocytes, and acts as an inhibitory checkpoint that blocks T-cell activation; ipilimumab, the anti-CTLA-4 monoclonal antibody, is able to restore T cell activity [7]. Similar to CTLA-4, the PD-1 receptor is expressed on T-cells, and it normally binds to the PD-1 and 2 Ligands (PD-L1, PD-L2); moreover, it is present on antigen-presenting cells and suppresses T-cell activation; pembrolizumab and nivolumab are two anti-PD-1 antibodies that block the interaction between PD-1 and its ligands, PD-L1 and PD-L2, thus reactivating the T-cell response [8]. Very recently, the first live oncolytic virus therapy against melanoma has been approved by the FDA. Imlygic is a genetically modified oncolytic herpes virus, able to replicate within cancer cells, destroy them, and produce the immunostimulatory protein GM-CSF (Granulocyte-Macrophage Colony Stimulating Factor) [9].

In the last few years, the treatment options for the advanced disease have expanded dramatically, thanks to the identification of activating mutations in the genes involved in melanoma progression. New molecular targeted therapies have been set up, with the development of small molecular inhibitors that target these mutated proteins. In 2011, the FDA approved the first targeted drug for the advanced melanoma disease, the B-RAF inhibitor Vemurafenib [10]. B-RAF is a Serine/Threonine Kinase, belonging to Mitogen-Activated Protein Kinases (MAPKs), one of the main signal transduction pathways involved in cell proliferation. Vemurafenib, with the more recently

Table 1: FDA-approved drugs in Immunotherapy and Targeted therapy.

Targeted therapy		
Drug	Activity	FDA-Approved
Cotellic (Cobimetinib, Genentech)	MEK inhibitor	Nov 2015
Trametinib (Mekinist, GlaxoSmithKline)	MEK Inhibitor	May 2013
Dabrafenib (Tafinlar, GlaxoSmithKline)	Inhibitor of V600E-mutated form of BRAF	May 2013
Vemurafenib (Zelboraf, Roche)	Inhibitor of V600E-mutated form of BRAF	Aug 2011
Immunotherapy		
Drug	Activity	FDA-Approved
Talimogene laherparepvec "T-Vec" (Imlygic, Amgen)	Oncolytic effects and immunomodulating effect by producing GM-CSF (granulocyte-macrophage colony-stimulating factor)	Oct 2015
Pembrolizumab (Keytruda, Merck)	PD-1 inhibitor	Set 2014
Nivolumab (Opdivo, Bristol-Myers Squibb)	PD-1 inhibitor	Dec 2014
Peginterferon alfa-2b (Sylatron, Merck)	Peginterferon alfa-2b with immunomodulating action	Apr2011
Ipilimumab (Yervoy, Bristol-Myers Squibb)	CTLA-4 inhibitor	Mar 2011
IL-2 (Proleukin-Chiron)	IL-2 with immunomodulating action	Jan 1998
Interferon alfa-2b recombinant (Intron A, Schering-Plough)	Interferon alfa-2b with immunomodulating action	Dec 1995

FDA-approved dabrafenib, targets the V600E/K mutated form of BRAF, which is carried in 50-60% of cutaneous melanomas [11]. The treatment with the BRAFV600E/K inhibitors has been successfully used, prolonging both progression-free and overall survival, when compared with DTIC [12-14]. Another member of the MAPK pathway is the Mitogen-activated protein kinase (MEK), which is the primary downstream target of B-RAF. MEK is mutated in about 8% of melanomas [15]. MEK inhibitors, such as cobimetinib and trametinib are now available on the market for the treatment of advanced melanoma (Table 1). Moreover, several clinical trials are on-going for testing inhibitory molecules against other activating mutations which have been identified in melanoma, such as C-KIT or mTOR (i.e.: Trial ID NCT02501551, NCT01280565 and NCT01960829).

Both targeted therapies and immunotherapies are promising for advanced melanoma. However, toxicity and/or drug resistance remain unmet clinical problems. For instance, after vemurafenib treatment, most patients develop resistance after 6 to 7 months [12-14]; the concurrent administration of ipilimumab and nivolumab, although producing a 2-year survival of 79% caused severe immune toxicities in 53% of patients treated with this combination, leading 30% of patients to discontinue this therapy [16,17]. These important limitations strongly suggest investigating novel methods of drug delivery. With the recent and rapid developments in nanotechnology, the incorporation of therapeutic agents into Nanoparticles (NPs) can be the possible answer. The use of drug-loaded NPs can improve the solubility of poorly water-soluble drugs, optimize pharmacokinetics, increase drug half-life, improve bioavailability, achieve targeting specificity, diminish drug metabolism, with the ultimate goals of improving efficacy, overcoming drug resistance, and reducing toxicity.

Several types of NPs for cancer treatment are currently under investigation, including liposomes, Solid Lipid Nanoparticles (SLNs), polymeric micelles, nanospheres, dendrimers, nanotubes, mesoporous silica NPs, quantum dots, super paramagnetic iron oxide NPs, and gold NPs [18-21]. Several nanomedicines for cancer treatment have been FDA-approved, such as pegylated doxorubicin

(Doxil) in ovarian and breast cancer, albumin-bound paclitaxel nanospheres (Abraxane) and liposome-encapsulated doxorubicin (Myocet) in breast cancer [22]. For melanoma, nanomedicines are not yet FDA-approved, but there are several on-going trials (Table 2).

Beside the therapeutical option, another interesting application for the nanotechnological platforms consists of their use for diagnostic purposes. Several types of NPs, containing non-radioactive tracers, can be useful for visualizing the disease site, such as the presence of metastatic melanoma cells into the lymph nodes. Moreover, the possibility of combining the therapeutic and the diagnostic abilities, referred to as theranostic, is receiving growing interest [23-24]. Table 3 also reports the on-going trials having a diagnostic or theranostic purpose.

The opportunities offered by nanomedicine for melanoma treatment, diagnosis, and theranostic applications have been extensively reviewed [18-24-27]. In this review we present an update on the latest pre-clinical studies (2015-2016) of the use of nanotechnology in melanoma treatment and diagnosis.

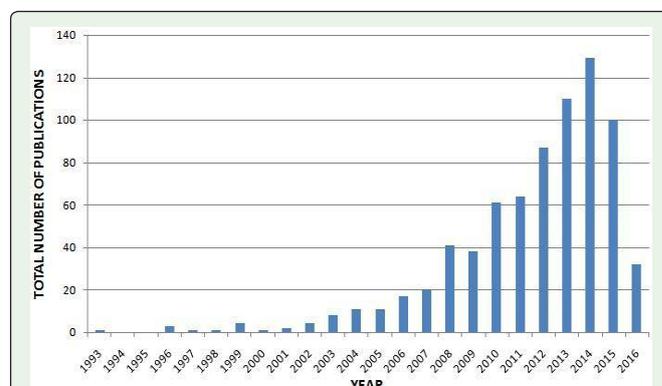


Figure 1: Number of Publications per year on Nanomedicine and Melanoma. Number of publications per year obtained on PubMed with the following query: "(Nanoparticle or Nanomedicine) and melanoma". A great increase is evident from the year 2008.

Table 2: Ongoing clinical trials involving NPs and melanoma (from www.clinicaltrials.gov and from www.isrctn.com).

Nanoformulations	Disease	Phase	Results	Trial ID
Cancer immunotherapy				
Pegylated Interferon-alpha-2a	Patients With Malignant Melanoma IIA-III B	III	ongoing, not recruiting	NCT00204529
Pegylated Interferon-alpha-2b	Melanoma stage I-III	0	recruiting	NCT00871533
Cytotoxic chemotherapy				
Albumin-bound paclitaxel (Nab-paclitaxel)	Pediatric Patients With Recurrent/ Refractory Solid Tumours, including melanoma	I - II	recruiting	NCT01962103
Targeted therapy				
Liposomes containing shRNA against human stathmin 1	Several metastatic tumours, including melanoma	I	ongoing, not recruiting	NCT01505153
Combined therapy				
Nab-paclitaxel and bevacizumab vs. ipilimumab	Patients With Stage IV Melanoma That Cannot Be Removed By Surgery	II	ongoing, not recruiting	NCT02158520
Nab-paclitaxel in combination with bevacizumab	Patients With Stage IV Melanoma That Cannot Be Removed By Surgery	II	ongoing, not recruiting	NCT01879306
Nab-paclitaxel in combination with bevacizumab	Patients With Stage IV Melanoma That Cannot Be Removed by Surgery	I	recruiting	NCT02020707
Rituxan and Nab-paclitaxel	Patients With Inoperable Stage III and IV Malignant Melanoma	II	recruiting	NCT02142335
nab-paclitaxel in combination with PLX7486 (tyrosine kinase inhibitor) and gemcitabine	Patients With Advanced Solid Tumours, including melanoma	I	recruiting	NCT01804530
Liposomal Cytarabine (DepoCyt™) in combination with lomustine and brain radiotherapy	Leptomeningeal Metastasis From Malignant Melanoma	I	recruiting	NCT01563614
Diagnosis and imaging				
Magnetic Nanoparticles	Using magnetic tracers to find the sentinel lymph nodes in patients with Melanoma skin cancer	III	recruiting	ISRCTN15768185
Silica-based nanoparticles labeled with the fluorophore cyanine 5.5, and functionalized with RGD	For Image-Guided Intraoperative Sentinel Lymph Node Mapping in Head and Neck Melanoma, and others cancers	0	recruiting	NCT02106598

The Use of Nanotechnology in Melanoma Treatment

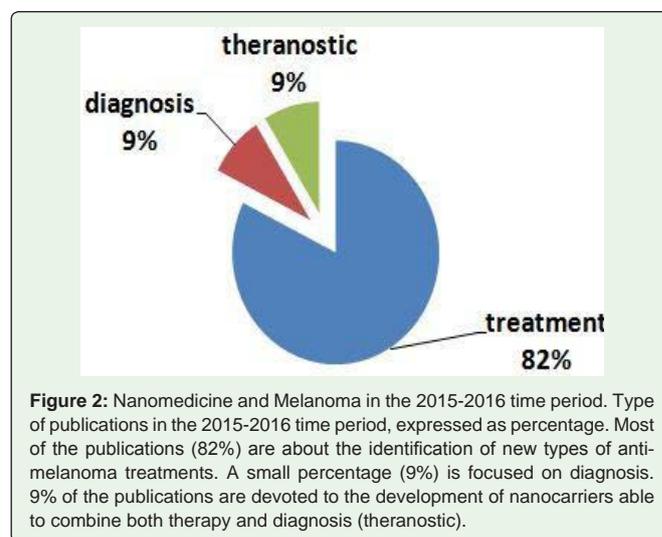
The interest in nanotechnology for the treatment and diagnosis of melanoma has grown exponentially over the past five years (Figure 1). We found 79 publications in the period 2015-March 2016, about latest pre-clinical studies on nanotechnology applied to melanoma research. Most of the publications (65, representing the 82%) are about the identification of new type of anti-melanoma treatments. A small percentage (7 publications representing the 9%) is focused on diagnosis. Seven publications (9%) are devoted to the development of nanocarriers able to combine both therapy and diagnosis (theranostic) (Figure 2).

By considering the publications related to new nanotherapeutic approaches, we can further subdivide them according to the type of treatment: immunotherapy, the treatments that can restore or enhance the immune system’s ability to fight cancer; cytotoxic chemotherapy, which employs the common cytotoxic drugs; new anticancer agents, which explore the anti-tumoral properties of new molecules of different origins; targeted therapy, using drugs that interfere with specific molecules involved in tumor growth and survival; physically-driven therapy, which involves the use of photosensitizing agents; combined therapy, which combines the contemporary use of two or more therapeutical strategies; cellular targeting, which explores several strategies to enhance the active tumor targeting, such as the functionalization of nanocarriers or the stimuli-responsive nanoformulations; and theranostic, nanocarriers able to combine both therapy and diagnosis. In the 2015-2016 period, the nanoformulations were most commonly employed for immunotherapy (21%), followed by cellular targeting (18%), physically-driven therapy (15%), and new

anti-cancer (14%) studies (Figure 3). A graphical overview of these nanotherapeutic approaches for melanoma in 2015-16 is showed in Figure 4.

Cancer Immunotherapy

In recent years, we have witnessed tremendous progress in the development of the cancer immunotherapy, especially for melanoma tumours. Several immunomodulators are already in clinic (Table 1) and the scientists are expecting further advances with nanomedicine applications.



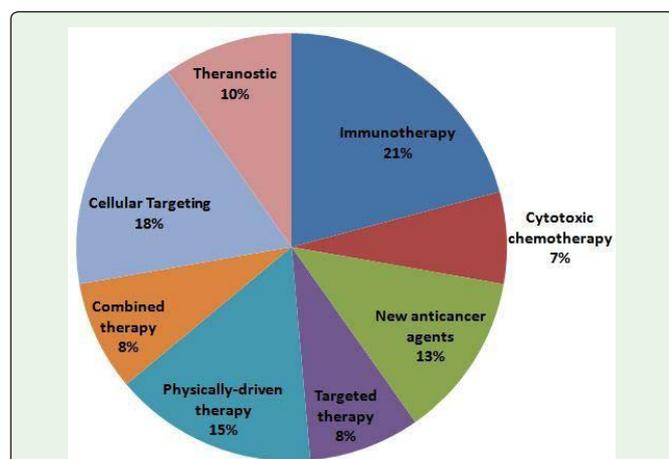


Figure 3: Different Nanotherapeutic approaches for Melanoma Treatment in the 2015-2016 time period. Number of publications in the period 2015-2016, expressed as percentage, relative to the different nanotherapeutic approaches for melanoma treatment.

Inhibition of the immunosuppressive cells: One of the possible strategies of cancer immunotherapy is to eradicate the immune suppressor cells, such as the regulatory T-lymphocytes (Treg) and the Myeloid-Derived Suppressor Cells (MDSCs), a heterogeneous population of immature myeloid cells that suppress effector T cell responses. Similar to anti-CTLA-4 drugs, Li and collaborators [28] developed NPs containing a siRNA targeting CLA-4 (NPsICTLA-4). They demonstrated, in the mouse model bearing B16 melanoma, that NPsICTLA-4 was able to activate the anti-tumour immune responses, as demonstrated by the increase of anti-tumour CD8+T cells, and the decrease of inhibitory T regulatory cells among tumour infiltrating lymphocytes [28]. An interesting strategy for the ablation of MDSCs was proposed by Jeanbart and collaborators [29], which have developed polymer micelles loaded with 6-Thioguanine (MC-TG), a cytotoxic drug used in the treatment of myelogenous leukemia, with the aim of killing MDSCs. After the injection of micelles in B16-F10 melanoma-bearing mice, they found a depletion of MDSC, as well as a higher efficacy of adoptive T cell therapy.

Toll-like receptors (TLR) agonists: Recently, tumour-specific immune activation was achieved with the agonist of Toll-Like Receptors (TLRs). Their engagement leads to innate and adaptive immune responses, which can have anti-tumoural effects [30]. For instance, it has been demonstrated that TLR agonists may indirectly kill chronic lymphocytic leukemia cells, by enhancing the activity of natural killer and tumor-reactive T cells [31]. Moreover, clinical trials demonstrated the successful treatment of cutaneous tumors, such as the basal cell carcinoma with imiquimod, an immunoresponse modifier affecting TLR-7, which is able to stimulate both the innate immune response and the cell-mediated immune system, via induction of cytokines [32].

With this aim, Zhu and collaborators [33] developed a new nanocarrier containing analogs of the unmethylated Cytosine-phosphate-Guanine (CpG), a class of potent adjuvants that activate TLR9, located in the endolysosome of many Antigen-Presenting Cells (APCs). This nanoformulation, obtained by self-assembling concatemer CpG analogs and Magnesium Pyrophosphate (Mg₂PPi), had a rapidly uptake by APCs. In the endolysosomes compartment,

Mg₂PPi was dissolved, due to the acidic environment, and CpG analogs were able to activate TLR9. Thus, APCs started to secrete proinflammatory and co-stimulatory factors, leading to the tumour growth inhibition in B16-F10 melanoma-bearing mice [33].

Cancer vaccines: The use of nanoparticle-based vaccines is a recent application of the field of nanomedicine [30]. A specific antigens, such as ovalbumin (OVA), or tumour-specific antigens with or without adjuvants, loaded in nanostructure, can be delivered to the “in situ” DCs for efficient antigen presentation and consequent stimulation of the antigen-specific response against cancer cells. Hong and collaborators [34] developed new antigen-encapsulating NPs, loaded with OVA, and coated with interLeukin-15 (IL-15) and its receptor IL-15R α (IL-15: IL-15R α), which functions as a vaccine adjuvant. After the treatment of DCs, they found an enhanced ability to stimulate antigen-specific CD8+T cell responses. Moreover, the treatment with (IL-15: IL-15R α)-coated NPs, in an animal model of murine melanoma, significantly increased the survival rate, in comparison with monovalent (IL-15: IL-15R α) treatment. Similar to IL-15, α -GalactosylCeramide (α -GalCer) has been regarded as a potent vaccine adjuvant. Dolen and collaborators [35] have encapsulated both in a single NP and they found that, in mice, a single immunization with OVA+ α -GalCer NPs provided substantial protection from melanoma tumour formation and even delayed the growth of already established tumours.

Poly Lactic-co-Glycolic Acid NPs (PLGA-NP) have been extensively studied for vaccine delivery and have been reported to target dendritic cells naturally through phagocytosis with efficient delivery of the vaccine components. OVA containing PLGA-NPs were loaded in thermoresponsive hydrogels, made of PolyEthyleneGlycol-PolyCaproLactone-PolyLactide-PolyCaproLactone-PolyEthyleneGlycol (PEG-PCL-PLA-PCL-PEG) [36]. The hydrogels stimulated both cellular and humoral responses, and stimulated effective anti-tumour responses in an animal melanoma tumour model. Tumour-Associated DCs (TADCs), compared to normal DCs, are less responsive to TLR stimulation, which has been related to STAT3 hyperactivity. Luo and collaborators [37] developed new nanovaccines with the aim to overcome DC dysfunction. They have co-encapsulated the Poly I:C (PIC), a TLR3 agonist, the OVA antigen,

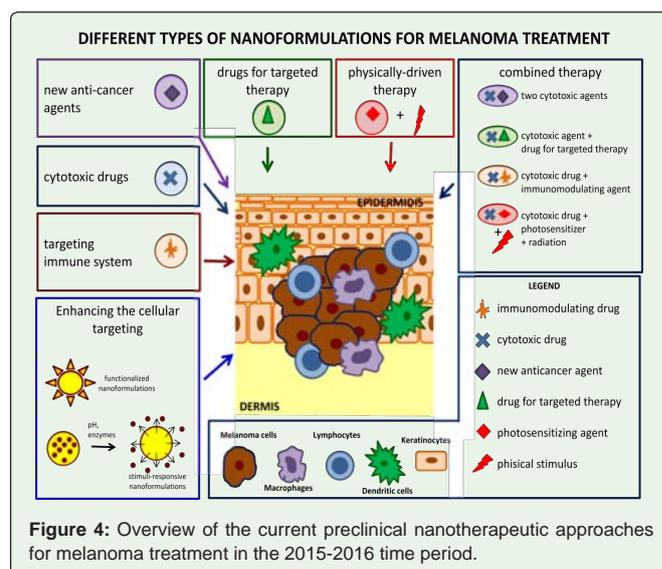


Figure 4: Overview of the current preclinical nanotherapeutic approaches for melanoma treatment in the 2015-2016 time period.

and the siRNA targeting STAT3, in PEG-b-Poly(L-Lysine)-b-Poly(L-Leucine) (PEG-PLL-PLLeu) polypeptide micelles. These micelles showed a strong tumour regression effect with prolonged survival, accompanied by anti-tumour immune responses in B16 melanoma-bearing mice. Silva and co-authors [38] co-delivered OVA and two TRL ligands, such as Poly(I:C) and CpG in mannose-functionalized NPs. The presence of this sugar, on the surface of the NPs, enhanced the up-take from the antigen-presenting cells, which have the mannose receptors. This nanovaccine decreased the growth rate of murine B16F10 melanoma tumours. A gene-carrier system based on chitosan NPs with immunomodulant activity was developed by Yan and collaborators [39]. These NPs were loaded with a plasmid containing the fusion of two genes: the extracellular domain of the activating receptor NKG2D (Natural-Killer Group 2, member D) and the IL-15 gene. The protein showed the ability to activate NK and CD8+T cells, thus enhancing the antitumour activity of the immune system. Indeed, intramuscular injection of fused gene NPs suppressed tumour growth and prolonged survival of melanoma-bearing mice.

The use of specific antigens for melanoma can improve the efficacy of nanovaccines. Several authors, in 2015-2016, developed new nanovector containing specific melanoma antigens and adjuvants. PLGA-based NPs (PLGA-NPs), carrying the melanoma antigen (hgp10025-33) and, as adjuvant, the TRL4 agonist MonoPhosphoryl Lipid (MPLA), were recently reported [40]. Interestingly, the PLGA-NPs were coated with erythrocyte membranes, by virtue of their easy isolation and intrinsic biocompatibility [41]. PLGA-NPs were further modified by adding the mannose on the surface, to actively target APCs in the lymphatic organ. This nanovaccine demonstrated superiority to an ordinarily used vaccine formulation against tumour prevention, growth, and metastasis in B16-F10 bearing mice [40]. Zhuang and collaborators [42] proposed a Lipid-coated Zinc Phosphate hybrid NP (LZnNP NP), able to co-deliver both the tumour specific antigens, represented by a multi-peptide (TRP2180-188 and HGP10025-33) and a TRL4 agonist (MPLA) as adjuvant. This nanoformulation, with the size of 30 nm, was intradermally injected in C57BL/6 mice. Ten days after the last immunization, C57BL/6 mice were inoculated subcutaneously B16-F10 melanoma cells. Mice exhibited antitumour immunity, as demonstrated by the secretion of cytokines and the increased CD8+T cell response. This antitumour effect elicited an inhibition of melanoma growth, more consistent when compared with the treatment of free antigens [42]. The gp100 melanocyte differentiation protein epitope was loaded into NPs, obtained by engineering the E2 subunit of pyruvate dehydrogenase [43]. Moreover, this non-viral cage contained CpG DNA molecules, able to increase the antigen-specific anti-tumour responses following immunization, since CpG sequences are similar to those found in bacterial DNA. They succeed in obtaining higher CD8+T cell activation, as well as an increased survival of animals bearing B16 melanoma by 40%, compared to PBS-treated animals [43].

The melanoma immunotherapy can also take advantage of the treatment with plant-derived Viral NPs (VNPs), since they are natural nanomaterials, biodegradable and biocompatible. They can be used as a carrier for drug delivery or for imaging applications, or they can use as an adjuvant immunostimulatory molecules, able to activate the anti-tumoural T-cell response [44]. For instance, it has been shown that the self-assembling virus-like NPs from Cow Pea Mosaic Virus (CPMV) [45] and the Papaya Mosaic Virus nanoparticle (PapMV) [46] suppress melanoma metastatic cancer in animals.

Cytotoxic Chemotherapy

In melanoma, the response rates to the common cytotoxic drugs are very low. Thus, the scientists do hope to develop effective anticancer treatments thanks to nanotechnology, which will increase the effectiveness of anti-cancer treatments. Gold NPs (AuNPs) conjugated with doxorubicin (Au-Dox) are receiving great attention. Zhang and collaborators [47] demonstrated that the intratumoural injection of ultra-small Au-Dox is effective against melanoma in immunocompetent mice bearing murine B16 melanoma cells and in nude mice bearing human SK-MEL-28 xenograft. Moreover, Tawagi and collaborators [48] compared the toxicity of Au-Dox in B16 melanoma cell lines and cardiomyocytes, measured by real-time growth assays and Fluorescence Lifetime Imaging Microscopy (FLIM). They demonstrated that cardiomyocytes were more sensitive than B16 cells to Dox alone, but were dramatically less sensitive to Au-Dox. On the contrary, Au-Dox was more effective in inducing cell death of B16 melanoma cells, compared to Dox alone. The different patterns of Au-Dox in the two cell types can explain the differential toxicity: while Au-Dox concentrated in the nuclei of B16 cells, it remained endosomal in cardiomyocytes. Kaiser and collaborators [49] demonstrated an enhanced antitumoural activity of docetaxel, when loaded in Acid-Prepared Mesoporous Spheres (APMS-TEG). This nano-formulation was effective in MelJuSo, UACC903, and WM1205 melanoma cell lines at a nanomolar concentration, thus suggesting a potential use in clinic. A Graphene Oxide (GO) sheet conjugated with paclitaxel (PTX) was successfully employed in B16 melanoma-bearing C57 mice [50].

An interesting study has determined the influence of nanoparticle size on targeting lymph node metastases [51]. The authors delivered micelles, loaded with the platinum anticancer drug, with different diameters, in a syngenic melanoma model. The targeting of lymph node metastases was compared with results obtained with the clinically used Doxil having a diameter of 80 nm. They found that the sub-50 nm micelles were more efficient in reaching lymph node metastasis, having a higher capability in extravasating from the blood and penetrating into the metastatic tumour.

New Anticancer Agents

The anti-melanoma properties of several plant extracts, or derivatives, have been explored in the last two years.

Dwivedi and collaborators [52] have studied the antitumoural effect of artemisone, an artemisinin derivative, isolated from the plant *Artemisia annua* and currently used as an antimalarial drug, on melanoma cells *in vitro*. They loaded Artemisone in nano-vesicular niosomes and in solid lipid NPs, demonstrating in both cases enhanced antiproliferative effects against human melanoma A375 cells, with respect to the free drug. Moreover, these nanoformulations had negligible toxicity towards normal skin cells. Later, they performed *in vitro* skin permeation studies with both nanoformulations. They found out that, in the stratum corneum-epidermis, artemisone-SLNs were found at higher concentration compared to than the artemisone-niosomes, and that, in the epidermis-dermis, artemisone was only detected after application of the SLN formulation. These results suggest the possible topical delivery of artemisone-SLNs in treatment of melanoma [53].

Hu and collaborators [54] have developed new NPs loaded

with saikosaponin, a biologically active compound extract from the *Bupleurum chinense*, with anti-melanoma activity “*in vitro*” and “*in vivo*”. They were able to demonstrate that saikosaponin-d NPs enhanced the antiproliferative activity against melanoma cells, and induced apoptosis through the mitochondrial pathway.

The plant-derived curcumin, loaded into chitosan-coated NPs was orally administered to the B16F10 metastatic melanoma bearing mice, obtaining an enhanced anti-tumoural activity [55].

Gismondi and collaborators [56] developed novel Detonation NanoDiamonds (DNDs), which are new nanoparticles produced by detonation of explosive Carbon materials. They loaded the Citropten (5,7-dimethoxycoumarin) agent, a plant secondary metabolite into DNDs, and demonstrated that this nanovehicle was able to reduce B16-F10 tumour cell growth.

The herb-derived compound triptolide (TP), with anti-angiogenic properties, was loaded into methoxy poly(ethylene glycol)-block-poly(ϵ -caprolactone) micelles by Wang and collaborators [57]. In B16-F10 melanoma-bearing mice they demonstrated that the nanovehicle enhanced the TP accumulation in tumour tissues, increased the survival time and inhibited angiogenesis.

4-Hydroxynonenal, an endogenous compound derived from lipid peroxidation was studied as an anti-melanoma agent [58]. A new type of lipid nanocapsule, based on β -Cyclodextrin-poly(4-acryloylmorpholine) conjugates, was designed to enhance its solubility and stability. HNE loaded in the nanocapsules was more effective than free HNE in inhibiting proliferation of several tumour cancer cells, including melanoma. Moreover, the effect of these new nanocapsules on a three-dimensional human reconstructed model of skin melanoma was evaluated. Two diverse treatments were performed: one in the medium, mimicking the parenteral administration, and the other onto the epidermal surface, mimicking the topical treatment. Both treatments were more effective than free HNE on melanoma cell growth inhibition. Interestingly, the encouraging results obtained with the topical administration on the epidermal surface could open new perspectives in melanoma treatments [58].

Other chemical compounds loaded into NPs were successfully employed for melanoma treatment, such as the novel synthetic tubulin inhibitor, 2-(1H-indol-5-yl)thiazol-4-yl)3,4,5-trimethoxyphenyl methanone (abbreviated as LY293) [59], and the Cerium oxide CeO_2 [60].

Targeted Therapy

Most recent preclinical studies in the field of targeted therapy explored the use of nucleic acid for melanoma treatment.

Li and collaborators [61] developed new NPs able to carry the DNA for the pro-apoptotic gene PUMA (p53 up-regulated modulator of apoptosis). In particular they used the cationic Polymer Polyethylenimine (PEI), widely employed for non-viral transfection, crosslinked with Sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate (Sulfo-SMCC) conjugating Trans-Activating Transcriptional activator (TAT). This nanovector enhanced the transfection efficiency of PUMA gene in malignant melanoma A375 cell, resulting in increased apoptosis.

Polycation based NPs (jetPEI) were used as carrier for the delivery of a plasmid expressing the short hairpin RNA (shRNA) against the CXC motif Chemokine Receptor 4 (CXCR4) [62], as an anti-

metastatic target. They succeed in obtaining a significant reduction in the number of pulmonary metastatic nodules (50%) in animals that received a retro orbital injection of jetPEI CXCR4 1 shRNA.

Hundt and collaborators [63] set up a method to monitor by MR imaging the antiangiogenic gene therapy in M21 melanoma-bearing mice. As the antiangiogenic gene, they chose the dominant-negative mutant form of Raf-1 (Raf-1-). The plasmid containing Raf-1- was loaded into RGD-targeted sNPs and was given to the animals. They found that the targeted gene delivery therapy induces significant changes in Magnetic Resonance Imaging (MRI) and that there was an excellent correlation between MRI and histological results, which were direct effect of the gene delivery therapy.

Beside the employment of the nucleic acids, specific inhibitors were also studied, such as the Sn-2 lipase-labile, an inhibitor of the oncogene c-myc [64] and glycomimetic (P-3F(ax)-Neu5Ac), an inhibitor of the sialic acid, frequently over expressed on cancer cell surfaces and contributing to the metastatic process [65].

A nanovector for the imatinib mesylate, a tyrosine-kinase inhibitor already in clinical use for several tumours, was developed by Labala and collaborators [66]. This nanoformulation consists of a layer-by-layer polyelectrolyte coated AuNP (LbL-AuNP) and it was specifically designed for the ionophoretic transport into skin.

Physically-Driven Therapy

Therapies inducing hyperthermia: Photo Thermal Therapy (PTT) involves the use of light and a photosensitizer to generate heat for therapeutic purposes. Gold-based NPs are widely used for this purpose, since after the irradiation, they generate a localized heat so as to damage a region of interest [67]. A Gold-Ferrite Nano Composite (GFNC) was obtained by Heidari and collaborators [68], as a photo thermal agent in melanoma-bearing mice. They have demonstrated a higher necrotic surface tumour area in mice receiving GFNC injection and laser irradiation. Wang and collaborators [69] developed gold nanoshell capsules, which easily penetrate melanoma cells. After a mild laser irradiation, they observed a consistent ablation of malignant melanomas.

Magnetic Field Hyperthermia (MFH) treatment has received great attention from the scientific community, since this therapeutic option elicits the heating of magnetic NPs by time-varying magnetic fields. Blanco-Andujar and collaborators [70] demonstrated that human melanoma cells undergo apoptosis upon exposure to citric acid-coated iron-oxide NPs, followed by a Magnetic Field Hyperthermia (MFH) treatment.

The ability of Radio Frequency (RF) radiation to heat human tissues has been known for a long time, and now this knowledge can be exploited in cancer therapy. Haghniaz and collaborators [71] have explored the potential use of Dextran-coated (Dex) Lanthanum Strontium Manganese Oxide (LSMO) NPs, as a hyperthermia agent in the treatment of cancer. B16-F1 melanoma cells were exposed to Dex-LSMO NPs and heated using a radiofrequency generator, finding that the cell death increased in a time-dependent and temperature-dependent manner.

Photo dynamic therapy (PDT): Photo Dynamic Therapy (PDT) uses nontoxic photosensitizing agents and a light source to treat cancers. Under light exposure, these chemical compounds are excited and are able to produce ROS, able to kill cancer cells. Ogawara and

collaborators [72] developed a new nanoformulation for cancer PDT. They encapsulated Photoporphyrin IX DiMethyl Ester (PppIX-DME), a hydrophobic porphyrin derivative, into polymeric NPs composed of polyethylene glycol and polylactic acid block copolymer (PN-Por). An “*in vitro*” phototoxicity study clearly indicated the significant phototoxicity of PN-Por for three types of tumour cells, (including B16-BL6 melanoma cancer cells), in a PppIX-DME concentration-dependent fashion [72]. Thus, the use of Zinc Oxide (ZnO) NPs in anticancer treatment has become a promising strategy, due to their excellent photo-oxidation activity.

Under light activation, ZnO, or a derivative, is able to induce ROS production, thus killing cells via oxidative stress. However, ZnO has a low photocatalytic decomposition rate and Arooj and collaborators [73] demonstrated that metal ions such as Silver (Ag) improve their activity. Under daylight exposure, ZnO: Ag nanocomposites induced cell death of human malignant melanoma (HT144) more efficiently than ZnO alone. Interestingly, these ZnO: Ag nanocomposites killed melanoma HT144 cells more efficiently than normal Human Corneal Epithelial Cells (HCEC). Wang and collaborators [74] succeeded in treating cultured melanoma cells and a B16 murine melanoma model with Near-InfraRed (NIR) Plasmonic copper sulfide (Cu₂-xS) Nano Crystals (NCs), followed by NIR irradiation. Interestingly, they concluded that the therapeutic effect was due to a combination of the photo thermal heat mechanism (Photo Thermal Therapy, PTT) and the photodynamic activity, via the production of high levels of ROS.

Araki and collaborators [75] explored the possibility that the Photo Dynamic Therapy (PDT) towards the tumour vasculature (Photo-triggered tumour Vascular Treatment, PVT) may enhance the vascular permeability, leading to augmented Enhanced Permeability and Retention (EPR). B16 tumour-bearing mice, with low permeable vasculature, were treated with liposomal paclitaxel (PL-PTX) and a hydrophobic porphyrin derivative in polyethylene glycol-block-poly(lactic acid) NPs was used as a photosensitizer. The authors demonstrated that the PVT treatment enhanced the anti-tumour activity elicited by PL-PTX, thus augmenting the EPR effect in a model of low permeable tumour vasculature.

Non-thermal atmospheric-pressure plasma: Non-thermal atmospheric-pressure plasma, also named cold plasma, is defined as a partly ionized gas and it is a new innovative approach to medicine [76]. Recently, its anti-tumour activity has emerged and gained attention. However, its action is not specific. Choi and collaborators [77] succeeded in enhancing the capability of the cold plasma in specifically killing melanoma cells. They have targeted NEU (human epidermal growth factor receptor 2) protein, which is frequently over-expressed in the cell membrane of melanoma cells, using anti-NEU antibody-labeled gold NPs. The labeled NPs preferentially targeted melanoma cells rather than normal keratinocytes. Both cells were exposed to the cold plasma and they found the death rate of melanoma cells was significantly higher than that of normal keratinocyte cells.

Combined Therapy

Several combination therapies have been FDA-approved for melanoma treatment (i.e. nivolumab plus ipilimumab; trametinib plus dabrafenib), and studies on other combinatory regimens are on-going in many trials. Thus, the researchers continue exploring several types of combinations in pre-clinical studies, involving nanotechnological carriers.

Combining two chemical agents: Ruttala and collaborators [78] have developed a liposome carrier containing two chemotherapeutic agents, paclitaxel (PTX) and curcumin (CUR). Via a thin-film hydration technique, they encapsulated the PTX-loaded Albumin NPs (APN) in PEGylated hybrid liposomes containing CUR (CL-APN). This co-loaded delivery system has shown a higher cytotoxic effect on several tumour cell lines, including B16-F10 melanoma cells, compared to single free chemotherapeutic drugs or single drugs encapsulated in the respective nanocarrier.

Recently, glutamate receptor antagonists, mainly used in the treatment of many neuronal diseases, have been proposed as anticancer agents. Tan and collaborators [79] developed Mesoporous Silica NPs (MSNPs) loaded with both an ionotropic Glutamate (iGlu) receptor antagonist, the 4-Hydroxyphenylacetyl spermine (L1), and Dox. Moreover, Dox was trapped within the MSNPs by a redox-cleavable linker, thus being able to be released upon exposure to glutathione. The authors demonstrated an enhanced antitumoural effect of L1 and Dox co-delivering on B16-F10 melanoma cells *in vitro*.

Combining chemical agent with targeted therapy: A porous silicon-based Micro/Nano Composite (MNC) has been designed, able to co-deliver a chemotherapeutic drug, such as Docetaxel, and a small interfering RNA (siRNA) against BRAF. The MNC was more effective in inhibiting tumour growth and reducing lung metastasis in a mouse melanoma model [80].

Doddapaneni and collaborators [81] designed a novel PEG-PCL polymer able to contain three drugs against melanoma: docetaxel (targeting microtubules), everolimus and LY294002 (two inhibitors of mammalian target of rapamycin, mTOR). They were able to modify the surface charge of the NPs, obtaining neutral, partially charged, or fully charged surface, with the aim of having preferential uptake and accumulation in the lymphatic system, in mice injected subcutaneously. Two metastatic melanoma mouse models with the two major mutations (NRASQ61K and RRR α) found in human melanoma, were used for the *in vivo* studies. After NPs injection, they found that the partially charged NPs have the highest potential in treating metastatic melanoma, demonstrating that the surface charge is a critical parameter for the lymphatic uptake [81].

Combining chemical agent with radiotherapy: Li and collaborators [82] succeeded in establishing a Pluronic® F127-based thermosensitive hydrogel (Au-Dox-Gel) containing gold NPs (AuNPs), used as radio sensitizer and Dox, the chemotherapeutic drug, to improve cancer chemo radiotherapy. Indeed, after radiation, tumour sizes in melanoma B16 bearing mice were significantly decreased by Au-Dox-Gel compared to control mice.

Combining chemical agent with immunotherapy: Chemotherapy with immunotherapy is a regimen generally referred to as ‘BioChemoTherapy’ (BCT). In this respect, Zhao and collaborators [83] have co-delivered two types of NPs in B16-F10 melanoma-bearing mice, one carrying the vaccine antigen and the second loaded with the chemotherapeutic agent. As anti-cancer drug, they have used the triterpenoid methyl-2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (CDDO-Me) loaded in PLGA-NPs. The antitumoural mechanisms of CDDO-Me include induction of apoptosis and modulation of several signal transduction pathways involved in tumour cell proliferation, but it can also block furthermore the

immune suppressive function of Myeloid-Derived Suppressor Cells (MDSCs) and improve the immune response to cancer. The second nanovector consisted of the self-antigen tyrosinase-related protein 2 (Trp2) peptide, a melanocyte differentiation antigen, loaded in a Lipid-Calcium-Phosphate NanoParticle (LCP-NP). The authors demonstrated that the intravenous delivery of CDDO-Me loaded in PLGA-NP, combined with the subcutaneous Trp2 vaccination, resulted in an increase of anticancer activity compared to Trp2 vaccine alone in B16-F10 melanoma-bearing mice [83].

Enhancing the Cellular Targeting

Two main strategies are in use to enhance active tumour targeting. The first consists of the decoration of the surface of the nanocarrier with ligands (i.e. antibodies, aptamers, peptides, sugars) to allow for the homing of the drug to a specific target site. The second approach is the stimuli-responsive delivery strategies, in which the drug release can be achieved within a tumour in response to a cancer-specific stimulus.

Functionalized nanocarriers: A well-known peptide used in targeting nanocarriers is the arginine-glycine-aspartic (RGD) peptide, which is the minimal binding domain of fibronectin necessary to recognize cell surface $\alpha\beta3/\alpha\beta5$ integrins, frequently over expressed on cancer cells and tumour vasculature. Zhao and collaborators [84] enhanced the hydrosolubility of the anti-cancer agent curcumin with a PEG-PLA micelle-based drug delivery system. These PEG-PLA micelles were functionalized with the $\alpha\beta3$ integrin-targeted peptide RGD. The authors showed that RGD-functionalized PEG-PLA micelles containing curcumin had a stronger inhibition of tumour growth in B16 tumour-bearing mice, compared with non-RGD modified PEG-PLA micelles. Similar results were obtained by Makino and collaborators [85] in B16-F10 melanoma-bearing mice with PEG micelles loaded with platinum anticancer drug and decorated with the RGD peptide. Moreover, they demonstrated the cyclic RGD peptide (cRGDs) have antitumour activities themselves, since it has been shown that they can inhibit tumour growth and metastasis by interfering with the angiogenesis or the integrin-dependent metastatic processes.

Hyaluronan (HA) is another interesting molecule which has been explored for active targeting. This non-sulfated polysaccharide is being recognized as an important regulator of cancer progression and is a ligand for CD44, a transmembrane glycoprotein abundantly expressed in many malignant tumours and present on many types of Cancer Stem Cells (CSCs) [86]. An enhancement of anti-melanoma activity was then observed after HA-decorated nanocarrier treatments in melanoma-bearing mice [87,88].

Interesting results on enhanced anti-melanoma activity have been obtained with several other molecules used for active targeting, such as anisamide, a small molecule of benzamide specific ligand for the Sigma-1 receptor, highly expressed by tumour cells [89]; the tumour-penetrating peptide RPARPAR [90], able to bind to the cell surface Neutrophilin-1 receptor (NRP-1), with essential roles in vascular biology and which is over expressed in angiogenic endothelial cells and in tumour cells; the tumour homing peptide GKRR [90], ligand for the receptor p32, a mitochondrial chaperone protein, aberrantly expressed at the surface of activated cells such as tumour blood and lymphatic endothelial cells, tumour cells, and

tumour-associated macrophages; and the Polydopamine (PDA), a mimic of the specialized adhesive foot protein Mefp-5 (mytilus edulis foot protein-5) secreted by mussels [91].

Stimuli-responsive particles: In the most recent period, scientists have focused their attention on pH-responsive nanocarrier and enzyme-responsive nanovehicles.

pH-responsive nanovehicles, from acetalated cyclodextrins loaded with docetaxel, have demonstrated a dramatically enhanced efficacy in a melanoma-bearing nude mouse model [92]. Xu and collaborators [93] have developed a pH-sensitive carrier, able to simultaneously deliver Dox and Bcl2 siRNA, specifically designed for local treatment of lung metastasis. Dox was conjugated onto Polyethylenimine (PEI) by using Cis-aconitic Anhydride (CA, a pH-sensitive linker) to obtain PEI-CA-Dox conjugates. At acidic pH the drug was released faster. Then, the anionic siRNA spontaneously formed a complex with the cationic PEI-CA-Dox NPs. This nanoformulation showed higher anti-cancer activity in B16F10 melanoma cells in vitro, with respect to the treatment with either Dox or Bcl2 siRNA alone. Interestingly, when it was directly sprayed into the lungs (with acidic pH), of B16-F10 melanoma-bearing mice, the PEI-CA-Dox/Bcl2 siRNA complex NPs exhibited enhanced antitumour efficacy compared with the single delivery of Dox or Bcl2 siRNA.

Among the enzymes, the Matrix MetalloProteases (MMPs) and gelatinases have been selected as the external stimulus for the nanocarrier opening, since these enzymes are more highly represented in the cancer microenvironment. Jallouk and collaborators [94] have designed a new perfluorocarbon NP delivery system activated by MMP-9 cleavage, able to carry mellitin derivatives (cytolytic peptides derived from bee venom) and obtaining enhanced tumour growth suppression in a mouse model of melanoma. Similar results were obtained with a novel gelatinase-stimuli nanoparticle, loaded with pemetrexed, a new antifolate medicine with antitumoural and antimetastatic activity [95].

Finally, a combination of the two types of active targeting treatments was presented by You and collaborators [96], which designed a complex nanocarrier with three layers: the innermost core of PCL loaded with the anti-cancer drug camptothecin (CPT), the medium layer containing the folate receptor, for the active targeting via functionalization, and the outer part consisting of a PEG layer sensitive to MMP2 and MMP9. In presence of the tumour cells, the PEG layer would detach from the NPs, due to the higher level of MMP2 and MMP9 in the cancer microenvironment, resulting in the exposure of folate to enhance the cellular internalization via folate receptor-mediated endocytosis, which accelerated the release rate of CPT in vivo. These nanovectors showed an enhanced anti-cancer activity on melanoma B16 bearing mice.

The Use of Nanotechnology for Imaging and Diagnosis

The presence of Lymph Node (LNs) metastasis is an important prognostic factor in melanoma. Nanotechnology can help in noninvasive, specific and sensitive detection of LN metastasis. Ultra small tumour-targeting inorganic (Silica) nanoparticles have been recently proposed as an intraoperative tool for guiding resection of sentinel lymph node metastases [97]. The specificity and sensitivity of contrast-enhanced MRI lymphography can be improved with the use of Gadolinium (Gd)-loaded NPs, as demonstrated by Partridge and

collaborators [98] in mice bearing B16-F10 melanoma. Interestingly, Zhou and collaborators [99] have demonstrated that Gd-embedded Iron oxide nanoplates (GdIOP), functionalized with Zwitterionic Dopamine Sulfonate (ZDS) molecules, were uptaken differently by B16 melanoma cells and by immune cells, such as macrophages and dendritic cells in B16 melanoma-bearing mice. After the addition of GdIOP NPs, they demonstrated that they were efficiently uptaken by immune cells, whereas melanoma B16 tumour cells showed a lower intake. Under T1-T2 dual-modal MRI, this difference generated pseudo contrast images, with potential use for the detection of tumour metastasis in LNs.

A possible application of nanotechnology is to detect angiogenesis *in vivo*. Melemenidis and collaborators [100] have developed RGD-targeted NPs of Iron Oxide (NPIO) for MRI of tumour angiogenesis, after injection, mice bearing subcutaneous melanoma (B16-F10) tumours underwent *in vivo* MRI. The authors demonstrated the specific binding of RGD-targeted NPs loaded with NPIO with the $\alpha v \beta 3$ expressing neo-vessels of the tumours.

Meir and collaborators [101] have found an interesting method to trace immune cells, with the aim to study their fate in cancer immunotherapy. T-cells were transduced to express a melanoma-specific T-cell receptor and then labeled with gold NPs (GNPs) as an X-ray Computed Tomography (CT) contrast agent. After injection, in mice bearing human melanoma xenografts, whole-body CT imaging allowed examination of the distribution, migration, and kinetics of T-cells.

Another interesting application of nanotechnology is the possibility of detecting and quantifying Circulating Tumour Cells (CTCs), since their presence at an early stage of cancer progression is of significant prognostic value, even in melanoma cancer [102]. However, most of the common methods to detect melanoma markers often lack sensitivity or selectivity and often produce false-positive results. Seenivasan and collaborators [103] proposed an electrochemical immunosensing method to detect melanoma cells, based on the affinity between cell surface MelanoCortin 1 Receptor (MC1R) antigen and anti-MC1R Antibody (MC1R-Ab). The MC1R-Abs were immobilized in amino-functionalized silica NPs and they achieved the very low detection limit of 20 cells/ml for melanoma cells.

Theranostic Applications on Melanoma

The possibility to load more than one molecule into a single nanocarrier opens the way to theranostic applications, since it allows the co-delivery of an anti-tumour agent and a tracer for imaging, useful for diagnosis. Bazylińska and collaborators [104] succeeded in developing a new nanoemulsion able to carry both DNA, useful for the gene transfer strategy in cancer treatment, and IR-780 indocyanine, a fluorescent marker for bioimaging analysis. They obtained good bioimaging of intracellular localization by Confocal Laser Scanning Microscopy (CLSM) and Total Internal Reflection Fluorescence Microscopy (TIRFM) in a melanoma MEWO cell line. Later, the same group developed new theranostic nanocarriers loaded with both colchicine, a cytostatic drug, and coumarin-6, as fluorescent tracer. These labeled nanovehicles were efficiently up-taken by several cell lines of different origins, including melanoma MEWO cells. The good fluorescent signal, as well as the biocompatibility, and the enhanced

antitumoural activity of colchicines of this nanoformulation suggest the potential use as nanotheranostic agents [105].

Vannucci and collaborators [106] developed PEG-based NPs functionalized with both the Human protein Ferritin (HFt), and the α -Melanocyte-Stimulating Hormone (α -MSH) and also contained fluorophore and magnetic resonance imaging tracers to detect “*in vivo*” localization of NP. After the treatment in a spontaneous metastatic mouse melanoma model, targeted HFt-MSH-PEG NPs accumulated mostly in the primary melanoma lesions, as well as at the metastasis level, with high selectivity with respect to other organs.

Interestingly, some metals, such as Au and Gd, can have both functions, thus they can have an anti-cancer activity and can be used as tracer for imaging. Gd is a paramagnetic metal, normally used in contrast agents for MRI. Moreover, it can enhance the efficacy of radiation therapy. Thus, the incorporation of Gd into nanoparticle allows for the simultaneous use of imaging to guide the radiation beams and to locate the tumour, as well as to enhance the toxic effect of radiation. Kolt and collaborator [107] have successfully treated animals bearing B16-F10 tumours with Gd-based NPs, AGuIX. After radiation therapy, in the AGuIX treated animals they observed an increase of tumour cell death, and improvement of the life spans of animals bearing multiple brain melanoma metastases. Kang and collaborators [108] have incorporated thermosensitive phospholipids, loaded with the drug docetaxel, onto the surface of gold NPs (AuNPs) or gold NanoRod (AuNR). Both nanovehicles showed enhanced tumour-cell suppression properties towards the melanoma B16-F10 cell line. A potential theranostic use is suggested by the authors. Indeed, two synergistic anticancer actions can be achieved after infrared photo thermal treatment and, thanks to the Au particles, they can have bioimaging applications.

An enhanced theranostic activity was achieved by conjugating functionalized gold NPs with Single-Wall Carbon NanoTubes (SWCNT) [109]. The authors demonstrated a selective imaging and efficient photo thermal therapy on UACC903 human melanoma cancer cells.

Wang and collaborators [110] have developed very complex multifunctional hybrid NPs, composed of gold nanocrystals coated on a magnetite-fluorescent porous carbon core-shell. The biomedical application of this theranostic nanocarrier includes the possibility of bioimaging in multicolor mode, the magnetic/NIR-responsive drug release, and the enhanced photo thermal therapy. Mouse melanoma B16-F10 cells have been used for this study, thus suggesting their potential use in melanoma treatment.

Conclusion

There is no doubt that nanotechnology may offer new therapeutic opportunities for the treatment of metastatic melanoma. Given the clinical success of immunomodulatory drugs, it is likely we will observe more and more pre-clinical studies on strengthening their effectiveness, thanks to nanotechnology platforms. Moreover, considering the currently on-going clinical trials, the combined therapy also seems promising in therapeutic advantages. However, it's difficult to identify the most promising nanomedicine to treat melanoma, relying on pre-clinical studies, since these studies present several concerns and limitations. The physico-chemical properties of delivery systems can modify pharmacokinetics, tumor accumulation, and biodistribution. Xenografts represent a useful

model to study these parameters for nanoformulations, however, tumor characteristics can vary with cell line and size, as well as the density and vascularization. Therefore, tumor uptake by the EPR effect is expected to be strongly dependent on the cell line used. On the other hand, the studies running on the murine melanoma model do not completely reflect the complexity of the human melanoma cell population. From this point of view, besides the implementation of nanotechnological therapies, it could be important to develop immunocompetent human models for melanoma research, such as three-dimensional human skin reconstruct models containing human melanoma cells, with the addition of the immune system cells.

Another concern regards the toxicity studies of NPs with clinical potential. Current research lacks a unifying protocol for the toxicological profiling of NPs, and studies on the long-term effects on human health are also needed [111]. As previously reported, in these years the majority of studies on melanoma therapy was regarding new platforms for immunotherapy. In melanoma treatments targeting the immune response of patients, it is necessary to pay particular attention to a possible interaction between nanoparticles and the immune system. Although it has been said that nanoparticles are unlikely to act as a hapten, inducing a specific IgE production, they can induce allergic sensitization (contact dermatitis) and they are likely to act as an adjuvant in inducing a specific pattern of cytokines, antibodies and cells that favor allergic sensitization to environmental allergens [112]. Moreover, the stimulation of inflammatory cytokines has been demonstrated to be a key point in nanoparticle-induced immunostimulatory reactions [113]. Since an adverse effect of immunostimulatory drugs is the risk of developing autoimmune disease, these aspects are very important considerations for the choice of a drug delivery platform. Therefore, further studies are needed in nanotoxicology, to provide safer nanoformulation for the melanoma treatment.

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