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Jacques Rouanet[1,2,3], Mercedes Quintana[1], Philippe Auzeloux[1], Florent Cachin[1, 3], Françoise Degoul[1]

[1] Université Clermont Auvergne, INSERM, Imagerie Moléculaire et Stratégies Théranostiques, UMR1240, 58 Rue Montalembert, 63005, Clermont-Ferrand Cedex, France.

[2] Department of Dermatology and Oncodermatology, CHU Estaing, 1 place Lucie et Raymond Aubrac, 63000 Clermont-Ferrand, France

[3] Centre Jean Perrin, Clermont-Ferrand, F-63011, France.

Corresponding author: Dr Françoise Degoul, PhD

IMoST, UMR 1240, 58 Rue Montalembert, 63005, Clermont-Ferrand Cedex, France

Present address: GRED UMR CNRS 6293-INSERM 1103, 28 Place Henri Dunant, 63000 Clermont-Ferrand

francoise.degoul@inserm.fr

Keywords

Metastatic melanoma; Melanin; Radiopharmaceuticals; Benzamides; Targeted radionuclide therapy; Imaging

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Abbreviations

Epithelial Mesenchymal Transition (EMT), External Beam Radiation Therapy (EBRT), Fluoro-deoxy Glucose (FDG), Immune Checkpoint Inhibitors (ICI), National Clinical Trial (NCT), Positron Emission Tomography (PET), Radio Chemical Yield (RCY), Reactive Oxygen Species (ROS), Single-Photon Emission Computed Tomography (SPECT), Targeted Radionuclide Therapy (TRT), Ultra-Violet (UV), Vascular Endothelial Growth Factor (VEGF).

Abstract

Cutaneous melanoma arises from proliferating melanocytes, cells specialized in the production of melanin. This property means melanin can be considered as a target for monitoring melanoma patients using nuclear imaging or targeted radionuclide therapy (TRT). Since the 1970s, many researchers have shown that specific molecules can interfere with melanin. This paper reviews some such molecules: benzamide structures improved to increase their pharmacokinetics for imaging or TRT. We first describe the characteristics and biosynthesis of melanin, and the main features of melanin tracers. The second part summarizes the preclinical and corresponding clinical studies on imaging. The last section presents TRT results from ongoing protocols and discusses combinations with other therapies as an opportunity for melanoma non-responders or patients resistant to treatments.

1. Introduction

Cutaneous melanoma is the malignant skin tumor caused by melanocytes (Liu et al., 2014; Miller & Mihm, 2006; Paluncic et al., 2016; Shain et al., 2015). These cells, located in the basal layer of the epidermis and the hair follicle, produce melanin, the pigment that protects the skin from DNA damage due to ultraviolet (UV) radiation and is responsible for tanning. Another hypothesis for the origin of primary tumor formation suggests that melanoma could arise from melanocyte stem cells residing in the bulge of the hair follicle, rather than differentiated melanocytes in the basal layer (Diener & Sommer, 2020; Moon et al., 2017; Sun et al., 2019). Cutaneous melanomas account for 10% of skin cancers with an incidence of 3.1 per 100,000 worldwide in 2018 and causing 47,200 deaths (Ferlay et al., 2019). They can appear on healthy skin (70-80% of cases) or as a result of the malignant transformation of a naevus (Liu et al., 2014; Miller & Mihm, 2006; Paluncic et al., 2016; Shain et al., 2015). Cutaneous melanoma is a term that covers different histological subtypes: superficial spreading melanoma, nodular melanoma, acral lentiginous melanoma and lentigo melanoma. Around eight percent of cutaneous melanomas are amelanotic (Thomas et al., 2014). Acral melanomas, usually located in the plantar and palmar areas and in sublingual zones, represent 2-3% of all melanomas (Saida et al., 2011). Their formation and progression are independent of UV exposure and have been associated with c-kit mutations (Rawson et al., 2017). Acral melanoma is often dramatic because diagnosed after the occurrence of metastases that did not respond well to current targeted therapies.

Other types of malignant melanocytic proliferation and pigmented tumors also exist in the mucosa (lips, oral and nasal cavities, vulva, vagina, uterus, etc.) and eyes, leading to mucosal and ocular (or uveal) melanomas, respectively. These cancers, characterized by specific oncogenic mechanisms, are less frequent than cutaneous melanoma (Goldemberg et al., 2019; M.-Y. Wu et al., 2020) and respond differently to treatments (Rossi et al., 2020). The prognosis is good for cutaneous melanoma detected early enough, i.e. with a thickness of less than 1 mm (Breslow index) and no identified metastases, allowing surgical removal. Delayed diagnosis reduces the survival rate considerably because of the high metastatic potential of this type of cancer.

A wide range of treatments is now available, metastasis surgery, radiation therapy and chemotherapy being the oldest. Indeed, until the early 2010s, the only systemic treatments available for metastatic melanoma were chemotherapy (Hodi et al., 2010) (dacarbazine) or immunotherapy (IL-2), with poor efficacy (5-10% survival at 5 years). Since then, the management of metastatic melanoma has seen significant therapeutic progress with therapies targeting the MAPK pathway, including BRAF V600E (Flaherty et al., 2010) and MEK-activated proteins (Long et al., 2015), as well as immunotherapy (Larkin et al., 2015) to eliminate the inhibition and escape mechanisms of the antitumor immune response. These antibodies are directed against immune checkpoint inhibitors, such as CTLA-4 (Hodi et al., 2010), PD-1 and its ligand PD-L1 (Robert et al., 2015). For more details, see reviews (Mason et al., 2019). Median overall survival has thus increased to 2 years (Luke et al., 2017), although more time is required to be able to confirm with any accuracy the encouraging increase in the number of long-term survivors (> 2 years). Furthermore, various protocols combining immunotherapy (Postow et al., 2015), targeted therapies (Long et al., 2015) and stereotactic radiotherapy (Williams et al., 2017) are in routine use or in the first or second phases of clinical trials. Although these new treatments have significantly improved the prognosis of metastatic melanoma, some melanomas do not respond or become resistant to treatment after just a few months.

Nuclear medicine can benefit cancer therapeutics through theranostics, i.e. the combined use of radiolabeled drugs for target identification (via imaging) and treatment delivery using dedicated radionuclides (Langbein et al., 2019). Another advantage of nuclear therapy relies on off-target effects, i.e.

bystander and abscopal effects (Pouget et al., 2015, 2018). Both nuclear imaging and targeted radionuclide therapy (TRT) involve the delivery of radiopharmaceuticals to melanomas using a specific vector coupled with a dedicated radionuclide. In addition to the small melanin radiotracers reviewed herein, there are a number of other vectors available for melanoma nuclear imaging and TRT, briefly discussed below. A recent review presents research on additional imaging tracers, such as the peptide ligands of integrin, (Wei et al., 2018). Peptide analogs of the α-melanocyte-stimulating hormone (α-MSH) targeting melanocortin receptor-1 (MC1-R) (Miao, Benwell, et al., 2007; Miao & Quinn, 2007) allowed imaging in preclinical models of murine melanomas and provided promising preclinical results in systemic TRT (Miao, Hylarides, et al., 2005; Miao, Owen, et al., 2005; Miao, Shelton, et al., 2007). However, their high renal concentration will result in renal toxicity, thus impairing their clinical use. Integrin targeting has also been proposed in a phase 1 clinical trial for melanoma imaging (Phillips et al., 2014) with RGD-functionalized nanoparticles, radiolabeled with Iodine-124. These nanoparticles were functionalized with α-MSH peptides in a Lutetium-177 TRT preclinical study with promising results for a future translational clinical study (Zhang et al., 2020).

The melanin pigment itself may also be a specific target for antibodies or peptides, as demonstrated in melanomas (Norain & Dadachova, 2016). Dadachova et al. successfully tested some TRT strategies in preclinical models with different radiolabeled melanin-binding antibodies (**[^{188}Re]6D2, [^{166}Ho]6D2**, **[^{90}Y]6D2**, **[^{188}Re]8C3, [^{213}Bi]8C3, [^{177}Lu]8C3**) (Dadachova et al., 2008; Klein et al., 2013; Revskaya et al., 2009; Thompson et al., 2014) and a melanin-binding decapeptide (**[^{188}Re]4B4**,**[^{177}Lu]4B4**, **[^{166}Ho]4B4**, **[^{153}Sm]4B4**) (Ballard et al., 2011; Dadachova et al., 2006). **[^{188}Re]6D2** mAb was also tested in one phase I clinical trial (Klein et al., 2013) and has shown good tolerance, localization in melanoma metastases and antitumor activity. Despite these promising results, this work did not lead to phase II trials.

Back in 1972, the first molecules reported to bind melanin were chlorpromazine (an anti-psychotic derivative of phenothiazine) and chloroquine (anti-malaria drug) (Lindquist & Ullberg, 1972). Our group came into the melanin ligand research field with a study on adiphenin, an inhibitor of nicotinic receptors showing high retention in pigmented tissues (eyes and skin) (Meyniel et al., 1990). Other compounds are also known to bind with melanin, such as methylene blue (**MB**) or acridine orange drugs (Mårs & Larsson, 1999). Comparison of these molecule structures reveals a common pattern of aromatic rings and a “tertiary

amino group" (Figure 1). The next step was to synthesize and select the best structures exhibiting these characteristics and a radiolabeling site, usually an iodine atom. This review starts by describing the key features of melanin and then recapitulates the benzamides and derivatives that have been tested in clinical trials, focusing on their radiosynthesis. The last part will be dedicated to therapeutic approaches in preclinical experiments and clinical trials with a specific focus on some promising combination strategies.

2. Melanin pigment

2.1. *Localization and melanogenesis*

Melanin is synthesized inside specialized melanocyte organelles called melanosomes under genetic and biochemical regulation (for reviews on pigmentation genes, see (Pavan & Sturm, 2019; Rocha, 2020; Sturm, 2009)). After maturation, the melanosomes are transferred to other cells, mainly to surrounding keratinocytes (Figure 2A). In the epidermis, melanocytes and keratinocytes form a melanin unit, by associating one melanocyte and around 36 keratinocytes (Haass et al., 2005). This pigment transfer is responsible for skin pigmentation and plays a central role in protecting the cells against ultraviolet (UV) radiation damage. Melanin is also responsible for the pigmentation of the hair, eyes, and mucosa. Melanin itself is a complex biopolymer made up of two chemically different melanins, called eumelanin (EM) and pheomelanin (PM). Eumelanin is a brown-black pigment, while PM has a yellow to reddish-brown color, ascribed to the cysteine. EM is constituted of 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) monomers, while benzothiazine and benzothiazole are the PM monomers produced by the melanogenesis pathway (Figure 2B). Considering the diversity of the DHI/DHICA ratio in EM, the degree of polymerization, and the availability of cysteine that governs PM synthesis, natural melanin constitutes a complex, heterogeneous, high molecular weight structure.

2.2. *Functions: photo-protection vs photo-toxicity*

The close association between UV exposure and its genotoxic effects that lead to the development of skin cancer, notably melanoma, is well established (Erdmann et al., 2013). Melanin photo-protection against UVB (290-320 nm) and UVA (320-400 nm) radiation has been demonstrated *in vitro* (human melanocytes) (Barker et al., 1995; Smit et al., 2001) and *in situ* (skin biopsies of healthy donors) (Tadokoro

et al., 2003; Yamaguchi et al., 2006; Mouret et al., 2006). The most studied pigment, EM, shows an exceptionally broad spectrum of UV-Vis absorption, with a decreasing exponential profile (Figure 2C) as well as an absorbed radiation dissipation capacity that supports its natural photo-protection action (for a review, see Meredith & Sarna, 2006). Melanin radioprotection is also mediated by radical scavenging and oxygen depletion throughout melanogenesis. Notwithstanding its photo-protective actions, melanin exhibits potential toxicity. In the presence of UVA irradiation, both pigments undergo oxidative degradation (Wakamatsu et al., 2012). However, the antagonistic effect of melanin on radiation protection is mainly attributed to PM. Among the main causes of cell oxidative stress mediated by PM are the antioxidant depletion (i.e. glutathione (GSH) and NADH) and the production of reactive oxygen species (ROS) and the formation of cyclobutane pyrimidine dimers (CPD) leading to DNA damage (Chedekel et al., 1978; H. Z. Hill et al., 1997; Helene Z. Hill & Hill, 2000; Panzella et al., 2014; Premi et al., 2015). *In vivo* demonstration of the spontaneous development of melanomas by $BRAF^{V600E}$ mutant mice that produce PM when not exposed to UV provides a link with the pro-oxidant potential of PM (Mitra et al., 2012).

2.3. *Melanin and melanoma*

Melanoma incidence is lower in dark-skinned than in light-skinned individuals (Bertrand et al., 2020; Erdmann et al., 2013; MacKie et al., 2009). Melanin is present in most primary melanoma tumors since only 1.8 to 8.1% are amelanotic (Koch & Lange, 2000). Amelanotic melanomas usually present a thicker Breslow index, a higher mitotic rate, and are associated with a lower survival rate than pigmented melanomas (Wee et al., 2018). It has been reported that melanoma cells lose their melanogenesis ability during metastatic dissemination, and it is assumed that about 50% of melanoma-derived metastases are pigmented (Cachin et al., 2014). The loss of pigmentation is usually considered as a loss of differentiation and a marker of disease progression (Miller & Mihm, 2006).

However, the presence of melanin in tumors can be deleterious to patients undergoing chemotherapy (Chen et al., 2006, 2009) or radiotherapy (Brożyna et al., 2013, 2016), related to intrinsic melanin trapping and radiation-protection abilities. Conversely, a beneficial role of melanin on melanomas has been also demonstrated *in vitro* (Sarna et al., 2013, 2014, 2018) and *in vivo* (Sarna et al., 2019) due to reduced cell elasticity. Interestingly, near-infrared pump-probe imaging showed structural changes to the

pigments in melanoma biopsies and pointed to DHICA EM fragmentation as a marker of melanoma metabolic activity and metastatic potential (Matthews et al., 2011; Ju et al., 2019). The relationship between structure, melanin function and changes depending on the melanoma stage deserves further investigation to understand its significance in biological terms.

As described above, melanin is an obstacle to external radiotherapy and chemotherapy for melanoma because of its photo-protection and scavenging properties. However, its presence in melanomas and the discovery of melanin drug affinities have promoted studies on radionuclide-labeled compounds to enable specific tumor targeting.

3. Radiotracers binding to melanin

3.1. *Discovery of strong affinities between certain drugs and melanin*

Ocular toxicology cases associated with a drug affinity for melanin were first reported in the middle of the 20th century, with a phenothiazine compound (Verrey, 1956) and chloroquine (Hobbs et al., 1959). In 1972, a biodistribution study on mice and monkeys confirmed the accumulation of radiolabeled chloroquine and chlorpromazine in pigmented tissues (Lindquist & Ullberg, 1972). These observations led to studies of the drug-melanin interaction mechanisms, mainly for the toxicological studies required for drug development (for a review, see Ings, 1984). The compounds with the highest affinity for melanin have two chemical structural similarities: firstly, an aromatic or heteroaromatic ring and, secondly, a protonated amine function at physiological pH (Figure 1). Early on, an analogue of chloroquine radiolabeled with iodine-125 was investigated for visualizing malignant melanomas and metastases in humans (Beierwaltes et al., 1968). Unfortunately, the physical properties of iodine-125 (half-life 59 days, main gamma emission energy 27 keV) are not suitable for low patient dosimetry and do not enable resolution sufficient for nuclear medicine imaging applications. In the late 1980s, our team worked on aromatic iodinated amino compounds, including adiphenine, to explore the central nervous system. Unexpectedly, an accumulation of radioactivity was observed in richly pigmented tissues, leading to the first radiotracer *N*-(2-diethylaminoethyl)-4-iodobenzamide (**BZA**), specifically designed to diagnose malignant melanoma (Michelot et al., 1991).

3.2. ***BZA*** *and benzamide derivatives with a high affinity for melanin*

BZA, like many benzamide structures, is known for its affinity for σ-receptors (Schmidt & Kruse, 2019). σ-Receptors are cellular receptors found in various organs and expressed by melanoma cells (Georgiadis et al., 2017; John et al., 1993). They were first identified as targets of benzamides. However, it is now assumed that **BZA** directly binds with melanin for three main reasons: 1) **BZA** fixation is independent of its molar activity, suggesting a non-saturable mechanism different from the receptor-ligand interaction (Dittmann et al., 1999). 2) *In vitro* **BZA** uptake in melanoma is not correlated with the presence of σ-receptors (M. Eisenhut et al., 2000; Oltmanns et al., 2009), but correlation was found between melanin and benzamide uptake (Labarre et al., 2002). 3) Secondary ion mass spectrometry showed that **BZA** co-localized with intracytoplasmic melanin (Guerquin-Kern et al., 2004; Chéhadé et al., 2005). However, the reversible interaction between **BZA** and melanin excludes a covalent bond (Labarre et al., 2002). The presence of the protonated cation on tertiary amine favors ionic interaction with carboxylates on one side, and a π-interaction between the aromatic ring and the heteroaromatic ring (indole) on the other (Figure 3).

This specific non-saturable interaction leads to significant pharmacomodulation for nuclear medicine. In the 1990s, various teams performed pharmacomodulation, mainly to investigate the position of iodine (Brandau et al., 1996; Nicholl et al., 1997), the length of the carbon chain between the two nitrogen atoms, and the terminal amine function (Dittmann et al., 1999; John et al., 1993; Labarre et al., 1999; N. Moins et al., 2001; Moreau et al., 1995; Nicholl et al., 1997). Almost all these molecules maintained strong affinity for melanoma in preclinical studies. More recently, the aromatic ring has been modified by adding phenyl substituents (M. Eisenhut et al., 2000; Nicholl et al., 1997), and using new heteroaromatic analogs (Chezal et al., 2008; Greguric et al., 2009).

Many of these structures have shown good potential as melanoma radiotracers in preclinical studies and have been reviewed elsewhere (Oltmanns et al., 2009; Wei et al., 2018).

3.3. Radiotracers binding with melanin polymers for clinical applications

3.3.1.*Radioisotope selection*

In parallel to chemical **BZA** pharmacomodulation, a number of attempts has been made to find a radionuclide replacement for iodine-123/131. For SPECT diagnosis, the more available technetium-99m has been extensively investigated with moderate success (Auzeloux et al., 1999; Auzeloux et al., 2000; Auzeloux et al., 1999; Auzeloux et al., 2001; Friebe et al., 2000, 2001; Michael Eisenhut et al., 2002; N'Dongo et al., 2010; Moura et al., 2012). The metallic nature of technetium requires the presence of a chelator group, resulting in partial loss of the melanin affinity. For PET imaging, fluorine-18 (S.-Y. Wu et al., 2014; Garg et al., 2017; Greguric et al., 2009, 2009; Pyo et al., 2020; Ren et al., 2009), carbon-11 (Garg et al., 2017; Igawa et al., 2017) and gallium-68 complex (Kim et al., 2012b, 2012a) have provided interesting results in preclinical studies. For radionuclide therapy, the alpha emitter, astatine-211, was considered in early studies, with a radio-analog of methylene blue in preclinical models (Link et al., 1989, 1996; Link, 1999).

Our team investigated both iodinated and fluorinated derivatives of quinoxaline. The same molecule can be radiolabeled with fluorine-18 for PET imaging or with iodine-131 for therapeutic applications (Billaud et al., 2013, 2015; Maisonial et al., 2013). This multimodal approach has shown promising preclinical results in theranostic approaches for pigmented metastatic melanoma: the best compound, [^{18}F]-*N*-(12-ethyl-1-fluoro-3,6,9-trioxa-12-azatetradecan14-yl)-6-iodoquinoxaline-2-carboxamide (**[^{18}F]ICF15002**), demonstrated high and early tumor uptake (14.33%ID/g, 1 hour after injection). After **[^{131}I]ICF15002** injection, tumor doubling time increased from 1.8 ± 0.3 to 2.9 ± 0.5 days (controls *vs* treated), associated with higher median survival in the B16-BL6 murine model (Rbah-Vidal et al., 2016). Until now, only radio-halogenated benzamide derivatives have been injected into humans, radiolabeled with iodine-123/131 or fluorine-18. The radionuclide properties are summarized in Table 1.

The iodine radioisotope pair offers a strategic advantage in terms of development as the same molecule can be used for both SPECT with iodine-123 or therapy with iodine-131 (Mier et al., 2014). However, for diagnosis applications, more studies are being conducted on fluorine-18 because of its availability (^{18}F is the radioisotope present in **FDG**, the 'gold standard' in nuclear medicine for tumor imaging) and because of benefits of PET *vs* SPECT (PET presents higher resolution and a better signal-to-noise ratio).

3.3.2. *Radiopharmaceutical production*

There are many chemical methods to incorporate a radio-halogen onto a small molecule (for radioiodination see Dubost et al., 2020 and for radiofluorination see Preshlock et al., 2016). These protocols involve two strong constraints: radiochemistry takes place at the trace scale (concentrations µM to pM) and the final product must comply with injectable drug regulations. Thus, for a radiopharmaceutical application, four main points must be respected: 1) the labeling time must be as short as possible; this parameter, while important for radioiodination, becomes crucial for the fluor-18 radiolabeling process; 2) radiochemical yields (RCY) must be as high as possible to reduce the activity, cost and radiation exposure involved; 3) the purification method must prefer single-use cartridges instead of a column; 4) the process must limit the formation of radioactive by-products.

For all these reasons, for the 10 molecules studied in clinical trials for malignant melanoma (Table 2), only four different radiolabeling methods were used. The oldest, and simplest one is the isotopic exchange used for **[^{123}I]BZA** and *N*-(2-diethylaminoethyl)-2-iodobenzamide (**[^{123}I]BZA2**). The chemical precursor corresponds to the last radiolabeled compound studied. However, final molecular activity levels are low (always < GBq/µmol). Nevertheless, this disadvantage is not a problem because the **BZA** derivative-melanin binding process is not saturable. Direct electrophilic aromatic substitution has been used with molecules involving an activating group. This method was successfully employed for the radioiodination of **MB**, *N*-((1-ethylpyrrolidin-2-yl)methyl)-2-hydroxy-3-iodo-6-methoxybenzamide (**IBZM**), *N*-(2-diethylaminoethyl)-3-iodo-4-methoxybenzamide (**IMBA**) and *N*-(2-diethylaminoethyl)-4-(4-fluorobenzamido)-5-iodo-2-methoxybenzamide (**MIP-1145**). Using a chloro- or bromo-chemical precursor, it is also possible to carry out a halogen exchange involving nucleophilic aromatic substitution. **BZA** and **BZA2** were thus radiolabeled with iodine-123 from the bromo-precursor, *N*-(2-diethylaminoethyl)-5-fluoropicolinamide (**P3BZA**) was radiolabeled with fluorine-18 from the bromo-precursor and *N*-(2-diethylaminoethyl)-6-fluoronicotinamide (**Mel050**) with fluorine-18 from the chloro-precursor. Note that the last two methods, direct substitution and halogen exchange, require a purification step to separate the chemical precursor from the final radiopharmaceutical. The most recently published method uses Iodo-destannylation of the butyltin precursor for both *N*-(4-((2-diethylamino-ethylcarbamoyl)-2-iodo-5-methoxyphenyl)benzo[1,3]dioxole-5-carboxamide (**[$^{123/131}$I]BA52**) and *N*-(2-diethylaminoethyl)-6-iodoquinoxaline-2-carboxamide

([^{131}I]**ICF01012**). This is a nucleophilic substitution reaction, requiring the presence of a strong oxidant. This regiospecific method is most often used for radioiodation because of its very soft conditions (18-25°C) and fast kinetics (only a few minutes). The limit of this method for clinical application is that it eliminates stannane compounds in the final formulation; a validated method for final purification (HPLC or cartridge) is therefore required.

4. Preclinical and clinical results

4.1. *Biodistribution, imaging, dosimetry and potential adverse effects*

The main concern with radiopharmaceuticals is dosimetry. Radiotoxicity must be minimized in imaging, non-targeted organs must be spared in therapeutic protocols, but enough activity must be delivered to ensure anti-tumor efficiency. The dose in internal radiotherapy is closely related to the amount of radiolabeled ligand in tissues, causing prolonged exposure to different radiations for a given radionuclide, inducing a low absorbed dose rate. This is quite different from EBRT, which involves a homogeneous high dose rate and short exposure (Pouget et al., 2015). Studies on melanin ligands, as for other radiopharmaceuticals, usually refer to the ratio of tumor to blood or muscle for pharmacokinetics and the percentage of injected activity per gram of tissues (Table 3). This percentage varied from 9 to 41% in preclinical model tumors for the most recent studies on structures transferred to clinical trials (Table 3). Dosimetry was calculated with the MIRD formalism, taking into account activity in the source and the S-factor derived from computed values (Bolch et al., 2009). S-factors are defined as the mean absorbed dose in the target organ per decay in the source organ (Stabin, 2003). These S-factors were calculated by MONTE-CARLO simulation to increase accuracy and to provide a closed fit with reality (Perrot et al., 2014); they are used for specific personal dosimetry in the ongoing MELRIV1 trial (NCT03784625).

Various melanoma cell lines were used in the preclinical models but most investigations were carried out on highly pigmented murine B16 cell lines grafted subcutaneously into C57Bl/6 mice (Poste et al., 1980). This syngeneic model demonstrates high fixation of radiolabeled benzamides in the eyes, particularly in the pigmented retinal epithelium, and choroid, both structures being close to the retina (Degoul et al., 2013). This specific targeting is not expected to have an impact in humans as dosimetry

extrapolations gave values below the maximum tolerated doses (Jouberton et al., 2018). Furthermore, the imaging and therapy protocols already performed on humans did not assess accumulation in the eyes, except in the case of uveal melanoma (Everaert et al., 1997; Sillaire-Houtmann et al., 2004). As mentioned previously, the melanin-binding of these molecules is not covalent and non-saturable, which allows high and low specific activities for these radiotracers. The kinetics of the radiolabeled ligands in pigmented tissues (mainly the eyes and melanomas) follow a curve that plateaus, indicating their affinity for melanin with the highest percentage of injected activity in pigmented tissues (Perrot et al., 2014). Radioiodinated benzamides can also affect skin and hair follicle melanocytes, and melanized neurons. Our results, and those from others obtained in preclinical models, did not evidence such toxicities. In the B16 preclinical model, cutaneous melanocytes only present in follicles were not modified qualitatively or quantitatively by **[^{131}I]ICF01012** (Degoul et al., 2013). Concerning neurons and the blood-brain barrier, we have already shown that the **[^{125}I]BZA2** radiotracer can evidence brain metastases (Cachin et al., 2014), proving that the radiotracer can pass through the blood-brain barrier in patients. However, dosimetry for the whole brain was not critical in preclinical models (monkeys and mice) (Joyal et al., 2010; Viallard et al., 2015) or also for extrapolated human doses (Jouberton et al., 2018). Finally, the most convincing argument for TRT safety is that the first TRT on humans did not cause adverse effects in patients receiving **[^{131}I]BA52** (Mier et al., 2014).

As far as SPECT imaging is concerned, the high specificity of the radiolabeled benzamides has been clearly demonstrated by various trials, with values above 86% (Table 3). However, when these tracers were tested for PET imaging with **[^{18}F]FDG**, sensitivity was lower (Cachin et al., 2014), resulting in the interruption of benzamide development for SPECT imaging. Nevertheless, the size of these molecules is compatible with fluorine radiolabeling for PET imaging, as exemplified with **[^{18}F]P3BZA** with 17% IA post injection, leading to good imaging quality in mice and subsequently in human PET scans (Ma et al., 2019). This tracer should be compared with **[^{18}F]FDG** to evaluate its benefits.

4.2. *Antitumoral effects of benzamide-based TRT*

TRT efficacy has been assessed in preclinical studies in both syngeneic (B16-F0, B16-F10 and B16-BL6) and xenograft (M4Beu, SK-MEL-3) models. We used different clones of B16 melanoma, selected for their

high metastazing capacities, for serial IV injection of cell lines from lung metastases, i.e. B16-F10 to B16-F0. B16-BL6 differs from its parent B16-F10 in that it is able to cross the bladder membrane (Poste et al., 1980). We observed that B16-BL6 exhibited higher melanin than B16-F0 and B16-F10 (Rondepierre et al., 2009). For instance, we showed that two injections of 18.5 MBq of **[^{131}I]ICF01012** significantly inhibited the growth of B16-F0 and B16-BL6 tumors (Bonnet et al., 2010; Bonnet-Duquennoy et al., 2009; Viallard et al., 2015). This therapeutic effect was also found with **[^{131}I]5-IPN367** on B16-F10 tumors with an identical therapeutic protocol (Xu et al., 2018). Similarly, in xenograft models, the injection of **[^{131}I]ICF01012** or **[^{131}I]MIP-1145** led to a significant slow-down of tumor growth in human pigmented tumors and increased survival (Bonnet-Duquennoy et al., 2009; Bonnet et al., 2010; Joyal et al., 2010; Degoul et al., 2013). TRT efficacy on tumor growth and survival was dependent upon the number and scheduling of radiopharmaceutical injections in both B16 and SK-MEL-3 models (Bonnet et al., 2010; Degoul et al., 2013; Joyal et al., 2010; Xu et al., 2018) and was obviously related to the amount of melanin (Viallard et al., 2015). **[^{131}I]BA52**, a molecule derived from **[^{131}I]MIP-1145**, was tested in metastatic melanoma patients. This unique TRT clinical trial showed that of the five patients, two were still alive 2 years after treatment (Mier et al., 2014). These patients received the higher activity, emphasizing the importance of dosimetry. Furthermore, a phase I clinical trial is currently assessing the safety and efficacy of the **[^{131}I]ICF01012** molecule (NCT03784625) (Table 3).

4.3. *Biological effects of benzamide-based TRT*

Compared with EBRT, the cellular and molecular effects of TRT are not so well documented and researchers are still working to decipher the TRT mechanisms, particularly with respect to dosimetry (Pouget et al., 2015). However, by analogy with EBRT, different non-mutually exclusive biological mechanisms are likely to be induced after TRT: 1) the DNA double-strand breaks and the cell cycle are altered, related to p53 mutational status (Baskar et al., 2014; Pawlik & Keyomarsi, 2004); 2) metastatic mechanisms involving VEGF expression are altered, and epithelial-mesenchymal transition (EMT) is altered and inhibited (Fujita et al., 2015; Moncharmont et al., 2014); 3) the antitumor immune response is stimulated (Rodríguez-Ruiz et al., 2018). The biological effects of TRT described below are summarized in Figure 4.

4.3.1.*Effects on tumor cells*

In B16-F0 and B16-BL6 tumors, TRT induces striking cellular abnormalities with anisocytosis and giant cells (Bonnet et al., 2010; Degoul et al., 2013). Both **[^{131}I]ICF01012** and **[^{131}I]5-IPN** induce a higher proportion of G2-phase blocked cells, suggesting that the main mechanism of TRT toxicity is the mitotic catastrophe (Degoul et al., 2013; Xu et al., 2018). This hypothesis is supported by morphological nuclear abnormalities, cytoskeleton alterations (tubulin-13 reduced) and consumption of metabolites (amino acids and glucose). In tumors following **[^{131}I]ICF01012** injection, DNA damage due to irradiation is indicated by the significant increase in SubG1 phase cells and Ser-15 phosphorylation induction of the p53 protein (Akil et al., 2019; Degoul et al., 2013). However, apoptosis is not the main mechanism of **[^{131}I]ICF01012** efficacy in this model (Degoul et al., 2013). With **[^{131}I]ICF01012** and **[^{131}I]5-IPN**, tumor cell proliferation was reduced (Bonnet-Duquennoy et al., 2009; Degoul et al., 2013; Xu et al., 2018). Reduced clonogenicity (Akil et al., 2019) and a decrease in the phosphorylated forms of ERK and AKT (proteins involved in cell survival) were observed after **[^{131}I]ICF01012** injection (Degoul et al., 2013).

4.3.2.*Effect on metastases: angiogenesis and epithelial-mesenchymal transition (EMT)*

Although the invasion modifications induced by EBRT have been described (Sundahl et al., 2018), few data are available on the impact of TRT on metastasis and particularly on EMT-like mechanisms. TRT with **[^{131}I]ICF01012** reduces the number of spontaneous lung metastases from subcutaneous primary B16-BL6 tumors (Bonnet-Duquennoy et al., 2009) and significantly decreases the number and size of B16-BL6 colonies in the lungs (Akil et al., 2019). This might be due to an anti-angiogenic effect, by VEGF down-regulation and up-regulation of an anti-angiogenic protein, IP10 (Degoul et al., 2013; Xu et al., 2018). A decrease in blood vessels has also been found in **[^{131}I]5-IPN** tumors (Xu et al., 2018). Alternatively, **[^{131}I]ICF01012** reduces the acquisition of the EMT-like phenotype by decreasing the expression of EMT-like-related genes (*Sparc, Brn2, Zeb2, Mitf*) and by reducing the protein expression of two EMT-like markers, N-cadherin and vimentine (Akil et al., 2019). Further investigations are required to improve understanding of the precise mechanisms underlying the effects of TRT on metastatic processes.

4.3.3.*Effect on antitumor immune response*

Antitumor immune response has been widely studied in melanoma, which is a tumor able to spontaneously regress due to a high intrinsic mutational burden (for a review, see Passarelli et al., 2017). In the same way, EBRT, which leads to DNA alterations and immunogenic cell death, has been shown to modify the antitumor immune response (for a review, see Rodríguez-Ruiz et al., 2018). A few studies report changes in the antitumor immune response after TRT (recruitment of specific antigen-presenting cells (Y. Wu et al., 2013), induction of a specific T cell immune response (Chakraborty et al., 2008; Keisari et al., 2014) and a memory T cell immune response (Hernandez et al., 2019). However, all these data were obtained in non-melanoma tumors and with different vectorization strategies.

In a B16-F10 murine model, **[^{131}I]ICF01012** efficacy has been found to depend on the immunocompetence of the mice (Rouanet et al., 2020)**.** Furthermore, **[^{131}I]ICF01012** modifies the antitumor immune response by inducing immunogenic cell death (exposure of annexin A1 and calreticulin at the cell membrane). This stimulates the recruitment of cytotoxic T cells, responsible for tumor immune destruction, regulatory T cells involved in immune tolerance, and innate immune cells (NK cells and macrophages) in the tumor environment (Rouanet et al., 2020).

4.4. *TRT combined with other treatments*

In spite of the promise of the antitumoral effects of TRT, the advent of targeted therapies and immunotherapy in the early 2010s cast doubt upon this strategy. Combining TRT with ICI and DNA repair inhibitors makes sense as TRT induces DSB and immune tolerance (see paragraph 4.3).

4.4.1. *Immune checkpoint inhibitors*

Combining TRT with ICI has been assessed using different approaches involving antibodies targeting melanin (Jiao et al., 2020; Nosanchuk et al., 2018), a peptide targeting the integrin VLA-4 (Choi et al., 2018) and melanin ligands (Rouanet et al., 2020). In this last study, **[^{131}I]ICF01012** combined with an anti-CTLA-4 antibody resulted in a significant increase in survival, confirming the importance of immune tolerance after **[^{131}I]ICF01012.** The association of **[^{131}I]ICF01012** with anti-PD-1 or anti-PD-L1 antibodies did not bring any significant benefit in terms of survival, showing that T cell exhaustion is not a major phenomenon after TRT. Further analyses, using the combination of **[^{131}I]ICF01012** with an anti-CTLA-4 antibody associated with an

anti-PD-1 or an anti-PD-L1 antibody, suggest that the **[^{131}I]ICF01012** + anti-CTLA-4 combination limits TRT-induced immune tolerance but induces T cell exhaustion. It is interesting to note that a similar observation was made on a B16 model following combined EBRT and ICI treatment (Twyman-Saint Victor et al., 2015). Finally, these results confirm that the combination of TRT and ICI is a promising approach for the treatment of metastatic melanoma and opens the prospect of clinical trials for this type of combination.

4.4.2.*coDbait*

coDbait is a small DNA molecule which mimics a DNA double-strand break and disorganizes the DNA repair mechanisms by trapping repair enzymes (Biau et al., 2014; Croset et al., 2013; Quanz et al., 2009). It has been shown that combining **[^{131}I]ICF01012** with coDbait results in a synergistic effect or additive effect on tumor growth and median survival, without increasing toxicity, in human SK-Mel 3 melanoma xenografts and in murine syngeneic B16-BL6 models (Viallard et al., 2016). However, there are no current plans for clinical trials on a DNA repair trap combined with TRT, even though Dbait associated with EBRT has proven its safety in melanoma patients (Le Tourneau et al., 2016).

5. Summary and perspectives

Benzamide-based radioligands targeting melanin were first developed for imaging. Although their specificity is well established, these structures are more suitable for therapeutic purposes because of the high capacity of **[^{18}F]FDG** to detect melanomas. In terms of TRT, we believe that, although many questions remain, the use of radiolabeled benzamides may constitute a therapeutic solution for melanomas resistant to therapy. TRT is an original strategy for the systemic targeting of not only pigmented lesions, but also unpigmented metastases through the abscopal effect. Furthermore, immunogenic modifications of treated TRT tumors can enhance the efficacy of ICI. Based on this observation, we are planning a future trial combining TRT with ICI inhibitors.

Conflict of Interest Statement:

The authors declare that they have no conflicts of interest.

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Figure 1

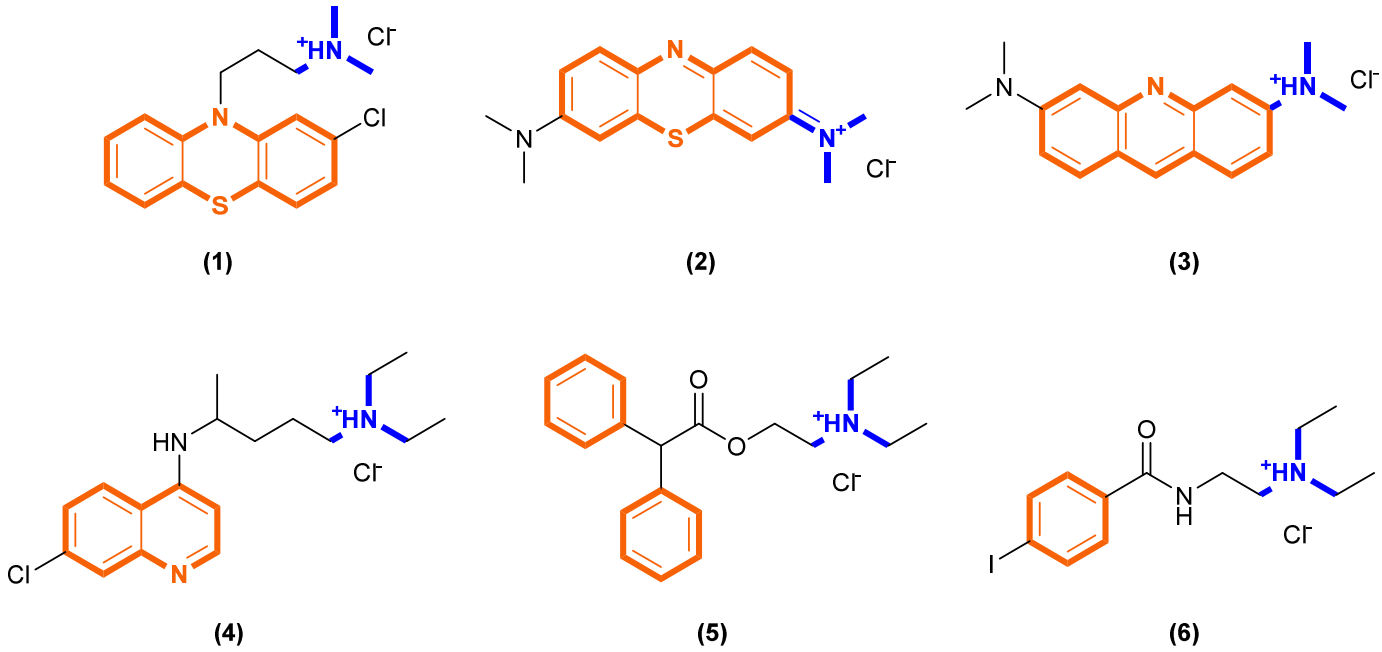


Figure 1. Examples of dyes or drugs with a high affinity for melanin. Chemical structures at physiological pH. (1) chlorpromazine; (2) methylene blue; (3) acridine orange; (4) chloroquine; (5) adiphenin; (6) *N*-(2-diethylaminoethyl)-4-iodobenzamide (**BZA**).

Figure 2

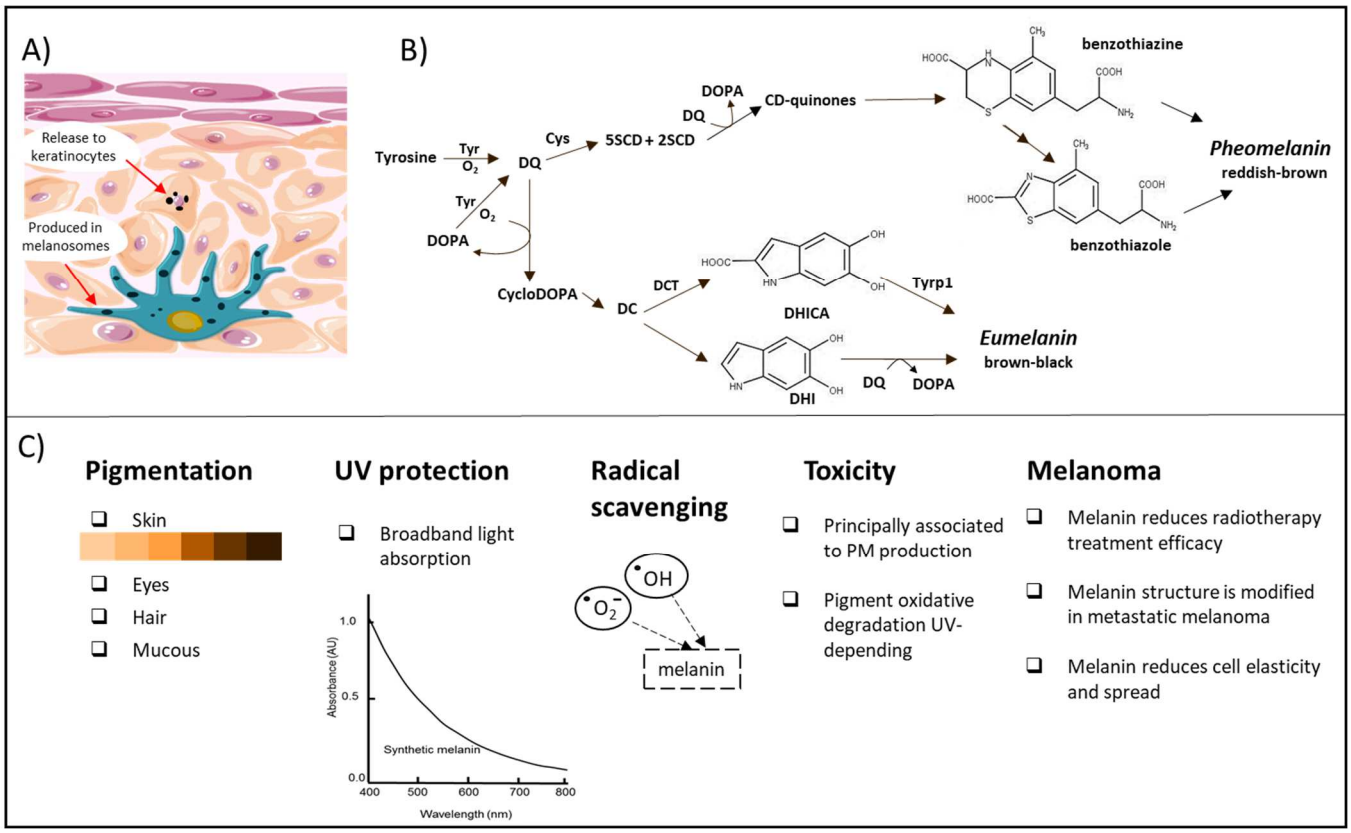


Figure 2. Melanin and melanogenesis. A) Localization of melanin synthesis within the melanosomes of melanocytes and transfer to keratinocytes. B) Melanogenesis pathway is mainly regulated by tyrosinase (Tyr) which produce dopaquinone (DQ) from tyrosine. If cysteine (Cys) is available, the DQ precursor first reacts with the Cys to generate cysteinyldopas 5-S and 2-S (5SCD, 2SCD), which are oxidized by DQ, producing benzothiazine, yielding PM after polymerization (Ito & Wakamatsu, 2008; Ozeki et al., 1997). After PM production, the benzothiazine is gradually converted to benzothiazole (Wakamatsu et al., 2009). The depletion of Cys therefore promotes the spontaneous cyclization of DQ, giving rise to DOPAchrome (DC) (del Marmol et al., 1996; Ozeki et al., 1997). This is rearranged to produce the constituent monomers of EM: 5,6-dihydroxyindole (DHI) and 5,6-dihydroxyindole-2-carboxylic acid (DHICA). The enzyme DOPAchrome tautomerase (DCT), also called tyrosinase-related protein 2 (Tyrp2), catalyses the production of DHICA (Aroca et al., 1992). Subsequent oxidation of both dihydroxyindoles forms the EM polymer. C) Overall view of the functions of melanin and its role on melanoma.

Figure 3

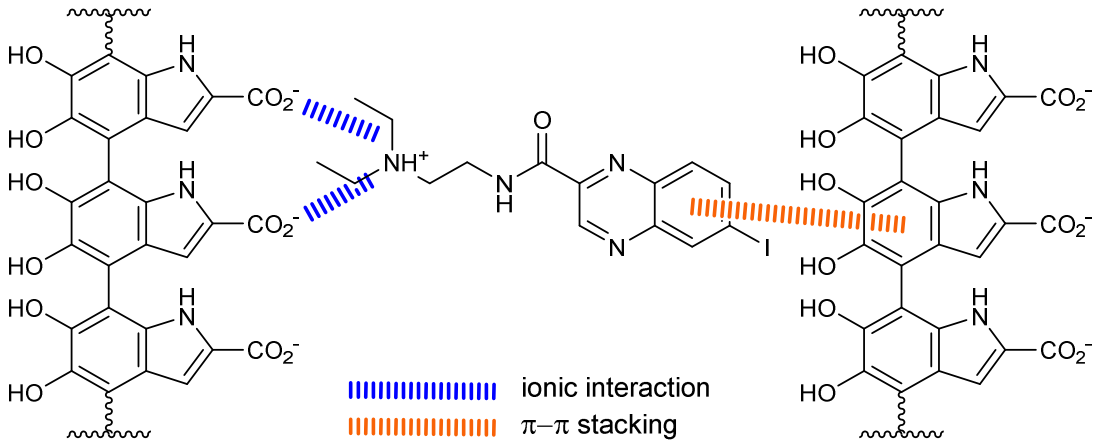


Figure 3. Example of **BZA** derivative-melanin binding: chemical interactions between **ICF01012** and melanin fragment.

Figure 4

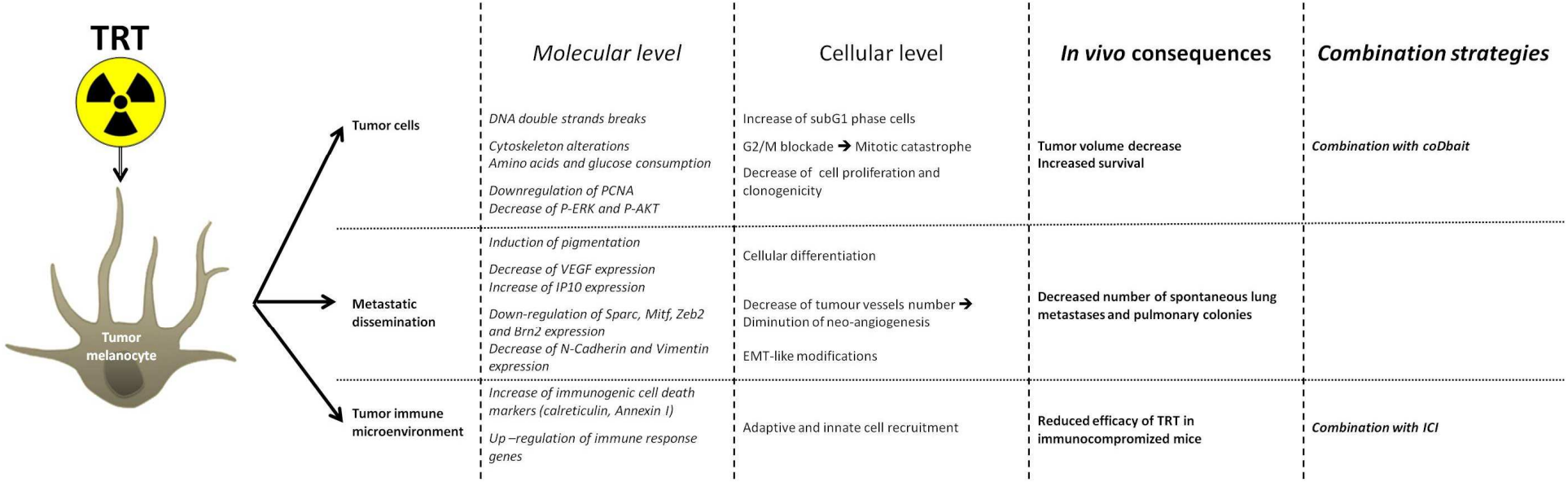


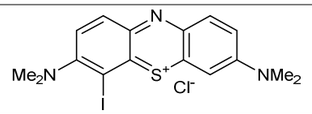
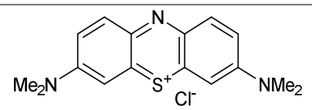
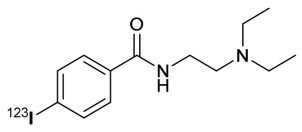
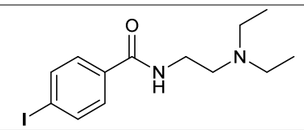
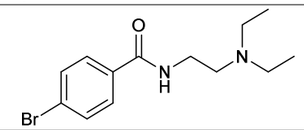
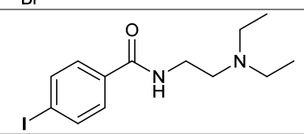
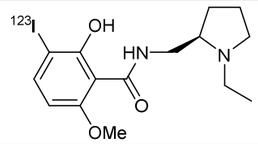
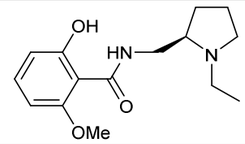
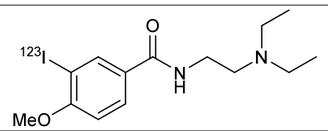
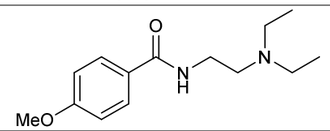
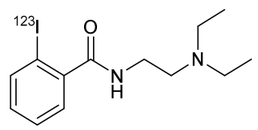
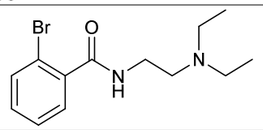
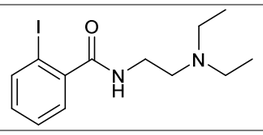
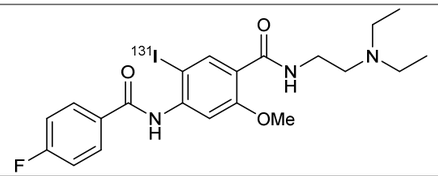
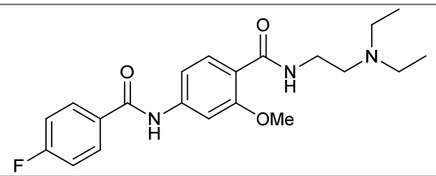
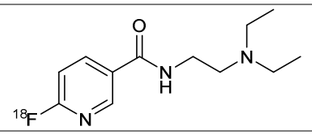
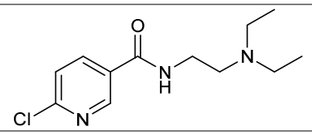
Figure 4. TRT biological effects from known preclinical molecular events to potential clinical applications.

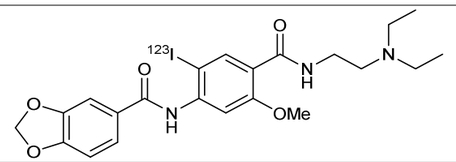
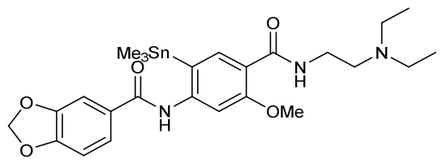
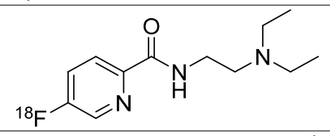
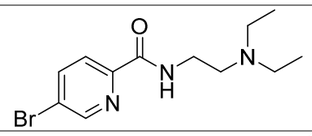
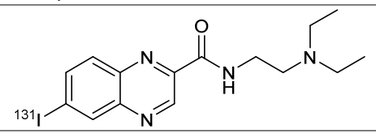
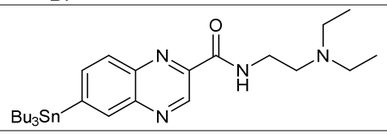
Tables

Table 1. Main properties of the three radionuclides used in clinical studies involving benzamide derivatives.

Radio-isotope	Half-life	Main decay (energy max)	Main γ emission (Intensity)	Production	Nuclear medicine application
Iodine-123	13.22 h	100% Electronic capture	159 keV (83%) 27.5 keV (46%) 27.2 keV (25%) 31.1 keV (13%)	Cyclotron	Diagnostic Imaging SPECT
Iodine-131	8.023 d	β^- (606 keV)	364 keV (81%) 637 keV (7%) 284 keV (6%)	Reactor	Therapy
Fluorine-18	109.8 min	β^+ (634 keV)	511 keV (194%)	Cyclotron	Diagnostic Imaging PET

Table 2. Main production characteristics of the radiopharmaceutical compounds involved in clinical trials.

Name	Chemical structure	Chemical precursor	Radiolabeling method	Conditions	Purification	Yield	Purity	Molar Activity	Reference(s)
MB Methylene blue			Direct Electrophilic Aromatic Substitution	KIO_3, HCl 100°C, 1 h	Anion exchange cartridge	60-70%	>99%	199 MBq/µmol	(Blower & Carter, 1990)
BZA *N*-(2-diethylaminoethyl)-4-iodobenzamide			Isotope Exchange	Citrate buffer pH=4 $CuSO_4$ 140°C, 40 min	none	>98%	>98%	9 MBq/µmol	(Michelot et al., 1991)
			Br-I Exchange	$PhCO_2H$ 240°C, 40 min	HPLC	40-60%	n.p.	1.4-1.5 TBq/µmol	(Brandau et al., 1993)
			Isotope Exchange	Acidic condition $SnSO_4/CuSO_4$ 100°C, 60 min	none	99.5%	>99%	256 MBq/µmol	(Everaert et al., 1997)
IBZM *N*-((1-ethylpyrrolidin-2-yl)methyl)-2-hydroxy-3-iodo-6-methoxybenzamide			Direct Electrophilic Aromatic Substitution	$AcONH_4$ buffer pH = 4 AcO_2H RT, 5 min	C4-cartridge	70-80%	95-98%	>185 GBq/µmol	(M.-P. Kung & Kung, 1989) (M. P. Kung et al., 1991)
IMBA *N*-(2-diethylaminoethyl)-3-iodo-4-methoxybenzamide			Direct Electrophilic Aromatic Substitution	$Tl(TFA)_3$ RT, 5 min	HPLC	80-98%	n.p.	90 GBq/µmol	(Nicholl et al., 1997)
BZA2 *N*-(2-diethylaminoethyl)-2-iodobenzamide			Br-I Exchange	$Na_2S_2O_5$ CuCl/AcOH ; 10-60 min à 180°C	HPLC	>80%	n.p.	5 TBq/µmol	(Brandau et al., 1996)
			Isotope Exchange	Acetate buffer pH=5 Dry evaporation 140°C, 50 min	Anionic resin	75%	>95%	6.4-15.4 MBq/µmol	(Nicole Moins et al., 2002)
MIP-1145 *N*-(2-diethylaminoethyl)-4-(4-fluorobenzamido)-5-iodo-2-methoxybenzamide			Direct Electrophilic Aromatic Substitution	$Tl(TFA)_3$ RT, 5 min	HPLC	70-90%	>95%	55.5 GBq/µmol	(Joyal et al., 2010)
Mel050 *N*-(2-diethylaminoethyl)-6-fluoronicotinamide			Cl-F Exchange	K_{222}/K_2CO_3 DMF 150°C 5 min	HPLC	<5%	>99%	150-220 GBq/µmol	(Denoyer et al., 2010) (Denoyer et al., 2011)

Name	Chemical structure	Chemical precursor	Radiolabeling method	Conditions	Purification	Yield	Purity	Molar Activity	Reference(s)
BA52 *N*-(4-((2-diethylamino-ethylcarbamoyl)-2-iodo-5-methoxyphenyl)benzo[1,3]dioxole-5-carboxamide			Iodo-destannylation	AcOH H_2O_2 RT, 10 min	HPLC	54-68%	>95%	n.p.	(Mier et al., 2014)
P3BZA *N*-(2-diethylaminoethyl)-5-fluoropicolinamide			Br-F Exchange	Automated radiosynthesis K_{222}/K_2CO_3 DMF 110°C 10 min	HPLC	12%	>99.5%	132 GBq/µmol	(Ma et al., 2019)
ICF01012 ***N*-(2-diethylaminoethyl)-6-iodoquinoxaline-2-carboxamide**			Iodo-destannylation	Automated radiosynthesis HCl, H_2O_2 RT, 15 min	Sep-pak cartridge	60-80%	>98%	24-84 GBq/µmol	(Chezal et al., 2008) This article

n.p.: not published

Table 3. Summary of radiolabeled benzamide derivatives injected into humans with corresponding preclinical studies on mice. The parameters given are for imaging the tumor-to-blood or tumor-to-muscle activity ratios (T/B, T/M) and the percentage of activity injected into the tumors allowing dosimetry to be calculated. For patient studies, the number of the clinical trial is indicated when available. For imaging, sensitivity and specificity calculations are based on lesion analysis.

Acronym	Preclinical models		Clinical trials			References
	Imaging T/M or T/B ratio or % of IA/gr	**Therapy** Dosimetry	**Number of patients / NCT**	**Imaging** Sensitivity Specificity	**Therapy** Dosimetry PFS	
[^{123}I]IBZM			**1** **11** **21**	*Case report* 87%, nd 80%, 100%		(L. S. Maffioli et al., 1993) (L. Maffioli et al., 1994) (Larisch et al., 1998)
[^{123}I]BZA	B16: 3.5% IA/gr (6 hrs p.i. 18 (6 hrs p.i.) 19 (6 hrs p.i.)		**110 (phase II)**	81%, 100%		(Michelot et al., 1991) (Michelot et al., 1993)
[^{123}I]IMBA	B16: 3.5% IA/gr (6 hrs p.i). 88 (6 hrs p.i.) 123 (6 hrs p.i.)		**2**	nd		(Nicholl et al., 1997)
[^{123}I]MB **[^{131}I]MB**	HX118: 3% (3 hrs p.i.) 20 (3 hrs p.i.) 14 (3 hrs p.i.)		**11**	80%, 100%		(Link et al., 1996) (Link et al., 1998)
[^{123}I]BZA2	B16: 5 (6 hrs p.i.) 10 (6 hrs p.i.)		**25** **40** **87**	100%, 95% 75% 23%, 86%		(Moreau et al., 1995) (Nicole Moins et al., 2002) (Sillaire-Houtmann et al., 2004) (Cachin et al., 2014)
[^{18}F]Mel050	B16: 9±0.6 (3 hrs p.i.)		NCT01620749			(Denoyer et al., 2010)
[^{131}I]MIP-1145 **[^{131}I]BA52**	SK-Mel-3: 9% (4 hrs p.i.) 25 (3 hrs p.i.)		 **9**		 12.2 Gy per GBq <2 years in 3/5 receiving >4.5 GBq	(Joyal et al., 2010) (Mier et al., 2014)
[^{131}I]ICF01012	B16-F0: 41% (6 hrs p.i.) SK-Mel-3: 27% (6 hrs p.i.)	108 Gy/37 MBq (B16-BL6) 52 Gy/75 MBq (SK-Mel-3)	NCT03784625 *Recruiting*			(Chezal et al., 2008; Bonnet-Duquennoy et al., 2009) (Bonnet et al., 2010; Degoul et al., 2013; Viallard et al., 2015)
[^{18}F]P3BZA	B16-F10: 17% (2 hrs p.i.) 88 (2 hrs p.i.) 37 (2 hrs p.i.)		NCT03033485 **6 controls** **5 patients**	T/M 60 minutes 18.5±4.8		(Ma et al., 2019)