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Receptor Recognition and Lysosomal Targeting to Enhance Cytotoxicity of Novel Anti-Cancer Agents that Bind Iron and Copper

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Abbreviations:

Bp4eT: 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone

Dp44mT: di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone:

HSA: Human Serum Albumin

PIH: Pyridoxal Isonicotinoyl Hydrazone

Introduction

Despite intensive research efforts, cancer remains a considerable health problem worldwide. To combat this devastating disease, novel drugs, known as chelators, are being developed as anti-cancer agents [1]. These compounds exploit crucial differences in the metabolism of iron and copper between cancer and normal cells to deliver selective anti-tumor activity by interfering with processes such as DNA synthesis and angiogenesis [1,2]. Moreover, tumor cells have increased iron and copper requirements that are necessary to sustain their generally highly proliferative nature [2-4]. Consequently, cancer cells are more susceptible to the deprivation of both these metal ions through chelation, making it an attractive chemotherapeutic avenue with a considerable therapeutic window.

Anti-cancer chelators have been shown to affect multiple cellular targets [1]. These agents are able to target a variety of molecules necessary for cell cycle progression, including ribonucleotide reductase, p53, cyclin D1 and cyclin-dependent kinase 2 [5]. Moreover, chelators have been shown to up-regulate the tumor growth and metastasis suppressor, N-myc downstream-regulated gene-1 [6]. After many years of development, a new series of chelators known as the dipyridyl thiosemicarbazones have been synthesized that show marked and selective anti-tumor activity [1]. One of the lead compounds from this group of agents is known as di-2-pyridylketone 4,4,-di-methyl-3-thiosemicarbazone (Dp44mT; Figure 1A) [7,8]. The anti-tumor activity of Dp44mT has been demonstrated in over 28 cancer cell lines, including drug-resistant and p53 mutant cells [8]. Importantly, its anti-cancer effects have been shown *in vivo* in multiple tumor xenograft mouse models, such as melanoma and lung carcinoma xenografts, and in a metastatic breast cancer model in severe combined immunodeficiency mice [8,9].

Interestingly, Dp44mT overcomes resistance to established chemotherapeutics and has been shown to target the lysosome to induce its cytotoxic activity [8,10]. Specifically, this ligand has been shown to accumulate within acidic lysosomes due to its ionization properties [10]. Due to the role of the lysosome in autophagy and the turnover of iron and copper containing proteins in this organelle, Dp44mT is able to bind these metals and form redox-active complexes that result in lysosomal membrane permeabilization and apoptosis [10].

Although an understanding of the mechanism of action of Dp44mT in the lysosome has been established, the initial uptake and membrane transport of the ligand into the cell has only recently been investigated [11]. An understanding of the membrane transport of drugs is crucial in the development of successful chemotherapeutics for three main reasons [12]. First, the membrane acts as a barrier, preventing drug entry into cells and inhibiting the interaction between drugs and their intracellular targets [12]. Second, cancer cells can develop drug resistance through the impairment of drug uptake and by enhancing drug efflux [13]. Third, an understanding of drug membrane transport has implications in the development of targeted drug delivery, which is important for the design of therapeutics with greater selectivity and anti-tumor activity [14].

To investigate membrane transport, recent studies have examined the uptake of ^{14}C-labeled Dp44mT by SK-N-MC cells in comparison to other structurally similar ligands, namely ^{14}C-2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (^{14}C-Bp4eT; Figure 1A) and ^{14}C-pyridoxal isonicotinoyl hydrazone (^{14}C-PIH; Figure 1A) [11]. These studies revealed that the transport of ^{14}C-Dp44mT occurred *via* a saturable, temperature- and energy-dependent carrier/receptor-mediated process (Figure 1B), which was subject to inhibition by competition with unlabeled Dp44mT [11]. In contrast, the uptake of the structurally-related ligands, ^{14}C-Bp4eT and ^{14}C-PIH, was not saturable, nor was it inhibited by their unlabeled counterparts, demonstrating that these compounds permeate cancer cells *via* passive diffusion [11]. Moreover, ^{14}C-Dp44mT competition studies with unlabeled structurally-related compounds revealed that both the pyridyl substituent and methyl groups of this ligand are important structural entities for recognition and binding to a putative Dp44mT receptor/carrier [11].

Several chemotherapeutic agents have been shown to utilise carriers/receptors to mediate uptake, including doxorubicin, paclitaxel and methotrexate [15]. Moreover, other thiosemicarbazones have been identified to bind receptors [16]. For example, salicylaldehyde thiosemicarbazones are agonists of the c-Mpl thrombopoietin receptor [16]. It is improbable that the c-Mpl thrombopoietin receptor is involved in Dp44mT uptake, considering the marked selectivity of the Dp44mT receptor/carrier and its requirement for both the pyridyl substituent and methyl groups of this chelator for ligand recognition [11]. Indeed, the salicylaldehyde thiosemicarbazones lack the latter substituents that are required for Dp44mT receptor/carrier binding.

Considering that human serum albumin (HSA) is the most abundant protein in the blood and is well known to affect drug bioavailability [17], studies have also examined the effect of HSA on the membrane transport of these ^{14}C-labeled ligands. Interestingly, in contrast to ^{14}C-Bp4eT and ^{14}C-PIH, HSA increased the uptake, toxicity and apoptotic activity of ^{14}C-Dp44mT [18,19]. Moreover, HSA led to the stimulation of a second saturable uptake process of lower affinity

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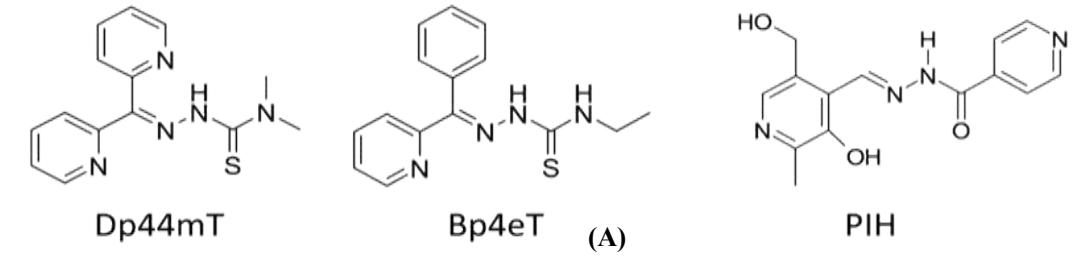
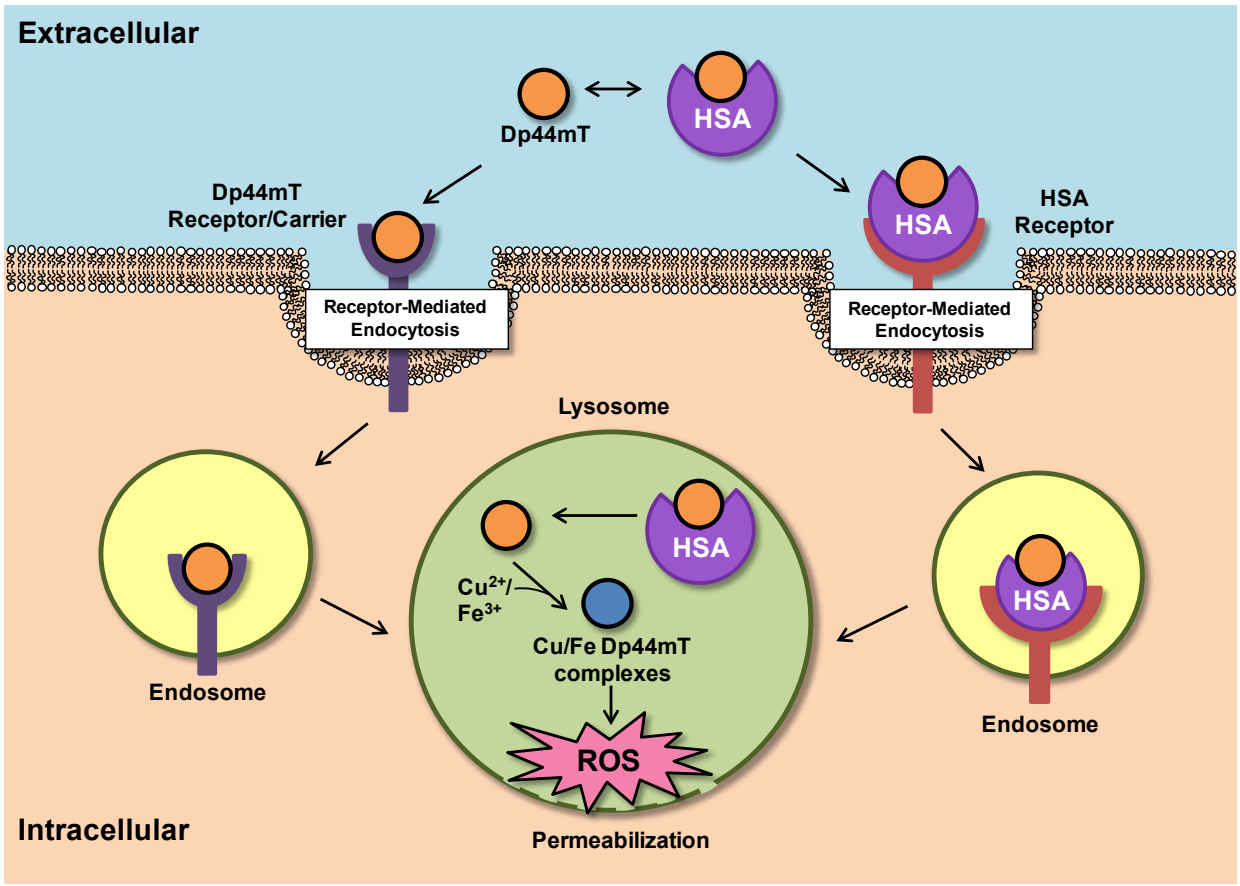


(A)



(B)

Figure 1: (A) Line drawings of the chemical structures of the chelators: 2-benzoylpyridine-4-ethyl-3-thiosemicarbazone (Bp4eT), di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT) and pyridoxal isonicotinoyl hydrazone (PIH). **(B)** Schematic of the internalization of Dp44mT through two pathways into the lysosome to induce cytotoxicity. The diagram shows the interaction of Dp44mT with its putative receptor/carrier and its internalization through receptor-mediated endocytosis (RME), where due to endosomal trafficking, the agent eventuates within lysosomes. Moreover, Dp44mT binds human serum albumin (HSA) and is potentially "piggybacked" into the cell *via* a putative albumin receptor by RME, leading to HSA degradation and the release of Dp44mT within the lysosome. The lysosome is a major target of Dp44mT and the formation of the Dp44mT-HSA complex results in enhanced delivery of Dp44mT to this organelle, leading to increased cytotoxicity.

and higher capacity than the putative high affinity Dp44mT receptor/carrier [18]. This finding is important in terms of the mechanism of action of Dp44mT, and it can be speculated that the Dp44mT-HSA complex may be internalized into cells by a HSA receptor (Figure 1B). In view of the fact that Dp44mT targets lysosomes to induce apoptosis by lysosomal membrane permeabilization [10], it is notable that HSA undergoes lysosomal catabolism in tumors [20]. Hence, it can be proposed that HSA-stimulated uptake of Dp44mT facilitates shuttling of this agent to lysosomes, thereby enhancing its anti-tumor activity (Figure 1B).

Importantly, the development of an albumin nanoparticle encapsulating Dp44mT could be foreseen as an effective lysosomal targeting strategy to improve the efficacy and selectivity of Dp44mT. In fact, previous studies utilizing other thiosemicarbazones have led to the successful generation of nanoparticles called "nanochelators" that have been shown to become localized within the lysosomal compartment [21]. Furthermore, albumin nanoparticles containing paclitaxel, known as Abraxane®, are clinically used for advanced and metastatic breast cancer and non-small cell lung cancer [22]. Recently, Abraxane® has also received FDA approval as a first-line treatment option for patients with metastatic adenocarcinoma of the pancreas in combination with gemcitabine (ClinicalTrials.gov, Identifier: NCT00844649). Several studies have demonstrated that patients treated with Abraxane® have a

greater response rate and experience less toxicity than patients treated with the traditional paclitaxel formulation [22]. Hence, it is of interest to develop a suitable albumin nanoparticle that could increase the lysosomal targeting of Dp44mT through interaction with albumin receptors.

In summary, increased targeting of Dp44mT to the lysosome *via* the interaction of this agent with the putative Dp44mT receptor/carrier and possibly a HSA receptor could potentiate the activity of this drug and enable the generation of novel therapeutics (*e.g.*, albumin-based nanoparticles). These studies examining Dp44mT underline the importance of the lysosome as a potential target for novel anti-cancer agents.

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