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The Anticancer Agent, Di-2-Pyridylketone 4,4-Dimethyl-3-Thiosemicarbazone (Dp44mT), Up-Regulates the AMPK-Dependent Energy Homeostasis Pathway in Cancer Cells

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ABSTRACT

Adenosine monophosphate-activated protein kinase (AMPK) is a cellular energy sensor that monitors ATP levels. There is also evidence that AMPK has onco-suppressive properties. Iron plays a crucial role in cellular energy transducing pathways and tumor cell proliferation. Therefore, metals (*e.g.,* iron) could play an important role in the regulation of AMPK-dependent pathways. Hence, this investigation examined the effect of the iron and copper chelator and potent anti-cancer agent, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), on the AMPK-mediated pathway. These studies demonstrated that Dp44mT, which forms intracellular redox-active complexes with iron and copper, significantly activated AMPK (*i.e.,* p-AMPK/AMPK ratio) in 5 different tumor cell-types. Furthermore, examination of the Dp44mT-metal complexes demonstrated that the effect of Dp44mT on AMPK was due to a dual mechanism: **(1)** its ability to chelate metal ions; and **(2)** the generation of reactive oxygen species (ROS). The activation of the AMPK-pathway by Dp44mT was mediated by the upstream kinase, liver kinase B1 (LKB1) that is a known tumor suppressor. Moreover, using *AMPKα1*-selective silencing, we demonstrated that Dp44mT activated AMPK, resulting in inhibition of acetyl CoA carboxylase 1 (ACC1) and raptor, and activation of Unc-51 like kinase (ULK1). These effects are vital for inhibition of fatty acid synthesis, suppression of protein synthesis and autophagic activation, respectively. Together, this AMPK-mediated repair response aims to rescue the loss of metal ions *via* chelation and the induction of cytotoxic damage mediated by redox cycling of the Dp44mT-metal ion complex. In conclusion, this study demonstrates for the first time that chelators target the AMPK-dependent pathway.

1. Introduction

Adenosine monophosphate-activated protein kinase (AMPK) is an important regulator of cellular energy and metabolic homeostasis [1,2]. In fact, AMPK belongs to a family of highly conserved serine-threonine kinases that are activated under stress and low energy conditions [1,2]. Once activated, AMPK enhances catabolic pathways, such as β-oxidation, to generate ATP, and inhibits anabolic processes that require energy, including fatty acid, cholesterol and protein synthesis [3]. The AMPK protein is activated, under different stimuli, through phosphorylation of T172 in the α-subunit by three main kinases [2], namely: **(1)** liver kinase B1 (LKB1) [4]; **(2)** calmodulin-mediated kinase kinase β (CaMKKβ) [5]; and **(3)** transforming growth factor-β activated kinase 1 (TAK1) [6].

Interestingly, AMPK has shown promise as an anti-cancer drug target [7]. In fact, metformin, which is a known activator of the AMPK pathway, has been demonstrated to positively increase the survival of cancer patients [8]. It has also been suggested that the anti-tumor activity of AMPK corresponds to its ability to regulate metabolic pathways [7,9,10]. As cancer cells generally have high energy requirements [11], AMPK could play a key role in cancer progression [7,9,10]. Additionally, LKB1, the key upstream kinase of AMPK, has also been shown to inhibit cancer cell proliferation as well as metastasis, possibly through AMPK activation [12].

A recent study has reported that AMPK can suppress the levels of oncogenic molecules, such as integrin β1, Src, FAK and p130Cas [13], which are known to participate in cell migration and invasion [14,15]. Notably, migration and invasion are critical steps involved in cancer

Abbreviations: AMPK, adenosine monophosphate-activated protein kinase; ACC1, acetyl CoA carboxylase 1; Bp4eT, 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone; BpT, 2-benzoylpyridine thiosemicarbazone; CaMKKβ, calmodulin-mediated kinase kinase β; DFO, desferrioxamine; Dp2mT, di-2-pyridylketone 2-methyl-3-thiosemicarbazone; Dp44mT, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone; DpC, di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone; DpT, di-2-pyridylketone thiosemicarbazone; ECAR, extracellular acidification rate; LKB1, Liver kinase B1; NAC, *N*-acetylcysteine; OCR, oxygen consumption rate; ROS, reactive oxygen species; TAK1, transforming growth factor-β activated kinase 1.

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metastasis [16]. Hence, AMPK has potential as a molecular target in terms of the development of novel anti-cancer strategies.

Metal ions such as iron and copper play significant roles in critical cellular processes [17]. Furthermore, these metal ions possess the ability to cycle between two redox states, allowing them to participate in a reaction as an electron donor or acceptor [18]. This permits metal ions to act as co-factors for enzymes involved in many cellular processes such as pathways involved in cellular energy metabolism (*i.e.*, the electron transport chain) [18]. Furthermore, iron is essential for rapid growth and proliferation of cancer cells [19–22] and this has resulted in the examination of chelators as anti-cancer agents [18,23–25].

Over the past 20 years, our laboratory has rationally and systematically designed chelators for the treatment of cancer [26–31]. In fact, studies over the past 10 years directly led to the development of the di-2-pyridylketone thiosemicarbazone (DpT)- and 2-benzoylpyridine thiosemicarbazone (BpT)-based metal ion chelators that demonstrate potent and selective anti-proliferative and anti-tumor activity *in vitro* and *in vivo* [32–37]. Notably, these agents lead to induction of a variety of apoptotic markers, such as cleaved caspase 3, caspase 4, cleaved PARP, *etc.*, in different cancer cell-types [33,38–40]. These compounds include the first lead agent, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), and its analogues, di-2-pyridylketone-4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC) [34,35] and 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT) [41] (Fig. 1i-iii). Due to the marked and selective anti-tumor activity of DpC *in vitro* and *in vivo* [34,35], appropriate pharmacokinetics [42], ability to overcome P-glycoprotein-mediated drug resistance [43], oral bioavailability and tolerability [35], this agent has entered Phase I clinical trials [44,45].

The activity of these thiosemicarbazone metal ion chelators (*e.g.*, Dp44mT) involves a "double punch" mechanism mediated by: (1) chelation of intracellular metal ions such as iron or copper; and (2) the formation of redox-active metal complexes within the cell to generate reactive oxygen species (ROS) [46]. Interestingly, a previous study demonstrated that skeletal muscle cells deficient in iron had higher levels of AMPK activity along with decreased expression of enzymes involved in the electron transport chain [47]. However, this latter study did not elucidate the molecular mechanisms involved. Thus, we examined the effect of the metal-binding DpT/BpT class of novel chelators on the AMPK-dependent energy homeostasis pathway. The effects of these agents were directly compared to the negative control compound, di-2-pyridylketone 2-methyl-3-thiosemicarbazone (Dp2mT; Fig. 1iv), that cannot bind metal ions [33,48]. A relative comparison was also made to classical, clinically used iron chelators, including desferrioxamine (DFO), Deferiprone and Deferasirox (Fig. 1v-vii).

The current investigation demonstrates that a range of iron chelators activated the AMPK-dependent energy homeostasis pathway. In fact, the effect of the chelator, Dp44mT, on activating AMPK (*i.e.*, the p-AMPK/AMPK ratio) was demonstrated in 5 different tumor cell-types. Further, the mechanism of AMPK activation by this agent was shown to be due to a dual effect, namely: **(1)** the ability to chelate intracellular metal ions; and **(2)** the generation of ROS. Notably, AMPK

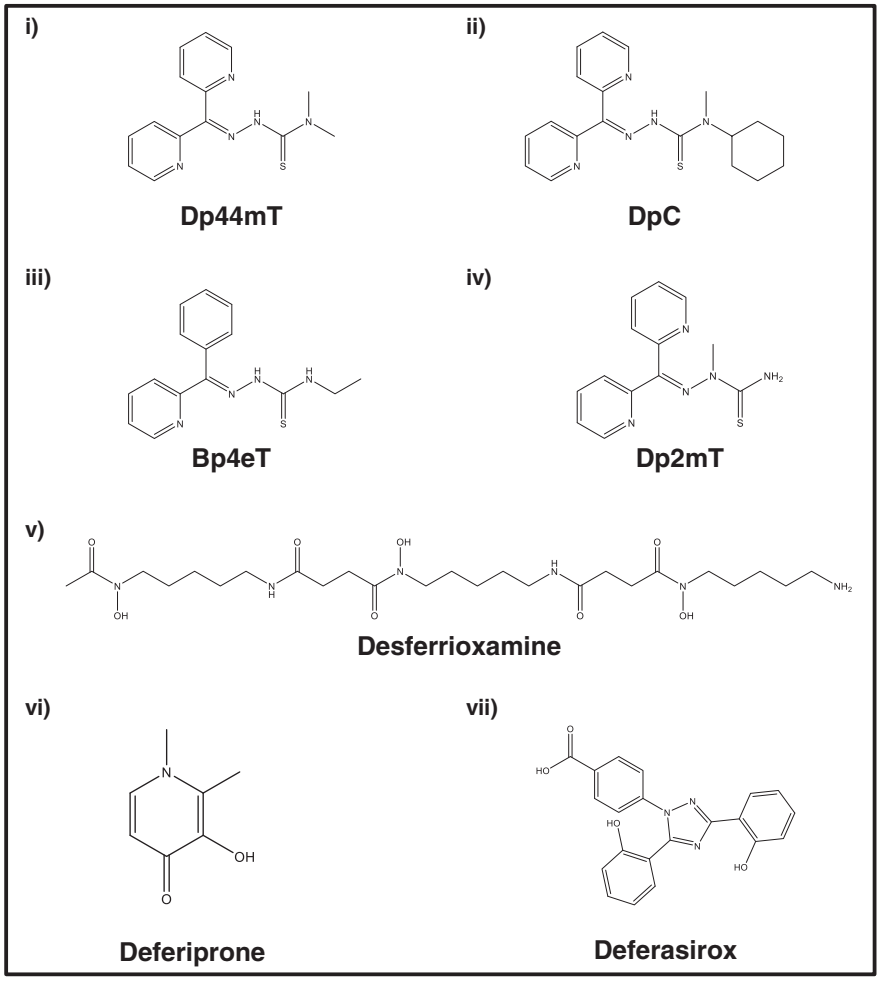

Fig. 1. Line drawings of the chemical structures of the iron chelators: **(i)** di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT); **(ii)** di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC); **(iii)** 2-benzoylpyridine 4-ethyl-3-thiosemicarbazone (Bp4eT); **(iv)** the related control compound, di-2-pyridylketone 2-methyl-3-thiosemicarbazone (Dp2mT); **(v)** Desferrioxamine (DFO); **(vi)** Deferiprone; and **(vii)** Deferasirox.