Comment

The redox-active, anti-cancer drug Dp44mT inhibits T-cell activation and CD25 through a copper-dependent mechanism

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We were particularly interested to read a recently published article in the *FASEB J* by Gundelach *et al.*[1] This study investigated the effect of the thiosemicarbazone iron chelator, di-2-pyridylketone 4,4-dimethyl-3-thiosemicarbazone (Dp44mT), which has been extensively investigated as an anti-cancer agent,[2–6] on the activation of isolated mouse splenic T cells *in vitro*. The authors observed that Dp44mT inhibited the up-regulation of surface CD25 expression after mitogenic stimulation, while CD69 expression was unaltered relative to the stimulated control.[1]

Although the exact mechanism responsible in the specific inhibition of CD25 up-regulation by Dp44mT was not identified, the authors suggest that the inhibition of T-cell activation after Dp44mT treatment occurred *via* a mechanism that was iron-independent.[1] In fact, it was proposed that the ability of Dp44mT to bind copper and/or the capacity of the Dp44mT-copper complex to redox cycle played a role in the inhibition of T-cell activation. Gundelach *et al.*[1] also observed that the non-toxic copper chelators, tetrathiomolybdate and bathocuprione disulfonate, were able to mitigate the inhibitory effects of Dp44mT.[1] In agreement with our previous studies demonstrating the effect of Dp44mT on cellular thiol systems,[5,7] this same mechanism was shown to be involved in inhibiting CD25 up-regulation.[1] Collectively, these authors suggest from their *in vitro* studies that Dp44mT may act as an immunosuppressive agent that could complicate its use as a novel anti-tumor drug.[1]

We would like to make several comments regarding the preliminary nature of these studies and the speculative nature of the conclusions. First, all of the studies reported by Gundelach *et al.* are limited to isolated T cells *in vitro* which provide a highly simplistic model of the intricate immune effector response against tumors. Indeed, a complex array of cellular interactions are associated with the immune response,[8–10] which cannot be predicted by utilizing isolated T cells alone. Hence, carefully performed *in vivo* studies should have been performed before speculating that Dp44mT interferes with the immune response and that this could affect the drug's potential as an anti-tumor agent. Such studies would need to include a comprehensive assessment of T cells (their various subsets), natural killer cells, macrophages, etc. and their tumor effector functions after administration of Dp44mT *in vivo*. With regard to the proposed effect of Dp44mT affecting T cells and then complicating its anti-tumor activity,[1] it is notable that we have demonstrated the marked efficacy of Dp44mT against tumors in nude mice, despite the fact that these animals have a significantly depressed immune system.[3] Moreover, we have also demonstrated the anti-tumor effects of Dp44mT *in vivo* using mice with a normal immune system.[4]

The deficiencies in experimental design described above, and particularly the lack of implementing an *in vivo* model, are underlined by the fact that Dp44mT acts differently *in vitro* and *in vivo*. We have previously demonstrated that Dp44mT can induce marked iron chelation and tumor cell iron depletion *in vitro*.[4] However, in clear contrast, a slight increase in tumor iron levels was observed upon administration of Dp44mT in mice *in vivo*.[3] Thus, it may be expected that the immunosuppressant role of Dp44mT observed *in vitro* in isolated T cells[1] may be different to that found *in vivo*. Of interest, our studies using Dp44mT *in vivo* in mice did not demonstrate any significant differences in white blood cell counts relative to the control.[3,4]

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In addition to the preliminary nature of this article, some of the interpretation appears unclear and the exact mechanism involved remains unknown, as the authors do not take into account the previous studies examining the chemical and biochemical properties of Dp44mT.[2,4,5,7,11] For example, Gundelach *et al.*[1] use iron supplementation (ferric ammonium citrate (FAC)) of the culture system to determine whether the block in T-cell activation by Dp44mT was dependent on the ability of this ligand to chelate iron. The authors report that FAC did not have any effect on the inhibition of T cells by Dp44mT and this observation led them to conclude that iron-binding was not implicated.[1] Instead, they indicate that copper chelation alone was involved in the mechanism on inhibiting T-cell activation,[1] but do not clearly dissect whether it was due to copper depletion and/or due to the ability of Dp44mT to form a redox-active copper complex.[2,11] In fact, they show that the antioxidant, *N*-acetylcysteine, rescues the inhibition of T-cell activation, suggesting that redox activity may be involved in the mechanism.[1] It is important to realize that unlike the ligand, desferrioxamine, Dp44mT forms redox active complexes with both iron and copper that mediate many of its biological effects.[2,11] Hence, the inability of iron supplementation to rescue CD25 inhibition by Dp44mT cannot be simply interpreted to indicate that iron complexation was not involved. In fact, both redox-active iron and copper complexes form in cells[2,11] and reactive oxygen species (ROS) generated by both of these are important to assess in terms of the effect observed.

Considering the deficiencies above, additional experiments are essential in order to clarify the interpretation of the roles of these complexes and their redox activity in the effects observed. These include (1) assessment of the ability of the antioxidant, *N*-acetylcysteine, to rescue T-cell activation in the presence of either the pre-synthesized iron or copper complex of Dp44mT; (2) examination of T-cell activation upon treatment with redox-inactive desferrioxamine in comparison to its redox-inactive iron complex to definitively determine if iron chelation alone is sufficient;[12] (3) determination of whether copper supplementation (as $CuCl_2$) acts similarly to iron supplementation and is unable to rescue T-cell activation in the presence of Dp44mT; (4) the use of cell-permeable scavengers of ROS to block their effects in the presence of the iron or copper complexes, and conversely, to implement agents that generate ROS to assess if it is responsible for the effects observed; and (5) implementation of Dp2mT, an analogue of Dp44mT which cannot bind iron, as an important control.[3,4] Collectively, these further studies would examine the potential role of ROS generation in the inhibition of T-cell activation by Dp44mT *via* the formation of iron and/or copper complexes and the role of iron and/or copper chelation.

Finally, Gundelach *et al.*[1] propose it is important to examine the immunosuppressive effects of compounds being considered as novel chemotherapeutics, and therefore, have chosen to study Dp44mT. However, while the anti-tumor activity of Dp44mT has been extensively documented by our group and others,[3,4,6] we have also demonstrated that this agent induces cardiotoxicity at high, non-optimal intravenous doses[3] and exhibits marked toxicity when given by the oral route.[13] Due to these side effects, better tolerated thiosemicarbazones (e.g. di-2-pyridylketone 4-cyclohexyl-4-methyl-3-thiosemicarbazone (DpC)) have been developed that do not demonstrate cardiotoxicity and are effective by both the oral and intravenous routes.[14,15] Therefore, it would have been preferable to assess the effect of this latter compound on T-cell effector function rather than Dp44mT that has unfavorable pharmacological properties.

In summary, the preliminary *in vitro* experiments conducted by Gundelach *et al.* are inconclusive. The studies with isolated T cells should have been complemented by *in vivo* studies in animal models with and without tumors to determine if appropriate thiosemicarbazones (e.g. DpC) demonstrate immunosuppressive activity.

References

1 Gundelach JH, Madhavan AA, Wettstein PJ, Bram RJ. The anticancer drug Dp44mT inhibits T-cell activation and CD25 through a copper-dependent mechanism. FASEB J 2013;27(2): 782–92.

2 Richardson DR, Sharpe PC, Lovejoy DB, Senaratne D, Kalinowski DS, Islam M, *et al.* Dipyridyl thiosemicarbazone chelators with potent and selective antitumor activity form iron complexes with redox activity. J Med Chem 2006;49(22): 6510–21.

3 Whitnall M, Howard J, Ponka P, Richardson DR. A class of iron chelators with a wide spectrum of potent antitumor activity that overcomes resistance to chemotherapeutics. Proc Natl Acad Sci USA 2006;103(40):14901–6.

4 Yuan J, Lovejoy DB, Richardson DR. Novel di-2-pyridyl-derived iron chelators with marked and selective antitumor activity: in vitro and in vivo assessment. Blood 2004;104(5): 1450–8.

5 Lovejoy DB, Jansson PJ, Brunk UT, Wong J, Ponka P, Richardson DR. Antitumor activity of metal-chelating compound Dp44mT is mediated by formation of a redox-active copper complex that accumulates in lysosomes. Cancer Res 2011;71(17):5871–80.

6 Liu W, Xing F, Iiizumi-Gairani M, Okuda H, Watabe M, Pai SK, *et al.* N-myc downstream regulated gene 1 modulates Wnt-beta-catenin signalling and pleiotropically suppresses metastasis. EMBO Mol Med 2012;4(2):93–108.

7 Yu Y, Suryo Rahmanto Y, Hawkins CL, Richardson DR. The potent and novel thiosemicarbazone chelators di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone and 2-benzoylpyridine-4,4-dimethyl-3-thiosemicarbazone affect crucial thiol systems required for ribonucleotide reductase activity. Mol Pharmacol 2011;79(6):921–31.

8 Buzas EI, Gyorgy B, Pasztoi M, Jelinek I, Falus A, Gabius HJ. Carbohydrate recognition systems in autoimmunity. Autoimmunity 2006;39(8):691–704.

9 Chew V, Toh HC, Abastado JP. Immune microenvironment in tumor progression: characteristics and challenges for therapy. J Oncol 2012;Epub 8 August 2012 PMID: 22927846.
10 Figge MT. Optimization of immunoglobulin substitution therapy by a stochastic immune response model. PLoS One 2009;4(5):e5685.
11 Bernhardt PV, Sharpe PC, Islam M, Lovejoy DB, Kalinowski DS, Richardson DR. Iron chelators of the dipyridylketone thiosemicarbazone class: precomplexation and transmetalation effects on anticancer activity. J Med Chem 2009;52(2): 407–15.
12 Kalinowski DS, Richardson DR. The evolution of iron chelators for the treatment of iron overload disease and cancer. Pharmacol Rev 2005;57(4):547–83.
13 Yu Y, Suryo Rahmanto Y, Richardson DR. Bp44mT: an orally active iron chelator of the thiosemicarbazone class with potent anti-tumour efficacy. Br J Pharmacol 2011;165(1):148–66.
14 Kovacevic Z, Chikhani S, Lovejoy DB, Richardson DR. Novel thiosemicarbazone iron chelators induce up-regulation and phosphorylation of the metastasis suppressor N-myc down-stream regulated gene 1: a new strategy for the treatment of pancreatic cancer. Mol Pharmacol 2011;80(4):598–609.
15 Lovejoy DB, Sharp DM, Seebacher N, Obeidy P, Prichard T, Stefani C, *et al.* Novel second-generation di-2-pyridylketone thiosemicarbazones show synergism with standard chemotherapeutics and demonstrate potent activity against lung cancer xenografts after oral and intravenous administration in vivo. J Med Chem 2012;55(16):7230–44.