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Response to Rosenthal *et al.*

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To the Editor:

We find the claims in the letter to the editor by Rosenthal *et al.* unfounded (as discussed in details below), and we do not think that any significant damage was done to the authors' reputation by the claims in our review article (3). Our remarks on their research do not go beyond well-intended scientific criticism. We feel that many researchers in the field do not always appreciate how long-lasting and costly damage can be made by careless claims and dissemination of compounds that are impure and/or of unknown chemistry.

Addressing the Matter of Opinion

We do not view the problems we have with the manuscript by Rosenthal *et al.* (12) as a matter of opinion. We may disagree with Rosenthal *et al.* on what Mn porphyrins do *in vivo*, whereas we agree that the scientific community still does not quite know whether there is a single or multiple actions of such compounds *in vivo.* Particularly as the compounds differ with respect to redox ability, charge, lipophilicity, shape, bulkiness, rotational flexibility, and so on; incoming data keep revealing new horizons. We, however, disagree with authors saying that Mn porphyrins do something (who knows what) while claiming that they are superoxide dismutase (SOD) and catalase mimics, particularly doing it so in the title without any hint about the ambiguity of such statement (12). Further, the authors claim that compounds are orally available SOD and catalase mimics, but (i) no *in vivo* efficacy data *via* oral route are provided (where, only, oral availability could be relevant), and (ii) compounds are essentially not SOD mimics (see below). Cellular studies were the only ones shown [Fig. 2 in (12)]. Although the possible actions may be discussed in a manuscript, the authors should not make such ungrounded and explicit claims in the title. Thus, the title of their 2009 paper (12) "Orally available Mn porphyrins with superoxide dismutase and catalase activities" is a misinterpretation, which may mislead the biological and medical readers who do not have sufficient chemical knowledge to critically look at the compounds described. A reader, trusting the claims stated in the title and manuscript, will be misled when choosing compounds for costly animal studies where the hypothesis is made that only a compound that is SOD mimic could play a role. The most appropriate title of the work by Rosenthal *et al.* might have been "Selected Mn porphyrins suppress oxidative stress in a cellular model."

To compare the SOD-like activity of a variety of compounds measured by different techniques, the k_{cat}, and not IC_{50}, values need to be provided, because IC_{50} is assay dependent. We recalculated the data in Table 1 (12) using IC_{50} 1.1×10^{-9} M of SOD enzyme provided by the authors (12), which gives k_{cat} of SOD enzyme as 2.36×10^{9} M^{-1} s^{-1} (log $k_{cat} = 9.37$); the value is the highest limit for k_{cat} reported in literature for SOD enzymes, which ranges from 8.84 to 9.30 [related references summarized in (4)]. Our calculations on the data by Rosenthal *et al.* (12) gave the average $k_{cat} = 5.5\times10^{5}$ M^{-1} s^{-1} for seven Mn porphyrins. Such k_{cat} is essentially identical to the rate constant for superoxide self-dismutation of $\sim5\times10^{5}$ M^{-1} s^{-1} at pH 7 (6). Only for EUK-418, the k_{cat} 1.5×10^{6} M^{-1} s^{-1} is somewhat higher than the self-dismutation rate constant. Based on the data for Mn salen (EUK-189 and EUK-207) (12), it seems that nitroblue tetrazolium (NBT) assay gives rise to lower IC_{50} values (for the reasons discussed below) and, in turn, higher k_{cat} values than does cyt *c* assay. Utilizing IC_{50} of SOD enzyme provided by authors (12), the average k_{cat} values for those two Mn salen derivatives are $\sim6.1\times10^{6}$ M^{-1} s^{-1}, whereas if one uses $IC_{50} = 1.3\times10^{6}$ M^{-1} s^{-1} of another Mn salen, EUK-8 given by cyt *c* assay (13), the k_{cat} value is 2×10^{6} M^{-1} s^{-1}. Such a comparison among the three Mn salen compounds is justified as Doctrow *et al.* showed that the SOD-like activity of all Mn salen derivatives synthesized is independent of ring substitution and bridge modification (5). The same conclusions may be also reached, if one compares IC_{50} of SOD enzyme and IC_{50} of compounds, which the authors obtained by the same assay in Table 1 (12). Therefore, given the considerations above, the estimated k_{cat} values for the Mn porphyrins by Rosenthal *et al.* represent, at best, the highest limits possible of k_{cat} values for such compounds, which are, nonetheless, not much different from superoxide self-dismutation, if at all.

In general, potent porphyrin-based SOD mimics are not good catalase mimics as they undergo rapid degradation in the presence of peroxide; moreover, they can even produce H_2O_2. For those reasons, and as no good data are provided on their purity (see below), we decided not to discuss the actions of the compounds by Rosenthal *et al.* in cellular models.

It is not clear what rational guided the authors to synthesize such compounds. If those compounds do "something" to neurons, how have the authors started targeting the structure of such molecules, are charges needed, should they be lipophilic; should they be redox-active? Easy to determine, the metal-centered reduction potential for $Mn^{III}P/Mn^{II}P$ redox couple would tell immediately whether those compounds are able to interfere with redox-based systems *in vivo.* Is the choice of compounds at the level of combinatorial chemistry?

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Addressing the Elemental Analysis

Elemental analyses were given as Supplementary Material, but their precision is outside of the acceptable limits (12). The difference in % of calculated and found carbon (C) and nitrogen (N) should not be more than 0.5%; the C/N ratio is also critical. The Mn porphyrins described by the authors show % difference between calculated and found carbon, reaching 5%. This should have been an immediate indicator for the authors that compounds are likely impure and not reliable for testing in biological experiments. For example, with EUK-425, no elemental analysis is shown; for EUK-423, calcd. C = 71.08% and found 67.48 (3.6% difference). With EUK-418, calcd. C = 66.93 and found = 65.15 (1.8% difference); further, as the percentage of calcd. and found nitrogen is nearly identical, the C/N ratio is, thus, very far off the calculated one. With EUK-452, calcd. C = 68.27 and found = 63.38% (4.9% difference). We routinely do not consider such compounds good enough for publishing and for use in *in vitro* and *in vivo* studies. We have had bad experiences with both small and large impurities in some Mn porphyrin preparations of various sources, some of them to such an extent that they obscured the identity or properties of the target compounds. Such impurities also compromised the conclusions of the *in vitro* and *in vivo* assays. Examples of such effects of impurities on obscuring the nature and properties of the samples with confusing biological outcomes have been well documented, addressed, and discussed elsewhere (2, 9–11).

Addressing NBT Assay

We have employed the cyt *c* assay with a large variety of Mn porphyrins that are cationic, anionic, and neutral at the periphery (bearing a single positive charge on Mn site) (3, 4, 7). We also analyzed water-soluble and insoluble compounds, without problems. The cyt *c* assay has been free of artifacts in large majority of cases and worked with the widely different porphyrins we had in our hands. The validity of cyt *c* assay has been checked by stopped-flow and pulse radiolysis. The problems that the authors experienced with cyt *c* might have been due to impurities (as authors' elemental analyses data suggest). Halliwell and Gutteridge (6) and Liochev and Fridovich (8) discussed the inadequacy of NBT assay for detecting superoxide dismuting ability and explained the artifact that could be produced (see details below). But regardless of the type of assay used, and based on the data the authors themselves presented in Table 1 (12), the Mn porphyrins synthesized by Rosenthal *et al.* (12) have essentially no SOD-like activity (see above). There is no significant electrostatic attraction of $O_2^{\bullet -}$ by such Mn porphyrins that could have significantly enhanced k_{cat} (3). Further, the reduction potentials for $Mn^{III}P/Mn^{II}P$ redox couple have not been determined to reveal whether there is any thermodynamic basis for the reaction of $Mn^{III}P$ or $Mn^{II}P$ with $O_2^{\bullet -}$ (3).

Commenting on Trova *et al.*

Using the paper by Trova *et al.* (14) as a reference can only be justified if the claims reported therein are correct. The discussion below addresses in details the problems we see with that paper. Trova *et al.* synthesized 33 Mn porphyrins. The SOD-like activity expressed in terms of specific SOD activity is shown for only 6 (1 of them is estimate) of 33 compounds. We are not accounting for eight single-digit values (of which three are estimates) as they are meaningless (see below the explanation). We recalculated the k_{cat} values for the catalysis of superoxide dismutation with Mn porphyrin using the units of SOD activity from Table 4 (14) and compared with the units of SOD-like activity of Mn(III) *meta*, *para*, and *ortho N*-methylpyridylporphyrins (1). All compounds are analyzed by the cyt *c* method. At best, the calculated k_{cat} values given by Trova *et al.* equal the k_{cat} of superoxide self-dismutation. For example, the specific activity of MnTM-2-PyP^{5+} is 8500 (1), whereas only one compound of Trova *et al.* has specific activity above 100 (129), which gives k_{cat} ($8.7\times10^5\ M^{-1}\ s^{-1}$) that is just ~70% higher than the k_{cat} for $O_2^{\bullet -}$ self-dismutation ($\sim5\times10^5\ M^{-1}\ s^{-1}$, pH 7) (14). Worth noting is that the SOD enzyme increases the $O_2^{\bullet -}$ self-dismutation by ~3.5 orders of magnitude ($k_{cat}\sim10^9\ M^{-1}\ s^{-1}$) (3, 4). A few of the Mn porphyrins reported (14) have specific SOD activity below 100, and either many have essentially no activity (single-digit values would give k_{cat} in the order of $10^3\ M^{-1}\ s^{-1}$ and ~2 orders of magnitude lower than O_2^- self-dismutation) or SOD-like activity has not been determined at all. Our calculation assumes that all compounds have similar molecular weight. No $Mn^{III}P/Mn^{II}P$ reduction potential was provided in the paper to show whether there is any thermodynamic basis for SOD-like activity. Claiming those compounds as SOD mimics in the title "*Superoxide dismutase mimics. Part II . . .*" is another example of misleading the readers. Further, and very importantly, the analytical data for the compounds clearly suggest that they are impure. For example, of 33 Mn complexes synthesized, elemental analysis was provided for 2 porphyrins only. This raises immediate doubts about the purity of other 31 compounds. No mass spectra were shown either; providing the information on the molecular ion observed in spectrum says nothing about the intensity of that ion and the presence of other ions. Melting point, a very strong indicator of purity, was reported for most compounds as being >300°C, which is meaningless. We understand that most porphyrins do not melt without decomposition below 300°C, and thus, melting point is not particularly an appropriate characterization technique for these compounds. In some cases, melting point data in the paper by Trova *et al.* (14) spread over several degrees (200–210), which also suggests an impure compound. The impurities of those compounds might have easily interfered with SOD assay. An unknown mechanism was claimed for interference of the compounds with the cyt *c* assay (14). In some cases, the ability of Mn porphyrin to reduce cyt *c* was mentioned as the reason for the failure to determine the SOD-like activity. One can hardly imagine that (pure) manganese porphyrins of the given structures could reduce cyt *c*. This would mean that Mn site undergoes oxidation to $O=Mn^{IV}$ or $O=Mn^{V}$ porphyrin. To the best of our present knowledge, only highly oxidizing species such as peroxides, peroxynitrite, or other strong oxygen donors (but not cyt *c*) can oxidize $Mn^{III}P$ to $O=Mn^{IV}$ or $O=Mn^{V}$ porphyrin. Years ago we were questioned by reviewers to justify whether the cyt *c* assay provides identical data as the direct chemical methods, stopped flow technique, or pulse radiolysis in order to claim the accuracy of our data. We provided such justification of cyt *c* method using pulse radiolysis (13) and the others did so using stopped-flow technique [reviewed in (3)].

NBT Assay

NBT can be reduced to $NBT^{\bullet -}$ radical (eq. [1]) (which disproportionates to blue formazan [2]) by superoxide and numerous

enzymes as well as by nonenzymatic reductants (6, 8). Thus, the assay is not superoxide specific (6, 8). Once reduced, $NBT^{\bullet -}$ radical can oxidize back with oxygen-producing superoxide and regenerating NBT (eq. [3]), which would falsely suggest that NBT reduction is inhibited. NBT assay is therefore prone to artifacts. The cyt *c* cannot auto-oxidize at any significant level.

$$NBT + e^- \rightarrow NBT^{\bullet -} \quad [1]$$

Disproportionation of $NBT^{\bullet -}$ (eq. [2]) results in formazan production, which is measured spectrophotometrically.

$$2NBT^{\bullet -} \Leftrightarrow NBT + NBT^{2-} (\text{ blue formazan}) \quad [2]$$

$NBT^{\bullet -}$ auto-oxidizes (eq. [3]), regenerating NBT and producing superoxide.

$$NBT^{\bullet -} + O_2 \Leftrightarrow NBT + O_2^{\bullet -} \quad [3]$$

$$2\ O_2^{\bullet -} + SOD + 2\ H^+ \Leftrightarrow O_2 + H_2O_2 \quad [4]$$

SOD enzyme or SOD mimic eliminates $O_2^{\bullet -}$ by eq. [4], pushing equilibrium [3] to more superoxide production, whereby regenerating NBT. Such data may be wrongly interpreted as inhibition of NBT reduction by SOD mimic.

All being said, based on the often missing acceptable analysis for most of the drugs synthesized, and on our personal experience, we believe strongly that caution needs to be exercised with compounds intended for clinical development and two questions must always be kept in mind: (i) what is the purity of the compounds, and (ii) what data do we have about the pure compounds in a simple aqueous system to correctly hypothesize their modes of action *in vivo*. There is a danger that increased pressure for funding may contribute to the abundance of misleading data. In a very mild form, it was our intention to warn those who will read ours and other articles to exercise caution when deciding which compound to choose for costly and timely animal studies and to aid on how to interpret the data obtained.

References

1. Batinić-Haberle I, Benov L, Spasojević I, and Fridovich I. The *ortho* effect makes manganese (III) *meso*-tetrakis(*N*-methylpyridinium-2-yl)Porphyrin (MnTM-2-PyP) a powerful and potentially useful superoxide dismutase mimic. *J Biol Chem* 273: 24521–24528, 1998.
2. Batinić-Haberle I, Cuzzocrea S, Rebouças JS, Ferrer-Sueta G, Emanuela Mazzon E, Di Paola R, Radi R, Spasojević I, Benov L, and Salvemini D. Pure MnTBAP selectively scavenges peroxynitrite over superoxide: comparison of pure and commercial MnTBAP samples to MnTE-2-PyP in two different models of oxidative stress injuries, SOD-specific *E. coli* model and carrageenan-induced pleurisy. *Free Radic Biol Med* 46: 192–201, 2009.
3. Batinic-Haberle I, Reboucas JS, and Spasojevic I. Superoxide dismutase mimics: chemistry, pharmacology and therapeutic potential. *Antioxid Redox Signal* 13: 877–918, 2010.
4. DeFreitas-Silva G, Rebouças JS, Spasojević I, Benov L, Idemori YM, and Batinić-Haberle I. SOD-like activity of Mn(II) *β*-octabromo-meso-tetrakis(*N*-methylpyridinium-3-yl) porphyrin equals that of the enzyme itself. *Arch Biochem Biophys* 477: 105–112, 2008.
5. Doctrow S, Huffman K, Bucay-Marcus C, Tocco G, Malfroy E, Adinolfi CA, Kruk H, Baker K, Lazarowych N, Mascarenhas J, and Malfroy B. Salen-manganese complexes as catalytic scavengers of hydrogen peroxide and cytoprotective agents: Structure-activity relationship. *J Med Chem* 45: 4549–4558, 2002.
6. Halliwell B and Gutteridge JMC. (Eds). Chemistry of free radicals and related reactive species. In: *Free Radical Biology Medicine*, 4th Edition, Oxford, Oxford University Press, 2007, Chapter 2.
7. Lahaye D, Muthukumaran K, Hung CH, Gryko D, Rebouças JS, Spasojević I, Batinić-Haberle I, and Lindsey JS. Design and synthesis of manganese porphyrins with tailored lipophilicity: investigation of redox properties and superoxide dismutase activity. *Bioorg Med Chem* 15: 7066–7086, 2007.
8. Liochev S and Fridovich I. Superoxide from glucose oxidase or from nitroblue tetrazolium. *Arch Biochem Biophys* 318: 408–410, 1995.
9. Pollard JM, Rebouças JS, Durazo A, Kos I, Fike F, Panni M, Gralla EB, Valentine JS, Batinić-Haberle I, and Gatti RA. Radioprotective effects of manganese-containing superoxide dismutase mimics on ataxia telangiectasia cells. *Free Radic Biol Med* 47: 250–260, 2009.
10. Rebouças JS, Spasojević I, and Batinić-Haberle I. Pure manganese(III) 5,10,15,20-tetrakis(4-benzoic acid)porphyrin (MnTBAP) is not a superoxide dismutase mimic in aqueous systems: a case of structure-activity relationship as a watchdog mechanism in experimental therapeutics and biology. *J Inorg Biol Chem* 13: 289–302, 2008.
11. Rebouças JS, Spasojević I, and Batinić-Haberle I. Quality of Mn-porphyrin-based SOD mimics and peroxynitrite scavengers for preclinical mechanistic/therapeutic purposes. *J Pharm Biomed Anal* 48, 1046–1049, 2008.
12. Rosenthal RA, Huffman KD, Fisette LW, Damphousse CA, Callaway WB, Malfroy B, and Doctrow SR. Orally available Mn porphyrins with superoxide dismutase and catalase activity. *J Biol Inorg Chem* 14: 979–991, 2009.
13. Spasojević I, Batinić-Haberle I, Stevens RD, Hambright P, Thorpe AN, Grodkowski J, Neta P, and Fridovich I. Manganese(III) biliverdin IX dimethylester. A powerful catalytic scavenger of superoxide employing the Mn(III)/Mn(II) redox couple *Inorg Chem* 40: 726–739, 2001.
14. Trova MP, Gauuan PJ, Pechulis AD, Bubb SM, Bocckino SB, Crapo JD, and Day BJ. Superoxide dismutase mimetics. Part 2: Synthesis and structure-activity relationship of glyoxylate- and glyoxamide-derived metalloporphyrins. *Bioorg Med Chem* 11: 2695–2707, 2003.

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Abbreviations Used

NBT = nitroblue tetrazolium
SOD = superoxide dismutase

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1. Ivana Ivanovi#-Burmazovi#, Milos# r. Filipovi#Reactivity of manganese superoxide dismutase mimics toward superoxide and nitric oxide **64**, 53-95. [CrossRef]