Preclinical safety assessment of the MRI contrast agent MnDPDP serendipitously revealed SOD activity, a potentially useful property in the treatment of several pathological conditions. MnDPDP and its successor $Ca_4Mn(DPDP)_5$ have proceeded into clinical Phase II studies.

Calmangafodipir [$Ca_4Mn(DPDP)_5$], mangafodipir (MnDPDP) and MnPLED with special reference to their SOD mimetic and therapeutic properties

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Reactive oxygen species (ROS) and reactive nitrogen species (RNS) participate in pathological tissue damage. Mitochondrial manganese superoxide dismutase (MnSOD) normally keeps ROS and RNS in check. During development of mangafodipir (MnDPDP) as a magnetic resonance imaging (MRI) contrast agent, it was discovered that MnDPDP and its metabolite manganese pyridoxyl ethyldiamine (MnPLED) possessed SOD mimetic activity. MnDPDP has been tested as a chemotherapy adjunct in cancer patients and as an adjunct to percutaneous coronary intervention in patients with myocardial infarctions, with promising results. Whereas MRI contrast depends on release of Mn^{2+}, the SOD mimetic activity depends on Mn^{2+} that remains bound to DPDP or PLED. Calmangafodipir [$Ca_4Mn(DPDP)_5$] is stabilized with respect to Mn^{2+} and has superior therapeutic activity. $Ca_4Mn(DPDP)_5$ is presently being explored as a chemotherapy adjunct in a clinical multicenter Phase II study in patients with metastatic colorectal cancer.

Introduction

Aerobic organisms exist in a catch-22 situation. Oxygen sustains them, but it also poisons them via reactive oxygen species (ROS) produced during respiration. Yet, as recently as 45 years ago, oxygen-derived free radicals were thought to be too reactive and unselective to occur in biological systems. That view changed dramatically when Irwin Fridovich and Joe McCord discovered superoxide dismutase (SOD) [1], an essential cellular defense against ROS. Furthermore, all free radicals are not bad. That is particularly true for nitric oxide (NO). The biological reaction of NO with superoxide to form the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$) was initially proposed during the studies that characterized the chemical nature of the endothelium-derived relaxing factor (EDRF) [2] as being NO [3–6]. Until 2005, PubMed listed close to 50,000 papers published on SOD [7]. Few other biochemists have had such an impact on the field of biochemistry [8].

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Jan Olof G. Karlsson has a PhD from Linköping University. Between 1990 and 1992 he held a position as a research fellow at the Swedish Medical Research Council. Thereafter, he worked as a senior scientist for Nycomed Imaging, which later became GE Healthcare. Between 2007 and 2013 he worked for PledPharma in Sweden. At present he works as scientific consultant for Karlsson–Tunér Invest in Norway. Dr Karlsson has published 75 scientific papers and is inventor of nine issued patent families. During the early 1990s, Dr Karlsson was involved in research showing that the MRI contrast agent mangafodipir possesses SOD-mimetic activity, a potentially useful property in the treatment of several pathological conditions.

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The route of O_2 reduction to H_2O in the mitochondrion occurs by a series of univalent electron transfers. Hence, intermediates will be encountered on this univalent pathway and these are O_2^-, hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). It is these intermediates that are primarily responsible for the toxicity of O_2 [9]. Oxidative stress can be defined as a situation where the production of ROS and RNS out-compete the endogenous cellular defense. There are multiple cellular sources of ROS. The most significant ones are the electron transport complexes I and III in the mitochondria, cytochrome P450 enzymes and NADPH oxidase. ROS production by each of these sources can be triggered by cytokines, inflammation, viral proteins, chemotherapy drugs, radiation, ischemia–reperfusion and iron and copper overload. Importantly, these processes initially generate O_2^- which is sequentially reduced to form H_2O_2, OH and, ultimately, H_2O. Under conditions of high oxidative stress and consequently high production of O_2^-, these reactive intermediates interact with other molecules to form secondary harmful ROS, such as lipid peroxidation products and nitrated proteins [10–13].

In recent years, it has become evident that ROS are not merely toxic byproducts but also serve important regulatory roles in numerous signaling pathways. Recent findings, for example, suggest that the intracellular redox environment fluctuates during the cell cycle, shifting toward a more oxidized state during mitosis [14,15]. After cell division, MnSOD activity increases in the G1 phase. The periodic fluctuation in MnSOD activity during the cell cycle inversely correlates with cellular O_2^-, as well as glucose and oxygen consumption [16]. On the basis of an inverse correlation between MnSOD activity and glycolysis during the cell cycle, it is proposed that MnSOD is a central molecular player in the Warburg effect (i.e. the increase in glycolysis followed by lactic acid fermentation as often seen in cancer cells) [17]. In general, loss of MnSOD activity results in aberrant proliferation [14,18,19].

Knowledge about cellular oxidative stress has provided us with important clues to treat and prevent disease. PledPharma and a few other companies look to exploit this knowledge through developing low molecular weight SOD mimetics. During development of mangafodipir (MnDPDP) as a magnetic resonance imaging (MRI) contrast agent it was discovered that MnDPDP and its metabolite manganese pyridoxyl ethyldiamine (MnPLED) possessed SOD mimetic activity. Preclinical studies have pointed out three medical fields where MnPLED derivatives have shown promising results: (i) cancer treatment; (ii) acute myocardial infarction (AMI) with percutaneous coronary intervention (PCI); and (iii) paracetamol (acetaminophen) intoxication. PledPharma has proceeded into clinical development with MnDPDP and its successor calmangafodipir [$Ca_4Mn(DPDP)_5$] in the two first fields.

MnSOD

The manganese form of the superoxide dismutase (MnSOD) is a homotetrameric protein found in the matrix of mitochondria, which is closely related, in terms of sequence, to the equivalent prokaryotic enzyme [9]. It is, however, unrelated to CuZnSOD in the surrounding cytosol. MnSOD and CuZnSOD (cytosolic as well as extracellular) are the most crucial enzymes in the cellular defense against oxidative stress. They catalyze dismutation of superoxide (O_2^-) to H_2O_2 and molecular oxygen [9]. Knockout mice that are unable to synthesize MnSOD are severely affected and live only a few days after birth. These mice express dilated cardiomyopathy that is a hallmark of chronic heart failure [20] and neurodegeneration [21]. The consequences of knocking out the cytosolic CuZnSOD are notable but much less dramatic [22,23]. Superoxide plays a central part in cellular production of the devastating hydroxyl radical (OH) by its high ability to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). As mentioned above, O_2^- readily reacts with NO to form toxic $ONOO^-$, revealing the 'ugly' side of NO [11].

No aerobic organism survives without having the ability to dismutate O_2^-. However, *Lactobacillus plantarum* and related lactic acid bacteria lack SOD enzymes. In these organisms Mn^{2+} forms complexes with various organic acids, including phosphoric acid, lactic acid and carbonic acid [24–26]. The complexed Mn^{2+} is first oxidized by O_2^- to Mn^{3+}. The Mn^{3+} thus formed is subsequently reduced to Mn^{2+} by a second O_2^-, making the reaction a true metal-catalyzed dismutation like that catalyzed by the SOD enzymes. Free Mn^{2+} ions in non-complexing buffers, by contrast, are poorly reactive with O_2^-. These Mn complexes have a high catalytic activity and serve the same function as SOD. However, in multicellular organisms the importance of SOD proteins is consistent with a selection process favoring organisms that elaborate a means of localizing transition metal catalyst for superoxide dismutation to parts of the cell where there is a high need for such dismutation. Results from myocardial ischemia–reperfusion in anesthetized pigs inevitably show that the intact MnPLED, but not manganese per se, protects against oxidative stress, seen as a profound reduction in infarct size [27].

The biological half-life of NO is prolonged by SOD that eliminates O_2^- at diffusion-controlled rates [9]. The rate constant of the reaction of O_2^- with NO is approximately six-times higher than that with SOD, and therefore NO could, under certain conditions, outcompete SOD for O_2^- [10–12]. During normal conditions, 1–5% of the total cellular oxygen consumption is estimated to leak out from the electron transport chain as O_2^- [11]. The concentration of O_2^- is however kept remarkably low by SODs, in particular by MnSOD.

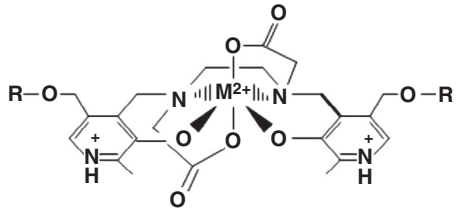
NO has erroneously been described as extremely short-lived and reactive. However, this is not true under normal cellular conditions where NO is not more reactive than molecular oxygen [11]. Otherwise, NO would not be able to fulfill its many important cellular signaling tasks [28]. During inflammatory processes, however, owing to induction of inducible nitric oxide synthase (iNOS), the NO concentration can increase to levels high enough to outcompete the endogenous MnSOD and CuZnSOD activities, revealing the ugly side of NO, namely $ONOO^-$ [11]. By nitrating tyrosine-34 of the human MnSOD, $ONOO^-$ irreversibly inactivates enzymatic activity, and hence causes a vicious circle that enhances the production of $ONOO^-$ further. Nitrated proteins are found in several pathological conditions, including inflammatory process [12], chronic rejection of human renal allografts [29], tumor-associated immunosuppression [30] and paracetamol (acetaminophen) intoxication [31]. Importantly, $ONOO^-$ is not capable of directly incorporating nitrate to the tyrosine residue but requires the contribution of a transition-metal-dependent (probably Fe^{2+}/Fe^{3+}) radical pathway [11,12].

SOD mimetics

Kensler *et al.* described anticarcinogenic activity of the first generation of a CuSOD mimetic in 1983 [32]. They were followed by

MnSOD mimetics, particularly of the so-called porphyrin, salen and cyclic polyamine types [33,34], as well as MnPLED derivatives [35–37]. During the development of the MnPLED derivative MnDPDP (Fig. 1) as an MRI contrast agent at the beginning of the 1990s, clinical studies showed that rapid intravenous injection into patients caused cardiovascular effects and facial flushing [38,39]. At that time, there was suspicion that these effects might have been caused by a Ca-channel 'blocking' effect of Mn^{2+}. In 1991 Matsunaga and Furchgott [40] demonstrated that low (30–100 μM) concentrations of Mn^{2+}, as well as Cu^{2+}, potentiated the vasodilator effect of photogenerated NO in endothelium-free rabbit aortas and suggested that the potentiating effects were due to O_2^- scavenging properties of these transition metals. On the basis of this finding, pharmacological studies were conducted in isolated bovine mesenteric arteries (BMA) with and without endothelial cells demonstrating that MnDPDP protected endothelium-derived NO from being destroyed by O_2^- [35,36]. These results further suggested that this effect was the result of a SOD-mimetic activity of MnDPDP (Fig. 2). In addition, a series of experiments in isolated rat hearts demonstrated that clinically relevant concentrations of MnDPDP did not cause any Ca-channel blocking effect [36]. Experiments in conscious [36] and anesthetized [41] dogs characterized MnDPDP as a cardiovascular safe drug.

In line with the results of Matsunaga and Furchgott, Asplund *et al.* [35] showed similar effects to MnDPDP for $MnCl_2$. The SOD-mimetic activity of $MnCl_2$ was presumably caused by Mn^{2+} complexed to phosphoric acid and carbonic acid of the used Krebs buffer, as described above. However, this effect of $MnCl_2$ is not expected to occur under *in vivo* conditions. The suggestion that MnDPDP possessed SOD activity was confirmed later by electron spin resonance (ESR) spectroscopy [37] (Fig. 3). It was furthermore shown that the metabolite MnPLED possessed SOD mimetic activity, whereas ZnPLED did not. It was realized that this property of MnDPDP could be employed in therapy. In 2003, the SOD mimetic activity of MnDPDP was confirmed by a photometric nitroblue tetrazolium reduction method by Bedda *et al.* [42]. They also demonstrated glutathione reductase as well as catalase activity



MnDPDP	**R = phosphate; M^{2+} = Mn^{2+}**
MnPLED	**R = H; M^{2+} = Mn^{2+}**
$Ca_4Mn(DPDP)_5$	**R = phosphate; M^{2+} = 1Mn^{2+}/4Ca^{2+}**

Drug Discovery Today

FIGURE 1

Chemical structure of manganese dipyridoxyl diphosphate (MnDPDP; generic name mangafodipir), manganese pyridoxyl ethyldiamine (MnPLED) and tetracalcium monomanganese penta(dipyridoxyl diphosphate) [$Ca_4Mn(DPDP)_5$; calmangafodipir].

of MnDPDP. However, in these experiments they used a ready-to-use contrast formulation of MnDPDP, which in addition to 10 mM MnDPDP also contained 6 mM ascorbic acid. Unfortunately, no ascorbic acid control was included, which makes these results somewhat questionable.

Another important property of MnPLED derivatives is their high affinity for iron. The ^{10}log formation constant [$^{10}\log(K_{ML})$] for Fe^{3+} and PLED is 36.88 [43], making PLED derivatives highly efficacious inhibitors of the Fenton reaction [44]. The formation constant for PLED is in fact six orders of magnitude higher than that of the clinically used deferoxamine [45]. Hence, MnDPDP, and particularly MnPLED, through their combined SOD mimetic activities and iron-binding properties, effectively inhibit iron-driven production of $ONOO^-$ as well as that of OH.

After intravenous administration, MnDPDP is rapidly dephosphorylated by alkaline phosphatases into the much more lipophilic MnPLED [46]. Indirect evidence from *in vivo* studies in pigs [27] and mice [46] suggest that MnPLED (molecular weight ~520 Da) but not its somewhat larger and more hydrophilic 'mother substance' MnDPDP (molecular weight ~680 Da) is taken up intracellularly – which probably is a prerequisite for the therapeutic activity. For example, the fact that MnPLED, but not MnDPDP, protects against anthracycline cardiotoxicity when added directly into an organ bath containing an electrically paced mouse atrium suggests intracellular uptake of MnPLED. Interestingly, in MnSOD-deficient *Cryptococcus neoformans* (an encapsulated yeast), the SOD mimetic Mn salen exerted antioxidant activity, but not $MnCl_2$ and none of a series of cationic and highly charged manganese porphyrins [47]. These results suggest that efficacy depends on intracellular uptake of the intact manganese complex. When it comes to MnDPDP, in addition to the dephosphorylation process a parallel dose-dependent transmetallation occurs where Mn^{2+} is displaced by endogenous Zn^{2+}. Zinc has a ~1000-times higher affinity than Mn^{2+} for DPDP or its metabolites [43]. When an MRI dose (i.e. 5–10 μmol/kg) is injected into humans or rats about 80% of the Mn^{2+} is released from DPDP or its dephosphorylated counterparts, and only about 20% stays bound to the chelator. Release of paramagnetic Mn^{2+} is in fact a prerequisite for the diagnostic MRI properties of MnDPDP [36,48], whereas the *in vivo* SOD mimetic activity depends on the intact manganese complex [37].

Manganese is an essential as well as potentially neurotoxic metal [49]. It has been known for many years that, under conditions of chronic exposure to manganese, a syndrome of extrapyramidal dysfunction similar to Parkinson's syndrome frequently occurs. The neurological symptoms correlate with accumulation of manganese in the basal ganglia, seen as hyperintensity on a T1-weighted MRI [49,50]. For MRI purposes and for occasional therapeutic use, dissociation of Mn^{2+} from MnDPDP does not result in an increase in T1-weighted MRI signal of the basal ganglia in humans [51]. However, for more frequent use, as in therapeutic use, accumulated manganese toxicity can represent a problem.

A considerable part of Mn^{2+} release from MnDPDP is governed by the presence of a limited amount of free or loosely bound plasma Zn^{2+} [46]. Replacement of 80% of $Mn^{2+}$with Ca^{2+}, generating a compound known as calmangafodipir [$Ca_4Mn(DPDP)_5$], is enough for binding a considerable amount of the readily available

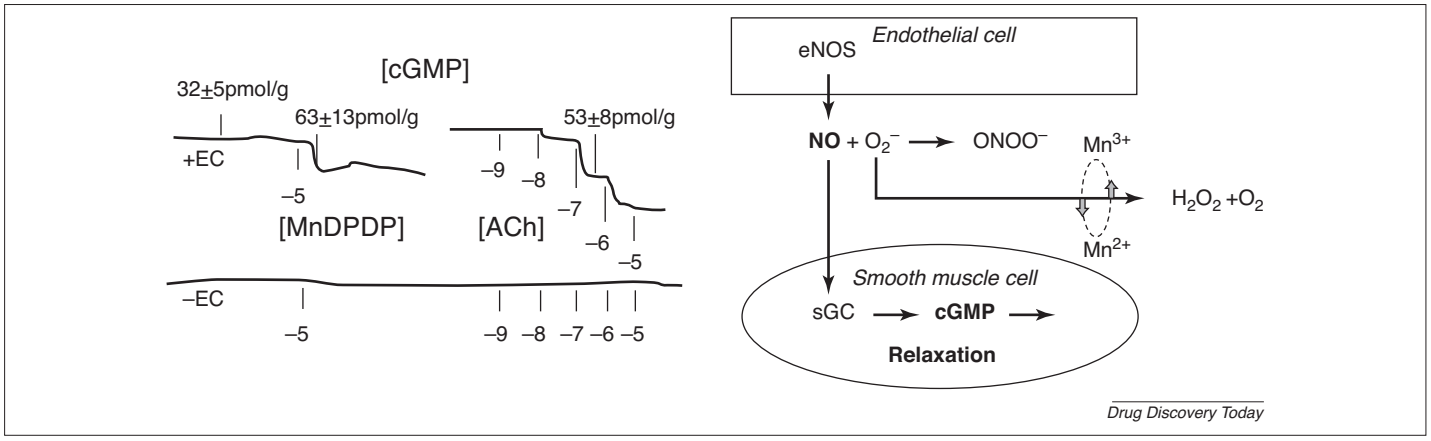


FIGURE 2

The vasodilator effects of manganese dipyridoxyl diphosphate (MnDPDP) and acetylcholine (ACh) and the cyclic (c)GMP content in the isolated bovine mesenteric artery (BMA) is shown to the left. MnDPDP or ACh was added to phenylephrine-contracted BMA rings. Tension was measured using isometric gauge transducers and polygraphs. The cGMP content of pre-contracted arteries was determined in the absence and presence of MnDPDP or ACh. The proposed mechanism is illustrated in the right part of the figure [35,36]. Vasodilator nitric oxide (NO) is produced by endothelial NO synthase (eNOS) in the innermost (endothelial) cellular layer of the artery, and NO stimulates guanylate cyclase (sGC) in smooth muscle cells to produce increasing amounts of cGMP which causes relaxation and hence vasodilation. Normally, a large fraction of NO reacts with superoxide (O_2^-), forming peroxynitrite ($ONOO^-$). MnDPDP preserves vasodilator NO by dismutating O_2^-. ACh causes vasodilation by increasing endothelial NO production per se by a receptor-mediated process. Neither MnDPDP nor ACh causes vasodilation in the absence of endothelial cells (EC).

plasma Zn^{2+}, resulting in considerably less Mn^{2+} release and retention in the brain and other organs [52]. Zn^{2+} has roughly 1000-times greater affinity than Mn^{2+} for DPDP [52], and replacement of Mn^{2+} with more loosely bound Ca^{2+} reduces the amount of Mn^{2+} released after injection. Calcium has approximately 10^6 times lower affinity than Mn^{2+} for DPDP [52]. Karlsson *et al.* [52] reported that replacement of 80% of the Mn^{2+} with Ca^{2+} more than doubles the *in vivo* stability of the Mn complex, and results in significantly (~40%) less retention of Mn in the brain compared with MnDPDP (at equimolar manganese doses) after repeated dosing (39 doses) at more than 30-times the assumed clinical dose (per dose) in rats. At equivalent Mn^{2+} doses, $Ca_4Mn(DPDP)_5$ is significantly more efficacious than MnDPDP to protect BALB/c mice against myelosuppressive effects of the chemotherapy drug oxaliplatin (Fig. 4).

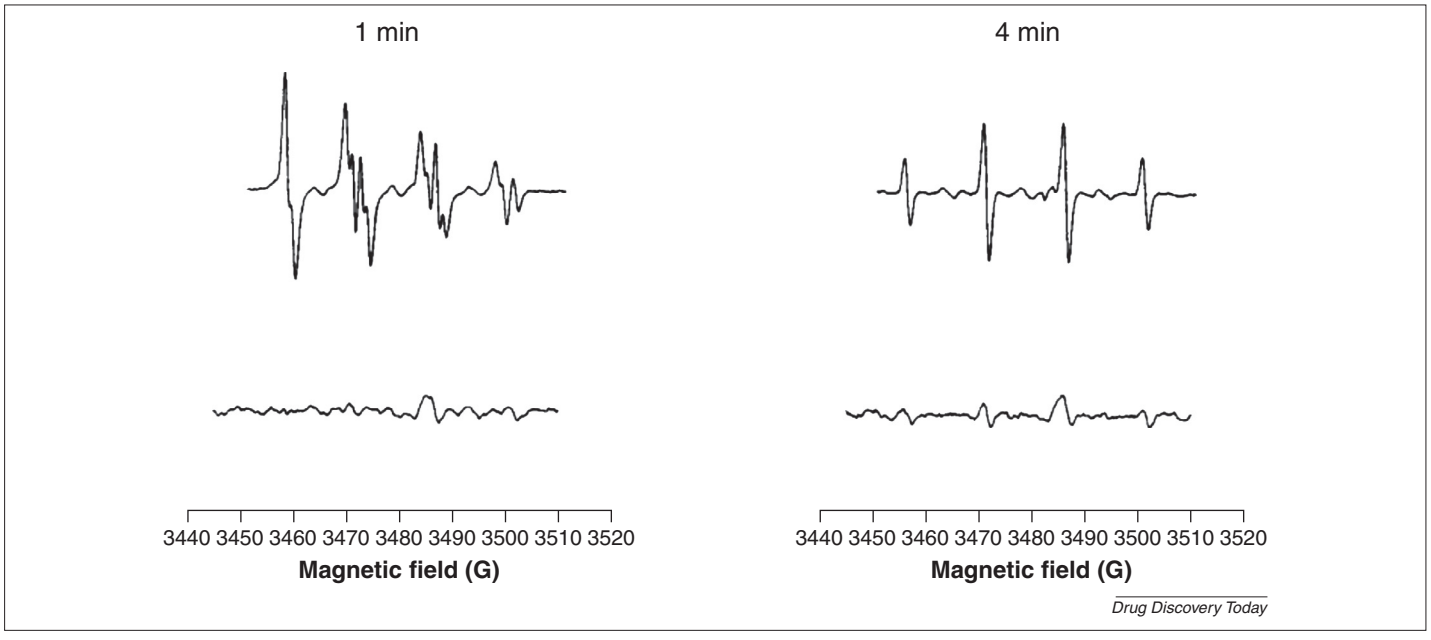


FIGURE 3

Electron spin resonance (ESR) spectra: O_2^- was generated in the xanthine oxidase reaction in the presence of the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). Control reaction (upper panel) revealed that the superoxide adduct DMPO-OOH evolved rapidly (within 1 min) toward the hydroxyl adduct DMPO-OH (4 min). 10 μM manganese dipyridoxyl diphosphate (MnDPDP; lower panel) markedly reduced the signal intensity indicating superoxide dismutase (SOD) mimetic activity [37].

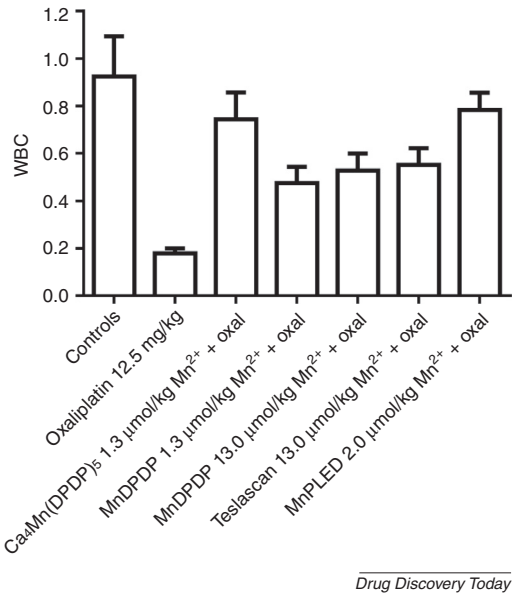


FIGURE 4

Myeloprotective effects of tetracalcium monomanganese penta(dipyridoxyl diphosphate) [$Ca_4Mn(DPDP)_5$], manganese dipyridoxyl diphosphate (MnDPDP), Teslascan (the commercial formulation of MnDPDP containing 6 mM ascorbic acid) and MnPLED (the MnDPDP metabolite), with regard to white blood cells (WBC) [52]. BALB/c mice were treated once i.p. with 12.5 mg/kg oxaliplatin. One day before (baseline) as well as 6 days after oxaliplatin treatment, blood samples were taken and analyzed for the content of WBC, and other blood cells. Thirty minutes before and 24 hours after oxaliplatin administration, mice received saline, 6.5 μmol/kg $Ca_4Mn(DPDP)_5$ (corresponding to 1.3 μmol/kg Mn^{2+}), 1.3 and 13.0 μmol/kg MnDPDP (corresponding to 1.3 and 13 μmol/kg Mn^{2+}), 13.0 μmol/kg Teslascan (corresponding to 13 μmol/kg Mn^{2+}) or 2.0 μmol/kg MnPLED (corresponding to 2 μmol/kg Mn^{2+}) i.v.; a control group received vehicle (5% glucose) instead of oxaliplatin and saline instead of test substance. The results are presented as relative differences in cell counts between baseline and day six in each treatment group. Results expressed as mean ± SEM ($n = 5$ in each group).

Therapeutic potential of MnPLED derivatives

Cancer treatment

Conventional chemotherapy and radiotherapy play important parts in palliation and cure of patients with cancer. However, the use of chemotherapy and radiotherapy is hampered by dose-limiting toxicity (DLT). To increase the therapeutic index of chemotherapy and radiation therapy, researchers have tried selectively to increase the sensitivity of tumor cells for a given treatment and, at the same time, protect normal cells. Rescue of normal cells through the use of cytoprotective agents could enable a much more intense therapy. Importantly, to be clinically useful as an adjunct to chemotherapy, a cytoprotectant should protect normal tissue from chemotherapy-induced toxicity but not tumor tissue (at least not to any great extent).

ROS are generated during chemotherapy with a series of structurally dissimilar anticancer agents [53]. The resulting increase in oxidative stress is believed to be a main cause of toxicity to normal cells, in particular to rapidly dividing cells, such as myeloid and mucosal cells. So far, two antioxidants (i.e. amifostine and dexrazoxane) have reached the clinic. However, their use has been restricted. When it comes to dexrazoxane, this restriction has mainly been due to suspicion of negative interference with the anticancer activity of chemotherapy. In addition to its cardioprotective use, dexrazoxane has been approved as treatment of extravasation caused by intravenous anthracycline chemotherapy [54] in Europe and the USA. Amifostine was mainly developed as a radioprotectant but also achieved approval for use in protection of the kidneys from harmful effects of cisplatin in patients with ovarian cancer. The clinical use of amifostine has been restricted as a result of hypotension and emesis. Furthermore, concerns about tumor protection have caused controversy regarding the appropriate use of amifostine [55].

The anthracycline doxorubicin (Adriamycin®) is one of the best agents for treating human hematological malignancies and solid tumors. Its use is, however, hampered by severe cardiotoxicity, resulting in chronic heart failure [44]. There is substantial evidence that O_2^- [56] and iron [57] play key parts in the underlying deleterious effects. Despite decades of research and testing of thousands of potentially protective agents, only one drug has been approved for clinical use, namely the iron chelator dexrazoxane in metastatic breast cancer treatment [56]. Interestingly, this drug is not an outcome of any sophisticated and rational drug design. Its cardioprotective activity was discovered accidentally during preclinical testing. However, dexrazoxane is not indicated at the beginning of doxorubicin therapy because of suspicion that it might reduce the anticancer effect [58].

An *in vitro* mouse model was employed by Towart *et al.* [59] to test the cardioprotective potential of MnDPDP and other compounds against doxorubicin toxicity. The toxicity is seen as a decrease in contractility of an electrically paced mouse left atrium. MnDPDP did not protect when added directly into the organ bath. However, after having been intravenously injected 30 min before the mouse was killed and the left atrium was dissected out; 1–10 μmol/kg MnDPDP was cardioprotective. MnDPDP was shown to be about 100-times more efficacious than dexrazoxane (i.e. 1 μmol/kg MnDPDP gave similar protection to that of 93 μmol/kg dexrazoxane). Most importantly, the fully dephosphorylated metabolite of MnDPDP, MnPLED, was about 100-times more efficacious than MnDPDP (unpublished data). Furthermore, when added directly into the organ bath, MnPLED but not MnDPDP protected against doxorubicin cardiotoxicity [44]. Indirectly, these results suggested that MnDPDP undergoes *in vivo* dephosphorylation before it can exert the therapeutic effect.

In vivo and *in vitro* experiments, utilizing human breast cancer cells (MX-1) and ovarian cancer cells (A2780), suggested that cardioprotection takes place without interfering negatively with the anticancer activity of doxorubicin [44]. *In vitro* experiments in fact suggested the opposite (i.e. MnDPDP exerted an anticancer activity). In addition to the above-described potential use of MnPLED derivatives in anthracycline-induced cardiotoxicity, preclinical studies have demonstrated that MnDPDP protects against myelosuppressive effects of paclitaxel [60]. This protective effect of MnDPDP on peripheral white blood cells was accompanied by a parallel protection of bone marrow cells, suggesting that MnDPDP exerts its protective effect at the level of myelopoiesis. Furthermore, co-administration of MnDPDP with paclitaxel dramatically improved the survival rate of mice infected with *Staphylococcus aureus* [60], revealing a possible preventive effect on febril neutropenia, a severe DLT of many chemotherapy

regimens. Importantly, the cytoprotective effect of MnDPDP was obtained without diminishing the anticancer efficacy [61]. Contrary, MnDPDP was shown to enhance the anticancer effect of oxaliplatin toward colon cancer cells under *in vitro* and *in vivo* conditions, and it displayed a distinct anticancer effect of its own [60,61]. These authors also demonstrated that *N*-acetylcysteine (NAC) had protective effects similar to those of MnDPDP but, in opposition to MnDPDP, NAC interfered negatively with the anticancer activity of oxaliplatin and paclitaxel.

The experimental SOD mimetic, MnTBAP, displayed similar *in vivo* as well as *in vitro* anticancer activity to MnDPDP toward murine colon cancer (CT26) cells in the presence of chemotherapy [60]. Whereas MnDPDP protected human leukocytes toward oxaliplatin, 5-fluorouracil and paclitaxel, MnTBAP did not. These phenomena were paralleled with the oxidative stress status of these cells. In the presence of oxaliplatin, 5-fluorouracil or paclitaxel, MnDPDP increased oxidative stress in the CT26 cells but decreased it in the leukocytes, whereas MnTBAP increased it in both cell types. The SOD mimetic activity of MnTBAP per se has, however, been challenged and is suggested to be caused by impurities [33].

As mentioned above, $Ca_4Mn(DPDP)_5$ was significantly more efficacious than MnDPDP at equivalent manganese doses at protecting BALB/c mice against myelosuppressive effects of the chemotherapy drug oxaliplatin [52] (Fig. 4). The efficacy of $Ca_4Mn(DPDP)_5$ was similar to that of MnPLED, at manganese equivalent levels, suggesting that surplus CaDPDP at clinically relevant therapeutic doses (1–5 μmol/kg) changes the metabolism of MnDPDP in a way that considerably more MnPLED is formed. $Ca_4Mn(DPDP)_5$ has also been shown to protect against myelosuppressive effects caused by carboplatin and docetaxel (unpublished data). Similar to MnDPDP, $Ca_4Mn(DPDP)_5$ increased the antitumor effect of oxaliplatin in CT26 tumor-bearing mice [52] (Fig. 5).

The reason why MnDPDP and $Ca_4Mn(DPDP)_5$ protect nonmalignant cells but kill cancer cells is poorly understood. One plausible explanation could be that MnDPDP and $Ca_4Mn(DPDP)_5$ in combination with chemotherapy increase oxidative stress in cancer cells to a level where apoptosis is induced, whereas at the same time lowering it in normal cells. As mentioned above, Kurz *et al.* [44] showed that the *in vitro* cytotoxic activity of MnDPDP is an inherent property of chelator alone and not of the intact Mn^{2+}complex. Speculatively, it could be that cancer cells preferably take up the dissociated chelator, whereas the intact Mn^{2+} complex is preferably taken up by nonmalignant cells, somewhat in analogy with that described for amifostine [62]. Alternatively, cancer cells might be more sensitive to the cytotoxic effect of DPDP and PLED than normal cells. As described in the SOD mimetic section, DPDP and PLED are strong iron chelators. That is particularly true for PLED having a $^{10}\log(K_{ML})$ of 36.88 [43], which is six orders of magnitude higher than that of the clinically used deferoxamine [45]. Interestingly, iron chelators are now gaining momentum as promising drugs in cancer treatment [63].

Some other clues can be obtained from research aiming to find selective radioprotectants. A body of evidence demonstrates that overexpression of MnSOD protects nonmalignant tissues against radiation-induced oxidative stress and, interestingly, simultaneously makes cancer cells more susceptible to radiation [64]. Recently, as mentioned above, MnSOD has been identified as a central molecular player for the Warburg effect (i.e. increase in glycolysis followed by lactic acid fermentation as often seen in cancer cells) [17].

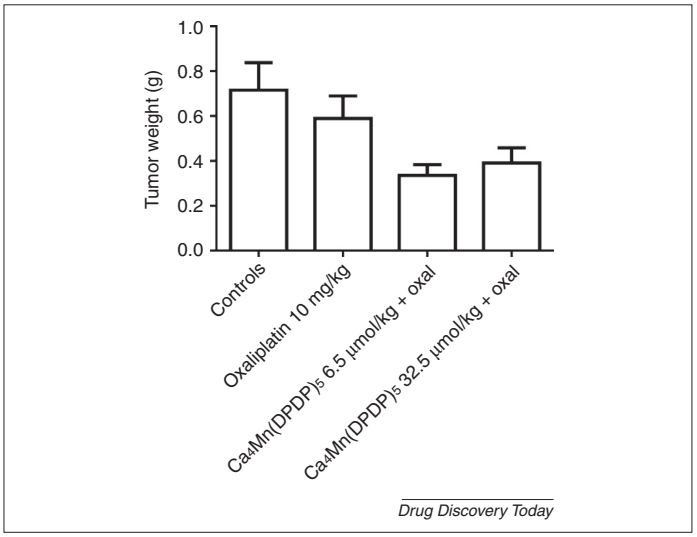


FIGURE 5

Antitumor effect of oxaliplatin in the absence and presence of $Ca_4Mn(DPDP)_5$ [52]. Briefly, syngenic BALB/c mice were injected subcutaneously in the back of the neck with 2×10^6 of CT26 (BALB/c colon cancer cells) at day zero. After 7 days (day seven) when the tumors were detectable, the tumor size was determined with a caliper and mice were grouped so that the sizes of the tumors were not statistically different by group. 10 mg/kg oxaliplatin ±6.5 or 32.5 μmol/kg $Ca_4Mn(DPDP)_5$ were injected and one group of mice received vehicle (0.9% saline + 5% glucose) treatment alone (controls). The mice were killed on day ten and the tumors were excised, and wet weights were determined. Results are expressed as mean ± SEM ($n = 5$ in each group).

Experimental as well as clinical use of SOD enzymes is restricted owing to their large molecular weight and poor cellular uptake [65]. To circumvent this limitation experimentally, transgenic animal models have been used to study the radioprotective potential of the MnSOD enzyme. These studies have demonstrated that treating radiated animal models with MnSOD, delivered by injection of the enzyme through liposome- or viral-mediated gene therapy or insertion of the human MnSOD gene, can ameliorate radiation-induced damage and simultaneously make cancer cells more susceptible to radiation [64]. It is known that reduced MnSOD expression contributes to increased DNA damage, and cancer cell lines often have diminished MnSOD activity compared with normal counterparts [14,18,19]. Mutations within the MnSOD gene and its regulatory sequence have been observed in several types of human cancers [14,19]. MnSOD is known to suppress cell growth in a variety of cancer cell lines and in mouse models. The MnSOD growth-retarding functions are at least partially caused by triggering of a p53-dependent cellular senescence program [66]. Transfection of human MnSOD cDNA into MCF-7 human breast cancer cells, UACC-903 human melanoma cells, SCC-25 human oral squamous carcinoma cells, U118 human glioma cells, HCT116 human colon cancer cells and DU145 human prostate cancer cells significantly suppressed their malignant phenotype [52]. Introduction of the normal MnSOD gene in cancer cells alters the phenotype and the cells lose the ability to form colonies in culture and tumors in nude mice [67].

A number of studies have reported that ROS play an important part in promoting tumor metastasis [52]. These data are consistent with the suggestion that the redox balance of epithelial tumor cells favors an elevated oxidant set point [53], including CT26 cells [60,61]. Although inflammatory processes secondary to oxidative stress damage normal tissue, they can, in fact, be beneficial to tumor tissue by creating a growth-factor-rich microenvironment [68,69]. Moreover, not only can such a condition promote tumor growth but it has also been shown to suppress activation of CD8+ T lymphocytes that are most efficient in tumor elimination. In fact, there is an increasing interest regarding cytotoxic-T-lymphocyte-mediated immune response for the outcome of cancer chemotherapy [70–73]. It is known that severe lymphocytopenia (<1000 cells/μl) negatively affects the chemotherapy response. Furthermore, a high neutrophil:lymphocyte ratio is associated with a low overall survival for patients with advanced colorectal cancer [71]. A collection of mouse cancers, including CT26 colon cancer, respond to chemotherapy with doxorubicin and oxaliplatin much more efficiently when they are implanted in syngenic immune-competent mice as opposed to in immune-deficient hosts (i.e. nude mice) [72]. Recently, ipilimumab (Yervoy®; Bristol-Myers Squibb), a monoclonal antibody targeting cytotoxic T lymphocyte antigen (CTLA)-4, received approval from the FDA for treatment of metastatic melanoma. CTLA-4 is a protein receptor that downregulates the immune system.

The interest for adoptive cell therapy (ACT) for cancer, in which T lymphocytes are extracted from a cancer patient, expanded *ex vivo* and re-administered to the same patient, is increasing. However, the efficacy of this approach is limited by several possible factors, including immune suppression of CD8+ T lymphocyte activity and tumor infiltration. The limited efficacy involves $ONOO^-$-induced tyrosine nitration of proteins essential for T lymphocyte function [30,73–75]. The mechanism behind immune suppression could in fact reveal new pharmacological possibilities to increase the efficacy of ACT. MnPLED derivatives are expected to inhibit tyrosine nitration via two powerful mechanisms; that is, through (i) their SOD-mimetic activities and (ii) their high affinity for iron. In addition, this class of compounds has been shown to possess (iii) a direct lymphocyte-protecting effect against chemotherapy in mice [52,60].

Acute myocardial infarction

A major concern in coronary heart disease is disabling heart failure after AMI. This is particularly true when it comes to ST-elevated AMI (STEMI). Current treatment by reperfusion using PCI provides clinical benefits. However, reperfusion per se could cause considerable cell death by a process known as reperfusion injury (RI), which can account for as much as 50% of the remaining infarct.

RI is a multifaceted syndrome [76] including arrhythmias, stunning, microvascular obstruction and necrosis. Oxidative stress with uncontrolled release of ROS and impaired calcium homeostasis followed by inflammation are the main causes of RI [76]. Previous attempts to target known mediators of myocardial RI in patients with antioxidant therapy, calcium-channel blockers, sodium–hydrogen exchange inhibitors and anti-inflammatory drugs have been disappointing, leading to calls for a re-evaluation of the current procedure for translating experimental interventions into clinical therapy [77].

In the late 1980s, Crompton and colleagues identified the immunosuppressant cyclosporine as an inhibitor of the mitochondrial membrane permeability transition (MPT) pore [76]. Piot and colleagues reported positive results from a clinical study involving 58 STEMI patients who were randomized to either intravenous cyclosporine or placebo treatment [78]. Their study suggested that the MPT pore could be a new target for reducing the myocardial infarct size. An ongoing confirmatory Phase III study including ~1000 STEMI patients randomized to either cyclosporine or placebo (CIRCUS; ClinicalTrials.gov identifier: NCT01502774) is estimated to be completed toward the end of 2015.

Another clinical study in STEMI patients also targeting the MPT pore, but with another compound, TRO40303 (NCT01374321), was completed October 2013. As recently disclosed [79], however, no differences were observed between TRO40303 and placebo. During the past few decades output of new drugs has in general been low [80]. Already more than 20 years ago, Sir James Black *et al.* [81] warned against this problem. This problem was, according to him and his co-authors, caused by a paradigm shift from a physiological/pharmacological one to a typical genetic sequential approach (i.e. A gives B which in turn gives C). According to the genetic sequential paradigm, creating a new drug is about identifying the right target, in this case the MPT pore, and the corresponding 'magic bullet'. However, according to the physiological/pharmacological paradigm life is much more complicated, where a multitude of signals are going on in parallel, creating nonlinear systems. It might very well be that attacking the oxidative stress upstream of the MPT pore is a much more efficacious approach when it comes to combating reperfusion injuries in the heart.

By making use of ESR spectroscopy Garlick and colleagues were able to show directly that re-oxygenation of a globally hypoxic *ex vivo* heart resulted in a sudden burst of oxygen-derived free radicals [82]. Bolli *et al.* [83], by also making use of ESR spectroscopy, were able to demonstrate direct *in vivo* evidence in dogs that reperfusion resulted in a burst of oxygen-derived free radicals. Treatment with a mixture of SOD and catalase (CAT) attenuated the burst. Interestingly, plasma 8-iso-PGF2α, a biomarker of oxidative stress, has been shown to peak 15 min after acute PCI in patients, whereas the biomarker of irreversible injury, plasma cTnT, peaked first after 6 hours [84]. This suggests a therapeutic window upstream of the MPT pore for a compound with SOD activity.

The idea of using SOD enzymes as adjuncts to reperfusion therapy was born in the 1980s. Preclinical studies using SOD alone or in combination with CAT showed, however, conflicting results [85]. The rationale of using SOD in combination with CAT is that the latter takes care of H_2O_2 formed during cellular dismutation of O_2^-. CAT catalyzes cellular decomposition of H_2O_2 into H_2O and O_2. Superoxide (O_2^-) participates in the devastating Fenton reaction ($Fe^{2+} + H_2O_2 + H^+ \rightarrow Fe^{3+} + OH + H_2O$) by reducing Fe^{3+} back to Fe^{2+}. The hydroxyl radical (OH) is probably the most devastating of all cellular formed ROS and RNS. Nevertheless, in several studies, addition of CAT was not felt necessary *in vivo* because of the presence of significant amounts in CAT-containing erythrocytes [85]. The conflicting results concerning whether or not SOD protects against ischemia–reperfusion injury could be due to the size of the enzyme which most presumably rules out intracellular uptake and access to the most crucial cellular compartments. Indirect evidence from studies in pigs [27] and mice

[44] suggests that MnPLED, but not MnDPDP, is taken up intracellularly.

Initial experiments in *ex vivo* rat hearts showed that MnDPDP significantly improved contractile function and reduced enzyme release lactate dehydrogenase (LDH) in hearts subjected to hypoxia-reoxygenation [37]. In anesthetized pigs with occlusion of the anterior descending branch of the left coronary artery (LAD) for 30 min and reperfusion for 120 min, MnPLED reduced infarct size by more than 50% (Fig. 6) [27]. MnPLED also improved cardiac function and abolished reperfusion-induced ventricular fibrillation. In this study, MnPLED was applied as an intravenous bolus at the end of ischemia followed by a continuous infusion over 120 min. The failure of MnDPDP to reduce the infarct size in this study is presumably caused by the fact that no or very little of the injected MnDPDP is transformed into MnPLED in the pig [27]. This is in contrast to humans, dogs and rats where about 30% of MnDPDP is present as MnPLED 5–30 min after injection [46].

Liver toxicity caused by paracetamol (acetaminophen) and ischemia–reperfusion

Paracetamol (acetaminophen) is a commonly used drug, and is the most prevalent cause of drug-induced acute liver failure [86]. Progression to hepatic failure is characterized by development of encephalopathy, coma, cerebral edema, coagulopathy, gastrointestinal bleeding and sepsis. The mortality rate is high. When given up to 8 hours after ingestion, NAC offers almost complete protection against developing acute liver failure, whereas more than 50% of retrospectively studied patients who only received supportive care developed severe liver damage [86,87]. After 8 hours, however, the antidote effect of NAC gradually fades and after 15 hours it gives no protection. An obstacle to achieve optimal protection with NAC is that 4–6 hours following a paracetamol overdose patients can be asymptomatic. This often results that NAC treatment is started after the crucial ingestion–treatment period of 8–15 hours has been passed. According to Prescott *et al.* [87], as many as 37% of patients started NAC treatment 10–24 hours after paracetamol ingestion.

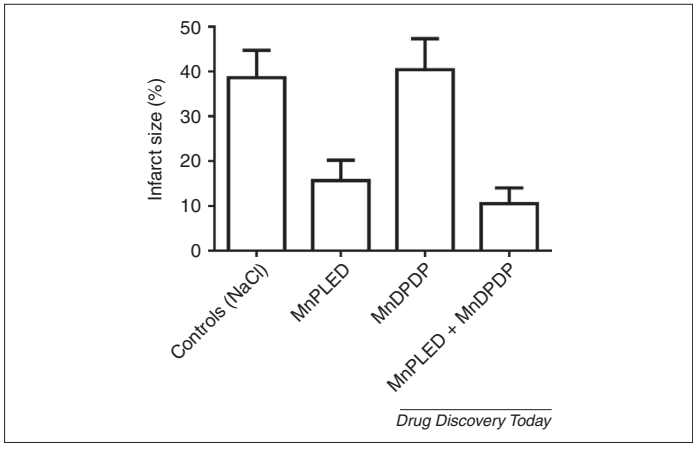


FIGURE 6

Infarct size in anesthetized pigs, expressed as % of area at risk [27]. Briefly, the left anterior descending artery (LAD) was dissected free between the second and third diagonal branches. After an initial 10-min recording, the LAD was occluded by a vascular clamp. After another 20 min, the pigs received 3 μmol/kg manganese dipyridoxyl diphosphate (MnDPDP) or 1 μmol/kg manganese pyridoxyl ethyldiamine (MnPLED) as a an i.v. injection followed by an i.v. infusion of 3 μmol/kg/hour MnDPDP or 1 μmol/kg/hour MnPLED throughout the experiment. To clarify whether MnDPDP (or a metabolite) interferes with the cardioprotective effects of MnPLED, an additional group of animals received a mixture of 3 μmol/kg b.w. MnDPDP and 1 μmol/kg MnPLED followed by an infusion of 3 μmol/kg/hour MnDPDP and 1 μmol/kg/hour MnPLED. Thirty minutes after the start of occlusion, the LAD was reperfused by removal of the vascular clamp. After another 120 min, the pigs were killed by an overdose of KCl, the heart excised and the area at risk and infarct sizes was histologically determined, with fluorescent cadmium sulfide microspheres and tetrazolium staining, respectively.

Paracetamol-induced hepatocyte death in mice occurs by two phases: a metabolic phase and an oxidative phase [88,89]. The metabolic phase occurs with glutathione (GSH) depletion, and the subsequent oxidative phase occurs with increased oxidative stress, $ONOO^-$-mediated protein nitration and inactivation of endogenous MnSOD, loss of mitochondrial membrane potential and hepatocyte death. Experiments in mice suggest that NAC offers protection when administered up to 2 hours after a toxic dose of paracetamol but not after 4 hours [90].

Bedda *et al.* [42] reported that 10 mg/kg (13 μmol/kg) MnDPDP administered 6 hours after a toxic dose of paracetamol protected mice, whereas NAC did not. NAC and MnDPDP protected when administered 2 hours before paracetamol. The fact that MnDPDP showed protective efficacy in mice as long as 6 hours after administration of a toxic paracetamol dose suggests that MnDPDP can offer liver protection during the oxidative phase, at a time-point when NAC has lost efficacy. These authors also presented convincing evidence that MnDPDP more or less abolished paracetamol-induced necrosis and apoptosis. Paracetamol is known to cause opening of MPT pores and breakdown of the mitochondrial membrane potential, which leads to ATP depletion and cell death by apoptosis and necrosis [88]. Interestingly, MnDPDP has been shown to inhibit the MPT pores and subsequent apoptosis [91]. Thus, strong preclinical evidence points to MnDPDP as an efficacious remedy in paracetamol intoxication. ROS play a central part in contributing to tissue injury after reperfusion of the ischemic liver. Coriat and co-workers [92] have shown that MnDPDP prevented experimental hepatic ischemia–reperfusion injuries in the mouse as indicated by a reduction in serum aspartate aminotransferase (ASAT) activity, and in markers of apoptosis.

SOD mimetics: clinical activities

There are four major types of manganese-based SOD mimetics, namely the porphyrin, cyclic polyamine, salen [33,34] and PLED types [35–37]. The porphyrin type is developed by Aeolus Pharmaceuticals (Mission Viejo, CA, USA). This company develops AEOL 10150, a Phase I clinical trial lead product for various indications, primarily as a medical countermeasure against the effects of acute radiation syndrome in the lungs and gastrointestinal tract. In January 2014, AEOL 10150 was granted an orphan drug designation for treatment of patients with acute radiation syndrome (ARS) (http://investor.aeoluspharma.com/releases.cfm). The cyclic polyamine type is developed by Galera Therapeutics (Malvern, PA, USA). At present, its lead candidate – the polyamine compound GC4419 (previously known as M40419) – is in a Phase I clinical trial as an adjunct to chemotherapy and radiation treatment for squamous cell cancer (a Phase I dose-escalation study of GC4419 in combination with chemoradiation for squamous cell cancer of the head and neck, ClinicalTrials.gov identifier:

NCT01921426). To the best of our knowledge, none of the salen type of compounds is in clinical development. When it comes to the PLED types, MnDPDP (mangafodipir) was originally developed as a contrast agent for MRI by Salutar (Sunnyvale, CA, USA), and achieved clinical approval in 1997. It was withdrawn from the market in 2010. The withdrawal was not based on any safety concerns. Nevertheless, during its time on the market more than 200,000 patients received it as a contrast agent (according to the producer).

Before the withdrawal, PledPharma initiated a feasibility study with MnDPDP in colon cancer patients on adjuvant treatment with folinate + 5-fluorouracil + oxaliplatin (FOLFOX; MANFOL study). Because of concerns that repeated use of MnDPDP could result in manganese accumulation in the brain and cause toxic effects, MnDPDP was only included in three out of 12 scheduled FOLFOX cycles, and at an accumulated dose of 6 μmol/kg previously shown to be safe with regard to brain accumulation [51]. In this study, Karlsson *et al.* [93] confirmed previous preclinical findings, namely that pretreatment with MnDPDP lowers DLT [44,59,60].

Oxaliplatin in combination with 5-fluorouracil has improved survival considerably in colorectal cancer patients [93]. However, the efficacy of this combination is compromised by toxicity, in particular oxaliplatin-induced sensory neuropathy. The use of intravenous calcium and magnesium (Ca/Mg) for prevention of oxaliplatin-induced sensory neuropathy became a common clinical practice after a report in 2004 that compared a series of patients treated with Ca/Mg with a historical control group suggesting that Ca/Mg decreased neuropathy by about 50% [94]. However, a confirmatory Phase III clinical trial determined that this treatment was ineffective [95]. Recently, Coriat *et al.* [96] provided results from a Phase II clinical trial using MnDPDP in patients with pre-existing oxaliplatin-induced neuropathy. The trial involved 22 patients with at least grade 2 sensory neuropathy after receiving oxaliplatin. After four additional cycles of oxaliplatin and MnDPDP, they reported that 17 patients had stable or improved neuropathy, and after eight cycles six patients had improvement in their neuropathy grade.

The paper by Coriat *et al.* [96] reported that the mean plasma manganese content increased from 11.8 ± 5.5 nM to 19.8 ± 4.3 nM after eight cycles of MnDPDP co-treatment. Although plasma manganese is considered as a weak predictor of manganese-induced neurotoxicity [50], this finding could in fact indicate an unacceptable high brain exposure for manganese released from MnDPDP. Furthermore, the paper by Coriat *et al.* confirmed the findings of Yri and co-workers, published as a case report in 2009 [97]. This report described a patient who underwent 15 palliative cycles of oxaliplatin plus 5-fluorouracil. In 14 of the cycles the patient was pretreated with MnDPDP. The patient received a cumulative dose of 15 × 85 mg/m^2 of oxaliplatin, which is a dose expected to cause sensory neuropathy. No symptoms of sensory neuropathy were detected, except in the fifth cycle, when MnDPDP was deliberately omitted, at which time the patient experienced sensory neuropathy. Although this reference was not included in the paper by Coriat *et al.* [96], both publications suggest that MnDPDP, probably as a result of its SOD mimetic activity, protects against oxaliplatin-induced sensory neuropathy. The patient in the case report of Yri *et al.* [97] received a dose of 10 μmol/kg at each of 14 cycles of chemotherapy, resulting in an accumulated dose of 140 μmol/kg. The overall clinical impression from this patient was positive, but an MRI scan of the brain after 14 cycles of MnDPDP showed increased T1-weighted signal intensity in the basal ganglia. This patient manifested symptoms similar to those previously seen in patients administered manganese-supplemented total parenteral nutrition [49,50].

PledPharma is at present testing $Ca_4Mn(DPDP)_5$ (calmangafodipir, PledOx®), a stabilized form of MnDPDP with superior therapeutic index [52] in an ongoing Phase II study (PLIANT study), which will include around 150 colon cancer patients. Before withdrawal of MnDPDP, PledPharma also initiated a feasibility study (MAMAMI study) in patients with acute STEMI going through PCI. The MANAMI study included ten patients randomized to MnDPDP and ten patients randomized to placebo treatment; and with an ischemic time (chest pain) less than 6 h. The study was recently reported to the Swedish Competent Authority, Läkemedelsverket and a manuscript describing major findings has been accepted for publication [98]. The ischemic time was significantly longer in the MnDPDP group than the placebo group (195 min vs 144 min). Despite this difference there were several findings suggesting that MnDPDP in fact could protect the human heart against reperfusion injuries, similar to that previously shown with MnPLED in pigs [27]. The most consistent finding in the study was a borderline statistical difference ($P = 0.0383$, one-sided Mann–Whitney) in ST-resolution (STR), in favor of MnDPDP. It is well established that early and complete STR is a powerful predictor of infarct-related artery patency, preserved microvascular integrity and low mortality in patients with STEMI [99].

Concluding remarks and future prospects

MnPLED derivatives possess pharmacological properties which makes them efficacious as therapeutic agents during conditions of pathological oxidative stress. They are small and lipophilic enough for entering crucial intracellular compartments. By dismutating O_2^-, MnPLED derivatives attack 'the root cause' of the problem (i.e. at the uppermost level of the chain of biochemical events that leads to cell death during oxidative stress). By high affinities for iron (and copper) they also attack further downstream by inhibiting $ONOO^-$-mediated tyrosine nitration (e.g. of endogenous MnSOD) and formation of OH.

Preclinical studies have pointed out (i) cancer treatment, (ii) acute myocardial infarction and (iii) paracetamol (acetaminophen) intoxication as particularly interesting fields of use. MnPLED derivatives have entered clinical Phase II trials for (i) and (ii), and $Ca_4Mn(DPDP)_5$ is at present being tested as an adjunct to FOLFOX treatment in patients with metastatic colorectal cancer in a multicenter Phase II study.

Conflicts of interest

Jan Olof G. Karlsson is a co-founder of PledPharma and a former employee of GE Healthcare and PledPharma. He owns shares in PledPharma. He is inventor on several granted patent families and patent applications covering the therapeutic use of PLED derivatives. Louis J. Ignarro is a co-founder of PledPharma. L.J.I. serves as a scientific advisor for PledPharma but has no financial interest in the company. Ingemar Lundström is a co-founder of PledPharma and own shares in this company, where he also serves as scientific

advisor. Per Jynge is a co-founder of PledPharma and owns shares in this company. P.J. is inventor on several granted patent families covering the therapeutic use of PLED derivatives. Torsten Almén is a co-founder of PledPharma and owns shares in this company, where he also serves as a scientific advisor.

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