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The emerging role of reactive oxygen species in cancer therapy

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Abstract

The generation of reactive oxygen species (ROS) can be exploited therapeutically in the treatment of cancer. One of the first drugs to be developed that generates ROS was procarbazine. It is oxidised readily in an oxic environment to its azo derivative, generating ROS. Forty years ago, Berneis reported a synergistic effect in DNA degradation when procarbazine was combined with radiation; this was confirmed in preclinical *in vivo* modes. Early uncontrolled clinical trials suggested an enhancement of the radiation effect with procarbazine, but two randomised trials failed to confirm this. The role of ROS in cancer treatments and in the development of resistance to chemotherapy is now better understood. The possibility of exploiting ROS as a cancer treatment is re-emerging as a promising therapeutic option with the development of agents such as buthionine sulfoximine and motexafin gadolinium.

Keywords: Procarbazine; Reactive oxygen species; ROS; Hydrogen peroxide; Apoptosis; Buthionine sulfoximine; Motexafin gadolinium

1. Procarbazine

1.1. Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radicals, and superoxide anion, are highly reactive molecules with unpaired electrons that are generated in normal physiological processes such as aerobic metabolism or inflammation. Cellular defences to ROS include antioxidant scavengers, such as ascorbate, glutathione and thioredoxin, and antioxidant enzymes, such as superoxide dismutase, catalase, glutathione peroxidase and thioredoxin reductase. The regulation of oxidation–reduction (redox) reactions is critical to a cell, as it influences metabolic and other signal-transduction pathways. When ROS generation exceeds the cellular antioxidant defences, cell damage ensues. It is now increasingly clear that the generation of ROS can be exploited therapeutically in the treatment of cancer and that the ability of a cell to defend itself against ROS is associated with resistance to chemotherapy.

One of the first drugs to be developed that generates ROS was procarbazine. The oxidation of procarbazine in aqueous solution leads to the production of hydrogen peroxide [1], which was thought to be essential to the cytotoxic effect of the drug. Berneis showed that isolated DNA could be degraded by procarbazine in the presence of oxygen. The addition of catalase or peroxidase prevented the degradation of DNA, indicating that hydrogen peroxide may be formed. Because it was known that the action of hydrogen peroxide on DNA proceeds via hydroxyl radicals, the effect of procarbazine on DNA was compared with the effect of ionising radiation. Berneis went on to publish, in 1966 in the *European Journal of Cancer,* that procarbazine synergised with ionising radiation in cleaving DNA *in vitro* through the formation of unstable peroxide products [2].

The first clinical trial of procarbazine was reported by Martz and colleagues in 1963 [3]. Subsequently, Mathe [4] reported on the use of procarbazine in the treatment of Hodgkin's lymphoma and other haematolymphoid malignancies. Two-thirds of the 22 patients with Hodgkin's lymphoma responded to single-dose procarbazine. The main toxicities were gastrointestinal and haematological: nausea and vomiting, neutropenia, and

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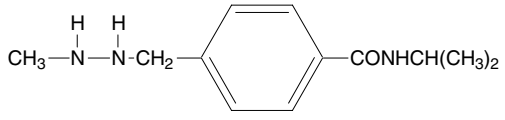
thrombocytopenia. Procarbazine was approved in the late 1960s as a cytotoxic drug and has been used since primarily for the treatment of Hodgkin's lymphoma, non-Hodgkin's lymphoma and primary brain tumours.

This article will review the data that have been generated since the Berneis paper in the use of procarbazine to enhance the effect of radiation, the role of procarbazine in cancer treatment today, and other attempts to develop cancer treatments through drugs that generate ROS.

1.2. Procarbazine and radiation therapy

In the search for monoamine oxidase inhibitors, scientists at Hoffmann–LaRoche synthesised hundreds of hydrazines and hydrazides [5]. Through *in vivo* screening, the methylhydrazine *N*-isopropyl-α-(2-methylhydrazine)-*p*-toluamide hydrochloride (procarbazine, Natulan®) was found to have antineoplastic activity and to be the least toxic [6,7]. The methylhydrazine derivates with antitumour activity have the chemical structure R–CH_2–NH–NH–CH_3, where R represents various organic groups, in particular benzyl groups. The structure of procarbazine is shown in Fig. 1. In early screening, procarbazine was found to prevent the growth of transplantable tumours in mice and rats, such as Walker carcinoma 256 and Ehrlich carcinoma (solid form and ascites carcinoma), and to reduce mitoses in Ehrlich ascites cells [5]. Chromatid breaks were observed [8]. Furthermore, procarbazine, in the presence of oxygen, was able to degrade DNA *in vitro*, as measured by DNA viscosity. When the experiment was carried out in nitrogen, no DNA degradation was observed. This effect was thought to be due to the auto-oxidation of procarbazine in the presence of oxygen to its azo derivative, producing hydrogen peroxide (Fig. 2). Hydrogen peroxide, in the presence of iron (Fe^{++}), generates hydroxyl radicals. The degradation of isolated DNA by procarbazine could be prevented by the addition of catalase to the reaction, or by the addition of an iron chelator such as desferrioxamine [1,9]. Catalase is an enzyme that catalyses the decomposition of hydrogen peroxide to water and molecular oxygen without the production of free radicals. Iron chelation prevents the generation of hydroxyl radicals from hydrogen peroxide. Thus the ability of procarbazine to cleave DNA *in vitro* seemed dependent on the production of ROS.

Because of the potential generation of hydroxyl radicals, the effect of procarbazine was compared to that of



Procarbazine (NSC-77213)

Fig. 1. Molecular structure of procarbazine.

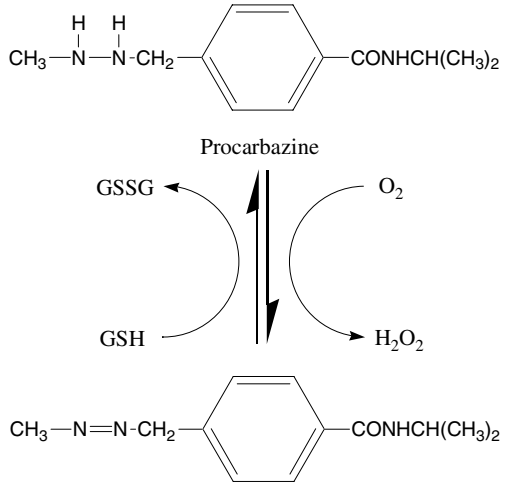


Fig. 2. Procarbazine oxidation to its azo derivative yields hydrogen peroxide.

ionising radiation. Berneis and colleagues reported a synergistic effect when procarbazine was combined with radiation [2]. DNA extracts were irradiated and then exposed to procarbazine, hydrogen peroxide or nitrogen mustard. The synergism between procarbazine and radiation in generating DNA breaks was greater than that between equimolar hydrogen peroxide and radiation. Thus the effect of procarbazine could not be explained solely by the formation of hydrogen peroxide. The effect of procarbazine was most pronounced when the drug was added to the solution at the completion of the 1-h irradiation experiment; the longer the interval between radiation and procarbazine treatment, the smaller the synergistic effect. This observation was assumed to be due to the peroxide products of the DNA that are formed during radiation, which are very unstable. In contrast, the addition of nitrogen mustard to irradiated DNA resulted in much less DNA degradation, and was not dependent on the time interval between radiation and the addition of nitrogen mustard.

Although the investigators made no claim that procarbazine was effective *in vivo* through the generation of ROS, the findings of Berneis and colleagues were reproduced subsequently under *in vivo* conditions [10]. Injection of Ehrlich ascites-carrying mice with hydrogen peroxide intraperitoneally significantly increased their survival. However, the amount of procarbazine required to generate the amount of hydrogen peroxide needed to be effective was well above the LD_{50} of procarbazine. *In vivo*, procarbazine did not produce DNA breaks. Injecting mice with a sublethal dose of procarbazine significantly decreased the LD_{50} of whole-body irradiation, whether the drug was administered before or after the radiation treatment [11], indicating that procarbazine is not a classical radiation sensitiser, but instead may have independent cytotoxicity or may act by inhibiting the repair of sublethal damage.

It was found that the terminal *N*-methyl group was critical for the activity of procarbazine [10]. Kreis determined that there was a direct transmethylation of the intact *N*-methyl group of procarbazine on to the seventh position of guanine, especially in transfer ribonucleic acid (tRNA), inhibiting the synthesis of tRNA, and subsequently of RNA, DNA and protein. However, the degree of nucleic acid alkylation with procarbazine was low compared with that of other alkylating agents [12]. Further investigation revealed that procarbazine can lead to a reduction in intracellular reduced glutathione and the generation of additional free radicals, including superoxide, in the metabolism of procarbazine and its azo derivative (Fig. 2) [12]. Thus procarbazine appears to be an agent with a multifunctional mechanism of action.

Based on the work of Berneis and colleagues, combined-modality treatment with procarbazine and radiation therapy was evaluated in a number of clinical trials. Sandison reported a study of 215 sequential patients with lung cancer treated with six different regimens, including radiation and radiation plus procarbazine [13]. There was no improvement in subjective and objective response rate (59.3% for radiation vs. 61.2% for radiation plus procarbazine), but there was an improvement in one-year survival (12.8% vs. 19%). Median survival was improved for stage III patients only (7 months vs. 9.6 months for radiation plus procarbazine; $P = 0.14$), and was unchanged for stage IV patients (5 months for both treatment arms). Falkson reported a study of patients with mesothelioma treated with either radiation alone or radiation plus procarbazine [14]. None of the nine patients treated with radiotherapy alone responded, whereas 14 of the 26 patients treated with procarbazine and radiation responded subjectively or objectively. Interestingly, the tumour shrinkage in the combined-modality arm was only observed within the radiation field, whereas procarbazine had no effect on the disease outside of the radiation port. This study was not randomised, and the radiation doses administered were not controlled in the treatment groups.

A randomised trial of 67 patients with inoperable non-small cell lung cancer treated with irradiation or irradiation plus procarbazine showed no benefit for procarbazine, favouring the radiation-only arm [15]. Palmer evaluated the use of low-dose radiation therapy (30 Gy) alone or in combination with procarbazine for the treatment of unresectable epidermoid carcinoma of the lung [16]; median survival times and median duration of remission favoured the radiation-only arm. The survival in both groups was similar to historical controls treated with higher doses of radiation (40–50 Gy). Thus the limited data from clinical trials did not support the suggestion that procarbazine enhances the effect of radiation in patients.

Because of these discrepant results, further preclinical experiments were conducted to clarify the potential of procarbazine as a radiation sensitiser [17]. In clonogenic assays *in vitro* of irradiated *Escherichia coli*, procarbazine was only a very modest hypoxic cell sensitiser, with enhancement rations of 1.15–1.4, compared with oxygen enhancement ratios of 2.8 in the same system. The concentrations of procarbazine required to achieve sensitisation in bacterial systems were far greater than the blood concentrations that could be achieved in man. Roberts concluded that unless procarbazine is a more effective sensitiser in mammalian cells, or is concentrated in tumours, far higher doses than the 100 mg/m^2 used clinically would be required to achieve radiation enhancement.

The work by Berneis stimulated the development of electron-affinic sensitisers [18]. Unlike procarbazine, which is easily oxidised (i.e. tends to donate rather than accept electrons), the hypoxic cell sensitisers are easily reduced (i.e. tend to accept electrons). These agents could oxidise free-radical damage caused by radiation therapy and thereby induce an irreparable lesion in the DNA, ultimately leading to increased breaks in double-stranded DNA. The nitroimidazoles, such as misonidazole, metronidazole and etanidazole, became the focus of investigations by radiation biologists and were extensively tested in the clinic [19]. These agents proved to be toxic and of little clinical benefit, as demonstrated by a number of large but negative randomised trials, such as those in glioma [20], head-and-neck cancer [21], lung cancer [22,23] and brain metastases [24].

1.3. The role of procarbazine in cancer therapy today

In early clinical testing of procarbazine, lymphomas were identified as particularly sensitive to the agent. In the first human trial, reported by Martz, 16 of 17 patients with malignant lymphoma responded, as opposed to none of 14 patients with solid tumours [3]. This was confirmed in a report of 44 patients with haematolymphoid malignancies treated by Mathe [4]: of 22 patients with Hodgkin's disease, 15 patients (68%) had a good response, with seven complete remissions. The other haematolymphoid malignancies responded less well, with no complete remissions and seven incomplete remissions. Remissions were brief, unless patients were previously untreated [25]. The combination of procarbazine with nitrogen mustard, vincristine and prednisone in the MOPP regimen led to responses in more than 80% of patients and to durable remissions [26,27]. MOPP was considered standard therapy for Hodgkin's disease until it was replaced by MOPP alternating with ABVD (adriamycin, bleomycin, vinblastine and dacarbazine), MOPP/ABV hybrid, and eventually by ABVD [28,29]. In Europe, procarbazine has been incorporated with good results in the BEACOPP regimen (bleomycin,

etoposide, doxorubicin, cyclophosphamide, vincristine, procarbazine and prednisone) for patients with unfavourable stage IIB or IIIA or stage IIIB or IV Hodgkin's lymphoma [30]. Procarbazine remains in use but less frequently for the treatment of Hodgkin's disease in regimens such as ChlVPP (chlorambucil, vinblastine, procarbazine and prednisone) [31], and BCVPP (carmustine (BCNU), cyclophosphamide, vinblastine, procarbazine and prednisone) [32].

Since procarbazine crosses the blood–brain barrier and rapidly equilibrates between plasma and the cerebrospinal fluid [33], it has been studied extensively in patients with primary brain tumours. To date, procarbazine is commonly used for many patients with glioma. The PCV regimen (procarbazine, lomustine (CCNU) and vincristine) has been used for primary or secondary treatment of anaplastic astrocytoma and oligodendroglioma [34,35].

Other uses of procarbazine are limited, even though it has activity in malignant melanoma, lung cancer (both small cell and non-small cell) and multiple myeloma [36].

1.4. Novel approaches to generate ROS as cancer therapy

Berneis and his colleagues recognised that drugs that generate ROS can be used as cancer treatments, and perhaps to enhance the antitumour effect of radiation therapy. This approach is now re-emerging as a topic of investigation in cancer research. A Medline search for the term 'reactive oxygen species' yielded 19,240 references.

Redox metabolism, the homeostasis of ROS and their detoxification, is critical in cell signalling and the regulation of programmed cell death, or apoptosis. An increase in the generation of ROS, or a reduction in their detoxification, such as the depletion of non-oxidised glutathione, results in the induction of apoptosis through depolarisation and permeabilisation of the mitochondrial membrane [37]. Soluble mitochondrial intermembrane proteins are released, such as cytochrome C and apoptosis-inducing factor, which activate downstream caspases and nucleases, ultimately leading to apoptotic cell death. The activation of apoptosis is regulated through a number of proapoptotic second messengers, such as ROS, nitric oxide, lipid messengers, such as ceramide, calcium, and proapoptotic Bcl-2 family members (e.g. Bax, Bid, and Bad). It is also regulated through a number of inhibitors, such as antiapoptotic Bcl-2 family members. Conventional chemotherapy drugs, such as doxorubicin, etoposide, cisplatin or paclitaxel, can induce apoptosis by inducing p53 expression, the ceramide pathway or the CD95/CD95L ligand system, affecting Bcl-2-like proteins, or by interfering with the redox or energy balance of the cell [37]. All of these pathways induce apoptosis through depolarisation of the mitochondrial membrane. Doxorubicin is a redox-cycling anthracycline that generates ROS through interactions with trace metals, such as iron or copper [38]. Biologics can also induce apoptosis through the generation of reactive oxygen. Rituximab, an anti-CD20 monoclonal antibody approved for the treatment of non-Hodgkin's lymphoma, induces a rapid and intense production of ROS followed by depolarisation of the mitochondrial membrane *in vitro* in human lymphoma cells [39]. Conversely, alterations in redox metabolism can lead to resistance to chemotherapy. For example, an increased cellular concentration of glutathione has been closely correlated with cisplatin resistance, even though it does not interfere with the extent of cisplatin–DNA adduct formation [40].

Several experimental agents are in development to increase apoptosis by introducing proapoptotic proteins or to induce apoptosis directly through the mitochondria; this can be accomplished by generating ROS, downregulating or inhibiting antiapoptotic pathways, or depleting glutathione.

2. Buthionine sulfoximine

Buthionine sulfoximine (BSO) inhibits the rate-limiting enzyme in the synthesis of glutathione (GSH), γ-glutamylcysteine sythetase, which is often upregulated in chemotherapy-resistant tumours. The depletion of cellular GSH can restore sensitivity to the oxidative cytotoxic effect of platinum compounds and alkylators. For example, Bcl-2-overexpressing MCF-7 breast cancer cells have a nearly threefold increase in glutathione levels, rendering them resistant to cisplatin. Pretreatment with BSO completely abrogated the Bcl-2-mediated resistance to cisplatin in these cells [40]. Further preclinical studies showed that depletion of GSH with BSO results in enhanced cytotoxicity of alkylating agents *in vivo* [41]. Phase I clinical trials demonstrated that BSO was well tolerated and that glutathione could be reduced to approximately 10% of pretreatment values in tumour samples and blood lymphocytes [42–44]. Grades 1 and 2 nausea and vomiting were the most common adverse events attributed to BSO, seen in 50% of patients. The antitumour effect of BSO in combination with cisplatin and alkylators is under investigation.

3. Motexafin gadolinium

Motexafin gadolinium is an expanded porphyrin that selectively localises in tumours. It has a multifunctional mechanism of action, including the generation of ROS and the depletion of reducing metabolites, such as protein thiols, thioredoxin, nicotinamide adenine dinucleotide phosphate (NADPH), ascorbate and glutathione

[45]. Electrons are transferred from the reducing metabolites to oxygen to generate ROS (Fig. 3). The drug inhibits ATP production by interfering with electron transfer [46]. The result is that the cells undergo apoptosis more readily [47], implying that motexafin gadolinium could be used as a single-agent treatment in haematolymphoid malignancies, cells that are very sensitive to oxidative stress, or in combination with chemotherapy and radiation. In preclinical models, motexafin gadolinium enhanced the effect of a variety of chemotherapy agents *in vitro* and *in vivo* [48], and the effect of radiation therapy [46,49,50].

The current thinking on the selectivity of motexafin gadolinium is that it is based on the abnormal metabolism in tumours. Compared with normal cells, cancer cells utilise primarily anaerobic glycolysis. Motexafin gadolinium is readily reduced (i.e. picks up an electron) in cancer cells. The resultant reduced species is trapped selectively in the tumour cell. The fact that motexafin gadolinium selectively targets tumours has been shown unequivocally by using radiolabelled drug [50] and by using magnetic resonance imaging (MRI) scans. Since motexafin gadolinium contains covalently bound gadolinium in its centre, the drug is detectable by MRI. MRI scans in patients treated with motexafin gadolinium have demonstrated selective drug uptake in primary and metastatic tumours, but not in normal organs, with the exception of the liver and kidneys, where the drug is excreted [51–54].

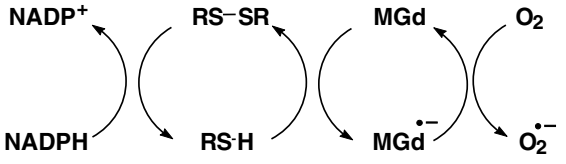


Fig. 3. Redox cycling of motexafin gadolinium (MGd) generates reactive oxygen and oxidises protein thiols (RS-H). Reduction of the oxidised protein thiols (RS-SR) to their reduced state requires energy in the form of NADPH.

Motexafin gadolinium is an experimental agent that has been administered to over 500 patients in a number of phase I, II and III trials. The drug is well tolerated, with dose-limiting reversible renal toxicity at high doses after single administration [53], and reversible hepatotoxicity after repeated administration [55]. A phase III trial in patients with brain metastases undergoing whole-brain radiation therapy has been completed recently. While the coprimary endpoints of survival and time to neurological progression were not met for the entire group of 401 randomised patients, the prespecified subgroup of 251 patients with non-small cell lung cancer and brain metastases showed a significant benefit in prolonging time to neurological progression in the radiation plus motexafin gadolinium arm (Fig. 4) [56]. The median was prolonged from 7.4 months in the radiation arm to not reached at 15 months in the radiation plus motexafin gadolinium arm ($P = 0.048$). In the lung cancer patients, a trend towards improved time to neurocognitive progression was observed as well [57]. The most frequently reported adverse events attributed to motexafin gadolinium were transient skin and urine discoloration, and grade 1 and 2 hypertension and nausea. These results are now undergoing confirmation in an international phase III trial in Europe, North America and Australia called the Study of Neurologic Progression with Motexafin Gadolinium and Radiation Therapy (SMART). This trial is evaluating time to neurological progression as the primary endpoint in 550 patients with non-small cell lung cancer and brain metastases. Patients are randomised to standard whole-brain radiation therapy (30 Gy in 10 fractions) or

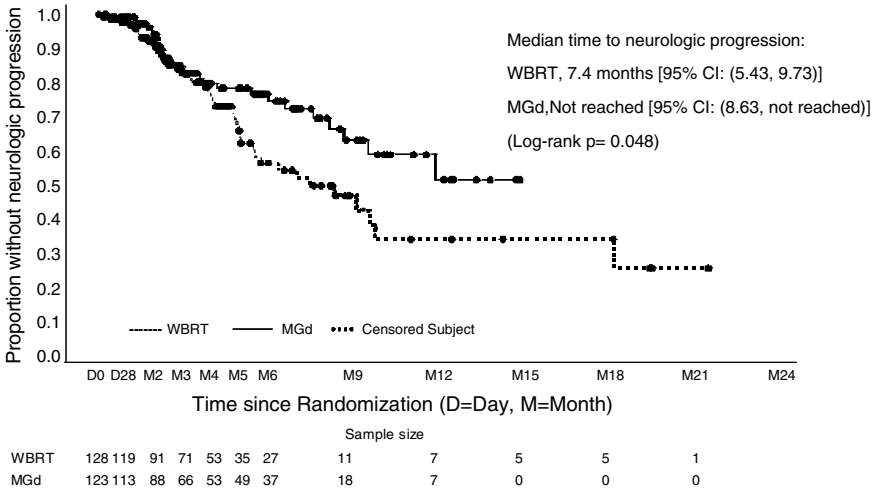


Fig. 4. Time to neurological progression as determined by a blinded events review committee in patients with non-small cell lung cancer and brain metastases by treatment arm. WBRT, whole-brain radiation (control) arm; MGd, motexafin gadolinium + WBRT (treatment) arm; CI, confidence interval; D, day; M, month [56] (reprinted with permission from the American Society of Clinical Oncology).

standard whole-brain radiation therapy plus motexafin gadolinium, administered intravenously each day before radiation therapy.

In conclusion, Berneis and his colleagues discovered 40 years ago that reactive oxygen generated by procarbazine increased the DNA degradation resulting from radiation therapy. Procarbazine remains an effect anticancer agent. The idea of utilising ROS in cancer treatment continues to be actively researched, most recently with the clinical testing of BSO and motexafin gadolinium.

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