Targeting Cancer Cells by an Oxidant-Based Therapy

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Abstract: Despite the progress achieved in chemo- and radiotherapy, cancer is still a leading life-threatening pathology. In that sense, there is a need for novel therapeutic strategies based on our current knowledge of cancer biology. Among the phenotypical features of cancer cells, two of them are of particular interest: their nearly universal glycolytic phenotype and their sensitivity towards an oxidative stress, both resulting from the combination of high anabolic needs and hypoxic growth conditions. By using menadione (vitamin K3) and ascorbate (vitamin C), we took advantage of these features to develop an original approach that consists in the exposure of cancer cells to an oxidant insult. When used in combination, these compounds exhibit a synergistic action and are devoid of major toxicity *in vivo*. Thus, this review is dedicated to the analysis of the molecular pathways by which this promising combination exerts its antitumoural effect.

Keywords: Ascorbate, menadione, cancer, oxidative stress, glycolysis, cell death.

MAIN FEATURES OF CANCER BIOLOGY

Normal cells perfectly fit their environment and respond to external signals *via* tightly regulated pathways that either trigger or repress growth. Cancer arises when a cell, for a variety of reasons, escapes the normal brakes placed on its growth and begins to divide in an uncontrolled fashion. Actually, tumorigenesis appears as a multistep mechanism that reflects the genetic alterations driving progressively a normal tissue to malignancy. The best known genes whose mutations are frequently associated with the arising of cancers are p53, c-myc, erb B or K ras [1]. Nevertheless, despite these classical mutations, no typical cancer cell genotype exists and each invasive cancer appears as the consequence of a particular genetic pathway travelled during carcinogenesis [2,3]. It is therefore quite surprising to note that the genetic diversity usually presented by cancer cells does not correlate with the clinical observations where a common invasive behaviour including uncontrolled growth and destruction of normal tissues are noted. Such a paradox can be explained by the selective barriers existing within a tumour (hypoxia, malnutrition, hormonal fluctuations, attacks of the immune system, etc) that lead to the selection of adapted cells [4]. Interestingly, this evolutionary process seems to be related to the manifestation of six main alterations in cell physiology, as nicely described in 2000 by Hanahan and Weinberg: self-sufficiency in growth signals, insensitivity to antigrowth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis [5]. Supporting this hypothesis, it has been recently shown that a simple network of well-defined genetic events is sufficient to convert a healthy cell into a tumorigenic state [6].

The main physiological changes presented above represent novel capabilities and are acquired during tumour development through multiple mutations. Besides these characteristics, two other secondary biochemical features are frequently presented by cancer cells: a strong glycolytic phenotype and a poor antioxidant status. Since they are of major interest for the rest of this work, they will be extensively described.

Glycolytic Phenotype

The up-regulation of glycolysis is probably the oldest feature of cancer described, since the first observations were made almost 80 years ago by Warburg [7]. Nevertheless, this nearly universal phenotype has never been fully investigated, with the exception of these last years and the widespread clinical use of [18]fluorodeoxyglucose positron-emission tomography (PETSCAN). PET imaging has now clearly demonstrated that glycolytic rates increase in neoplasms, a fact that can be directly related to tumour aggressiveness and prognosis [8].

Different hypotheses are proposed to explain the persistent metabolism of glucose to lactate even under aerobic conditions, the so-called "Warburg's effect". Among them, hypoxia is frequently suspected. Hypoxia arises from the uncontrolled proliferation of cancer cells that leads to the colonization of areas at increasing distance from blood vessels. Due to the poor limit of oxygen diffusion (less than 100 μM), a gradient of oxygen rapidly occurs within the growing tumour [9]. This fact, coupled to the increasing metabolic demands of the growing mass of cells provokes a chronic hypoxia, even in tumours of only a few cubic millimetres. In addition to chronic hypoxia, the uneven distribution of erythrocytes within the tumour microvasculature can generate a fluctuant hypoxia. These temporal fluctuations in red cell flux occur in the normal microcirculation but are exacerbated in the tumour microenvironment, due to its particular features (low pH, low pO_2, high vascular permeability…) Coupled to the tortuous tumour vasculature, this process results in a very unstable blood flow that provokes the appearance of areas of fluctuant hypoxia in tumours.

The adaptation of cancer cells to hypoxia notably arises from the activation of transcription factors such as HIF-1 [10]. HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1α subunit and a tightly regulated HIF-1α subunit. The expression of the latter is controlled by the levels of O_2 since its degradation is controlled by O_2-dependent

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mechanisms. HIF-1-regulated genes are particularly relevant to cancer and can be divided into four groups: those that encode angiogenic factors, glycolytic enzymes, survival factors and invasion factors. Therefore, the activation of HIF-1 provides an explanation for the high levels of some key glycolytic proteins found in cancer, such as glucose transporters (GLUTs) [11].

The overexpression of the mitochondrial-bound isoforms of hexokinase (HK-I and HK-II) is another hypothesis that could explain the glycolytic phenotype exhibited by cancer cells [12]. Such an upregulation occurs by epigenetic changes (hypomethylation) that allow an open conformation of the promoter, thus enhancing the binding of transcription factors. Since the mitochondrial-bound isoforms of hexokinase have an easier access to ATP, they are less susceptible to inhibition by their product (glucose-6-phosphate) and present a low Km for glucose. They act as a trap mechanism for glucose capture and greatly increase the rate of aerobic glycolysis [13].

Finally, it has been proposed that the acquisition of the glycolytic phenotype is the consequence of mitochondrial defects, leading to the impairment of oxidative phosphorylation. This is likely due to the high sensitivity of the mitochondrial genome to mutations. Indeed, the mitochondrial genome lacks histones and relative protective systems, and it has no introns as well and is therefore fully required to maintain mitochondrial functions [14]. While the occurrence of a respiratory defect in cancer cells is still hardly debated [15], significantly reduced levels of β-F$_1$-ATPase have been observed in several types of cancer, linked to an altered bioenergetic phenotype of mitochondria [16].

Although the up-regulation of glycolysis is usually considered as the consequence of cancer cell adaptation to extreme microenvironmental conditions, its role in invasiveness is now suspected [17,18]. Indeed, the constant release of acid from the altered tumour metabolism leads to an extracellular acidification in the neighbouring cancer cells resulting in the death of normal cells [19,20]. This is explained by the fact that cancer cells are more resistant towards acidification than normal cells, due to mutations in p53 or other components of the apoptotic pathway [21]. Then, cancer cells can progress following the peritumoral acid gradient and progressively replace the surrounding healthy tissue where cells are dead. In addition, the acidification promotes several mechanisms essential for tumour growth like angiogenesis, through acid-induced release of VEGF and IL8 [22], extracellular matrix degradation by proteolytic enzymes like cathepsin B [23], or the inhibition of the immune response [24]. Since the acquisition of a glycolytic phenotype represents a key event for both survival and progression of cancer, the inhibition of glycolysis appears a novel promising target for cancer therapy [25,26].

Deficiency in Antioxidant Defences

The antioxidant status of tumours has been a matter of debate for decades. This is mainly due to the difficulties encountered in the interpretation of these studies: intratumour heterogeneity, compartmentalization of the enzymes, absence of comparison with healthy tissues, artificial conditions of cell culture *in vitro*, few inhibitors available, etc

[27]. Nevertheless, the overall data from several publications support the alteration of the antioxidant status during tumour progression, a finding that is not really surprising if we consider the close relationship existing between the oxidants/antioxidants and cancer, at several steps. Indeed, at the beginning of the cancer process, oxidative conditions are often associated with carcinogenicity [28]. During its progression, some oxygen species like hydrogen peroxide can mediate signal transduction and promote cell proliferation [29]. Finally, the antioxidant capacity of cancer cells is described to be modified during tumour progression, and influences their resistance to both chemotherapy and radiotherapy [30-32].

Thus, copper-and zinc-containing superoxide dismutase (CuZnSOD) levels as well as those of catalase and gluthatione peroxidase appear to be decreased in tumours, compared to the tissues of origin [33-37]. Such a decrease was even related to cancer progression in oral squamous cell carcinoma [38]. The situation is more complex with manganese superoxide dismutase (MnSOD) which appears to be frequently overexpressed in human tumours, although its overexpression *in vitro* leads to cell death and cancer regression [39]. In the same way, the recently discovered thioredoxin (Trx) system perfectly reflects this ambiguity since many human tumours exhibit an overexpression of thioredoxins, possibly linked to a resistance to chemotherapy [40]. Nevertheless, thioredoxins have been shown to possess various cellular activities including growth-stimulating properties and it is difficult to evaluate whether these features are linked or not with their antioxidant capacity. Despite the controversial data obtained with some particular enzymes, a poor antioxidant status can generally be considered as another classical feature of cancer cells. Up to now, there is no real explanation of the loss of the antioxidant defences by cancer cells. This could reflect a normal process in cancer progression if it is assumed that this latter process is associated with a constant adaptation of cancer cells to their environment. Since the cancer environmental growth conditions generally lack oxygen (half of all solid tumours have a median oxygen concentration less than 10 mm Hg instead of 40-60 mm Hg in normal tissues) [9], the expression of antioxidant enzymes would have no utility and should therefore be repressed.

THE ASCORBATE/MENADIONE COMBINATION: AN ORIGINAL WAY TO INDUCE AN OXIDATIVE STRESS

Before starting the discussions about the combination of ascorbate and menadione, a brief overview of each compound will be given in the following pages.

Ascorbic Acid

Vitamin C was first isolated in 1928 by the Hungarian biochemist Dr. Szent-Gyorgyi who received the Nobel Prize for its discovery in 1937 [41]. In many species, ascorbic acid (Fig. (**1**)) can be generated de novo through the hexuronic acid pathway of the liver or the kidney. This is due to the activity of a particular enzyme, the gulonolactone oxidase. Since humans (as well as other primates, guinea pigs and a few bat species) lack this enzyme, they cannot synthesize

ascorbic acid and must satisfy a high requirement in foods, notably in fruits and vegetables [42]. That is the reason why ascorbic acid is considered as a vitamin. Ascorbic acid is a potent water-soluble antioxidant in biological fluids, and also possesses a variety of other functions. It acts as a cofactor in a number of hydroxylation reactions, by transferring electrons to enzymes that provide reducing equivalents. Thus, it is notably required for the conversion of certain proline and lysine residues in procollagen to hydroxyproline and hydroxylysine in the course of collagen synthesis, a role that explains its trivial name of "ascorbic acid" which designates its function in preventing scurvy.
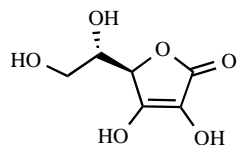
Fig. (1). Ascorbic acid

Ascorbic acid is transported by sodium-dependent transporters SVCT1 and 2 [43]. SVCT1 is largely confined to the bulk transporting epithelial systems (intestine, kidney, liver) and other epithelial tissues (lung, epididymus and lacrymal gland), whereas SVCT2 is widely expressed. Despite the existence of these transporters for ascorbic acid, several tissues (e.g. the erythrocytes) prefer the transport of the oxidized form of ascorbate, dehydroascorbate (DHA), by the glucose transporters (GLUTs) [44]. DHA is then rapidly reduced on the internal side of the plasma membrane, thus preventing its efflux and allowing the accumulation of ascorbate against a concentration gradient. Since tumours frequently overexpress GLUT, this mechanism explains why they present high amounts of vitamin C by comparison to their tissues of origin [11, 45-47].

With the exception of scurvy prevention, vitamin C possesses few pharmacological actions. Nevertheless, an extensive literature exists on the use of this vitamin in a wide variety of diseases, notably for cancer, where it has a controversial history (for review, see Gonzalez *et al.*, 2005) [48]. In 1954, W.J. McCormick, a Canadian physician, observed that the generalized stromal changes of scurvy were identical with the local stromal changes observed in the immediate vicinity of invading neoplastic cells. Following his observations, he formulated the hypothesis that cancer is a collagen disease, secondary to a vitamin C deficiency [49]. Twenty years after McCormick, Linus Pauling and Ewan Cameron proposed the use of vitamin C supplementation in large doses for the prevention and treatment of cancer [50] but the attempts to duplicate the amazing results obtained by Cameron and Pauling showed that high-dose vitamin C therapy (10 g per day orally) was not effective against advanced malignant disease [51,52]. Although new pharmacokinetic data claim that i.v. administration of vitamin C could act as a new non-toxic adjuvant therapy by achieving higher plasma concentrations than those reached orally, the use of only vitamin C in cancer therapy remains controversial and is yet to be proven [53-58].

Menadione

Menadione (also known as vitamin K_3) (Fig. (**2**)) is a synthetic derivative of the naturally occurring vitamins K_1

and K_2. Vitamin K is an essential nutrient for the production of clotting factors II, VII, IX, and X in mammals by acting as a cofactor in the enzymatic carboxylation by gamma-glutamyl-carboxylase of glutamic acid residues forming gamma-carboxyglutamic acid in plasma proteins [59]. Actually, menadione acts rather as a provitamin since it has to be converted into active homologues by the liver [60,61]. It is generally considered now that menadione is ineffective for the treatment of excessive anticoagulation [62].
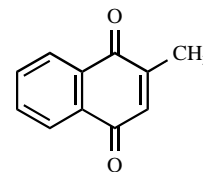
Fig. (2). Menadione

As for vitamin C, a sizeable amount of research has been done on vitamin K and cancer (for review, see Lamson *et al.*, 2003) [61]. Actually, most of these studies have focused on menadione. Indeed, menadione clearly presents a greater toxicity than its congeners, both *in vitro* and in tumour-bearing mice models [63,64]. *In vitro*, menadione is effective against a wide variety of tumour cells at concentrations that are clinically achievable (IC50 values usually ranging from 10 to 50 µM) [65,66]. Basically, two phenomena explain the toxicity of menadione towards cancer cells: oxidative stress and covalent binding (also known as arylation process). Oxidative stress is considered to be a mechanism of action of quinoid compounds because toxic oxygen species can be generated during redox cycling involving the quinoid structures. In the case of menadione, the oxidative stress generated appears to be dose-dependent and capable to induce both apoptosis and necrosis, depending on the doses used [67]. A second mechanism of toxicity for menadione and related quinones is the direct arylation of cellular thiols resulting in glutathione (GSH) depletion. Once GSH is depleted, cellular macromolecules start to be alkylated, resulting in their inactivation [68]. This is particularly true for sulfhydryl-dependent proteins such as Cdc25 phosphatases. These latter enzymes are the key regulators of the cell cycle since they control the phosphorylation of the cyclin-dependent kinases, thereby regulating their activity and cell cycle progression. Menadione, as well as other vitamin K derivatives, was notably described to covalently bind to the catalytic domain of Cdc25A phosphatase, leading to the formation of an inactive hyperphosphorylated Cdk1 [69]. In addition, menadione was reported to also inhibit cyclin E expression at the late G_1 phase and cyclin A expression at the G_1/S transition. Since Cdc25A phosphatase regulates both G_1/S and G_2/M cell-cycle transitions [70], its inhibition by vitamin K derivatives explains how these compounds can inhibit cancer cell growth [71,72]. These mechanisms were particularly well studied in the case of hepatoma cell lines, a kind of tumour for which menadione and other vitamin K derivatives seem to be particularly effective [73,74]. The encouraging results of these *in vitro* investigations have led to different human trials in patients suffering from gastrointestinal and lung cancer. These phase I and II trials were conducted in the 90's and established the maximum tolerated dose of menadione at 2.5 g/m^2 as a continuous intravenous

infusion [75-78]. It must be noted that the administration of high doses of menadione (4 and 8 g/m^2) was associated with hemolysis, despite the presence of red blood cell glucose-6-phosphate dehydrogenase. Interestingly, no resulting coagulopathy was recorded, a fact already observed in clinical trials conducted with vitamin K_1 [79].

The Ascorbate/Menadione Combination

The first description of a synergistic antitumoural effect developed by the combination of ascorbate and menadione was made nearly forty years ago. In 1969, a team from the National Cancer Institute observed that menadione at a very low dose (1 ppm) was able to greatly potentiate the cytotoxicity of ascorbate towards Ehrlich ascites carcinoma cells *in vitro* [80]. The authors already postulated that the cytolytic effect was due to the intracellular production of hydrogen peroxide. These observations are remarkable since they are still the basis of many recent studies dealing with the toxicity of ascorbate [53, 58]. At the same time, the reports of Daoust and collaborators described the deficiency of nuclease activity in both animal and human tumours [81]. Since this decrease was associated with cancer progression, several attempts were made in order to reactivate these enzymes. Indeed it was thought that such a reactivation could lead to cancer cell necrosis as well as cancer regression. A few years later, Taper discovered that vitamins C (ascorbate) and K_3 (menadione) were able to reactivate alkaline (DNase I) and acid DNase (DNase II) respectively [82].

In 1987, Taper *et al.* reported a non-toxic potentiation of cancer chemotherapy in TLT (Transplantable Liver Tumour) bearing mice by combined sodium ascorbate and menadione pre-treatment [83]. This potentiation appeared to be non-specific for a particular chemotherapy. Indeed alkylating agents (cyclophosphamide, procarbazine) as well as an antimetabolite (5-fluorouracil), an enzyme (asparaginase), a mitotic inhibitor (vinblastine) or an anthracycline derivative (adriamycin), all appeared to be potentiated by the ascorbate/menadione association. It should be noted that a similar potentiation of gemcitabine (an antimetabolite) was recently reported, both in *in vitro* and *in vivo* experiments in which human urothelial tumours were used [84]. The first results obtained by Taper highlighted an anticancer effect produced by the combination itself, a fact that was later confirmed by the demonstration that the ascorbate/menadione combination completely suppressed the tumour cell growth of three different cell lines (namely MCF-7, a breast carcinoma; KB, an oral epidermoid carcinoma and AN3-CA, an endometrial adenocarcinoma) [85]. In this *in vitro* study, Taper and colleagues reported a synergistic inhibition of cell growth at 10 to 50 times lower vitamin concentrations than that required when either agent was used alone. A few years later, using the same cell lines, a synergism was also described between ascorbate/ menadione and various chemotherapeutic drugs [86], a fact already described *in vivo* [83]. Although the precise mechanism explaining this synergy remained obscure, a hydrogen peroxide-mediated oxidative stress was suggested since the addition of catalase to the culture medium suppressed the antitumour effect [85]. The sensitisation of cancer chemotherapy by ascorbate/menadione pre-treatment in a mouse tumour resistant to Oncovin (vincristin) was also described in 1992 [87]. As for chemotherapy, a potential synergism was investigated between ascorbate/menadione and radiotherapy [88]. Mice with intramuscularly transplanted solid liver tumours were orally and parenterally pretreated with combined ascorbate and menadione before being locally irradiated with single doses of X-rays (ranging from 20 to 40 Gy). For all the X-ray doses tested, a synergism with the combination was described. Finally, an inhibition of the development of metastases was reported in 2004 [89]. In this article, Taper investigated the effect of a dietary ascorbate/menadione combination on the metastasis of mouse liver tumour (TLT) cells implanted in C3H mice. Reductions of 50 % and 63 % for lung and lymph node metastases, respectively, were recorded, demonstrating that the oral ascorbate/menadione combination significantly inhibited the metastases of TLT tumours in C3H mice.

Three major conclusions arise from the several studies conducted by Taper during these last twenty years. First, the synergism presented by the ascorbate/menadione combination seems to be related to the production of hydrogen peroxide. Second, the potentiation of both radio-and chemotherapy suggests a non-specific process. This is further confirmed by the fact that various chemotherapeutic agents (acting through different pathways) can be potentiated in a similar way. The third conclusion (and maybe the most important) is the fact that the ascorbate/menadione combination appeared to be selective for cancer cells. Indeed, histopathological examinations did not indicate any sign of toxicity in normal organs and tissues of ascorbate/menadione-treated mice. It should be noted that cumulative evidence suggests that this "apparent selectivity" is more a "differential sensitivity" between healthy and cancer cells, as described by Zhang *et al.* [90]. Actually, both vitamins are able to induce either apoptosis or necrosis depending on the dose, the incubation time, and the cell type utilized [91]. Nevertheless, at the doses at which they were used in our *in vitro* and *in vivo* studies, neither menadione nor ascorbate alone exhibit cytotoxicity. Only the ascorbate/menadione combination was toxic for cancer cells.

In our studies we have sought to examine the mechanisms that could explain the interesting results obtained by Taper *et al.* As further described, the synergistic anticancer effect of the ascorbate/menadione combination is likely explained by the redox-cycling that occurs between these compounds. This generates reactive oxygen species and leads to an oxidative stress particularly deleterious for cancer cells since they exhibit a poor antioxidant status. Among the different cellular pathways that can be impaired, glycolysis is well-known to be rapidly inhibited by an oxidative stress [92]. Since cancer cells have a high energetic dependence on glycolysis, we therefore postulate that this fact, coupled to the poor antioxidant status, is the rationale that explains the preferential killing of cancer cells by the ascorbate/menadione combination, this latter appearing as an interesting strategy that takes advantage of the biochemical features of cancer cells.

It should be noted that the salt form of vitamin C, known as sodium ascorbate, was always used in our studies (*in vitro* as well as *in vivo*), in order to avoid any pH interference. Menadione was used in the form of sodium bisulphite salt because of the poor solubility of menadione.

THE RATIONALE THAT SUPPORTS THE ASCOR-BATE/MENADIONE ANTITUMOURAL EFFECT

A Redox-Cycling to Explain the Synergistic Cytotoxicity

Menadione, like the other quinoid compounds, may be cytotoxic through either electrophilic arylation or redox-cycling. Menadione redox-cycling is greatly increased by the addition of ascorbate; a fact that explains the synergistic antitumour activity of the ascorbate/menadione combination. Actually, menadione is non-enzymatically reduced by ascorbate to form semidehydroascorbate and a semiquinone free radical (reaction 1). Such a semiquinone is rapidly reoxidized to its quinone form by molecular oxygen thus generating ROS (reaction 2). It is noted that the one-electron reduction of menadione driven by ascorbate is not favoured thermodynamically. Indeed, it occurs because the semiquinone product is continuously removed by autooxidation [93].

$$AscH^- + Q \rightarrow SQ^{\cdot -} + Asc^{\cdot -} + H^+ \tag{1}$$

$$SQ^{\cdot -} + O_2 \rightarrow Q + O_2^{\cdot -} \tag{2}$$
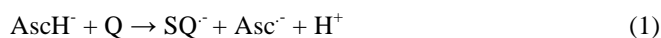
Various compounds without arylation sites (such as 2,3-dimethoxy-naphtoquinone and dichlone) were able to produce the same profile of cytotoxicity as observed for the ascorbate/menadione combination, underlining the key role of redox cycling in the cytolytic process [94-95]. It should be noted that we cannot refute the possible occurrence of some arylations within ascorbate/quinone-treated cells. However, the occurrence of such a pathway, if any, cannot be a worthwhile explanation for the cytotoxicity developed by these associations. Supporting the importance of the oxidative stress, we were able to detect the intracellular presence of ROS in K562 ascorbate/menadione-treated cells [96]. This was further confirmed by the inhibitory effect exhibited by the antioxidant N-acetylcysteine (NAC) that abolishes both the formation of ROS and cell death [95].

Since the higher redox potential of quinone molecules has been correlated with enhanced cellular deleterious effects, we studied the ability of the association of ascorbate with several quinone derivatives to cause cell death. Besides its interest by providing us with new mechanistic insights, this study demonstrated that menadione can be easily replaced. Indeed, several quinoid compounds are able to produce a synergistic cytotoxic effect in the presence of ascorbate, depending on their half-redox potentials. We observed that only quinones having half-redox potentials between −250 and +50 mV were able to be reduced by ascorbate, thus generating a redox cycling and the formation of ROS [95]. Such associations resulted in decreased levels of both ATP and GSH, *in vitro* cell death and decreased tumour cell proliferation in TLT-bearing mice. As for menadione, all tested quinones were devoid of toxic effects by themselves, at least at the concentrations we used. Interestingly, we noted that the type of cell death driven by these combinations between ascorbate and quinone was sharing the same pattern as described for ascorbate/menadione (necrotic-like, caspase-independent). This is in agreement with many previous studies reporting a correlation between quinone potential, reactivity with ascorbate and antitumoural action [97,98].

After having defined redox cycling as the mechanism supporting the cytolytic activity of the ascorbate/menadione combination, the involved oxidizing agent remains to be determined. Among the various ROS that can be generated, the role of hydrogen peroxide was early evoked due to the fact that the addition of catalase completely suppressed cell death [85]. Supporting this hypothesis, our results show a key role of hydrogen peroxide. Firstly, among different antioxidants tested on several cell lines, only catalase and NAC were able to suppress cytolysis [94,95]. Secondly, the absence of lipid peroxidation is consistent with a mechanism of toxicity based primarily on the generation of hydrogen peroxide since this latter agent is ineffective in oxidizing lipids. Thirdly, since hydrogen peroxide is detoxified by the enzyme catalase, we observed that co-incubation in the presence of aminotriazole (a potent catalase inhibitor) leads to the potentiation of the ascorbate/menadione-driven cytotoxicity, as evidenced by the increased release of lactate dehydrogenase into the incubation medium (Fig. (**3**)) [96, 99]. Taken together these data definitely confirm the key role played by hydrogen peroxide. Since cancer cells exhibit a poor antioxidant status, this fact explains their preferential killing by the ascorbate/menadione combination.
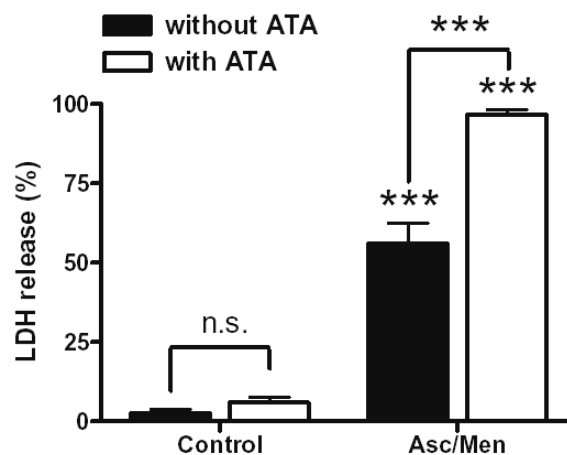

Fig. (3). K562 cells were incubated for 18 h at 37 °C in the absence (control) or in the presence of a mixture of sodium ascorbate and menadione (Asc/Men, 2 mM and 10 mM, respectively). When present, aminotriazole (ATA) was used at 5 mM and preincubated for 1 h. The viability of cells was estimated by measuring the activity of lactate dehydrogenase (LDH), according to the procedure of Wrobleski and Ladue [99] both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as 100 minus the ratio of released activity to the total activity, a percentage that reflects the loss of cell viability. In ascorbate/menadione-treated cells, the addition of ATA reinforces significantly the loss of cell survival, underlining the key role played by H_2O_2 in the cytolytic effect developed by ascorbate/menadione. The results represent the mean ± S.E.M of at least three independent experiments. The effects of ascorbate/menadione and aminotriazole + ascorbate/menadione were significantly different (p < 0.001, using one-way ANOVA) as well as compared to the control group (p < 0.001, using one-way ANOVA).

What are the Intracellular Targets of the Ascorbate/Menadione Combination?

The involvement of H_2O_2 in the cytolytic process driven by the ascorbate/menadione combination is no longer a matter of discussion. Since the exposure to an oxidative stress leads to the activation of MAP kinases, their role in the ascorbate/menadione-induced cell death has been investigated. Since no activation was observed, the MAP kinase pathway probably does not play a major role in the mechanisms underlying the cytotoxicity of ascorbate/menadione [100].

Another classical feature that generally appears after an exposure to H_2O_2 is the activation of the transcription factor NF-κB [101]. However, our results showed that the combination of menadione and ascorbate is inhibiting rather than activating NF-κB, that is the opposite of the expected effect [102]. This observation is likely explained by the fact that most studies performed on H_2O_2 use a bolus instead of a continuous production, as in the case of the ascorbate/menadione combination. Since these two ways of production are known to have completely different effects, this result is finally not surprising [103]. Whatever the cause of this inhibition, it could be an interesting mechanism since cancer cells often exhibit a constitutive activation of NF-κB that contributes to their aggressiveness [104].

Interestingly, we observed that sodium orthovanadate, a well-known inhibitor of protein tyrosine phosphatases, completely suppresses the cytotoxicity induced by the combination of menadione and ascorbate, while other inhibitors of protein kinases and phosphatases failed [105]. The mechanism underlying the suppressive effect of vanadate remains unclear but three different explanations have been postulated: the first one involves a potential interfering reaction by vanadate in redox-cycling. However, this is very unlikely because vanadate did not modify the oxygen uptake observed during the ascorbate/menadione reaction (unpublished data). The second is related to the formation of peroxovanadate, a possible reaction of vanadate with H_2O_2. Such a reaction might consume most (or all) of H_2O_2, thus avoiding its interaction with the intracellular putative targets. Again, this mechanism is very unlikely since a suppressive effect on cytotoxicity was also recorded by the addition of potassium bisperoxo(1,10-phenanthroline)oxovanadate, an oxidized form of vanadate unable to react with H_2O_2 (unpublished data). Finally, the third possibility is that vanadate, by inhibiting tyrosine phosphatases, modifies the phosphorylation state of some critical proteins. The results obtained with another tyrosine phosphatase inhibitor, namely Et-3,4-dephostatin, confirms that the protective effect by vanadate may be attributed to an inhibition of tyrosine phosphatases [105]. Nevertheless, the specific proteins that are involved in this process have not been yet identified.

Since lactate and ATP are severely and rapidly depressed following the exposure of cancer cells to ascorbate/menadione, we suggest that the major intracellular event is related to the impairment of glycolysis. The arrest of glycolysis occurs at the step catalyzed by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and appears as the result of NAD+ depletion [106]. Indeed, we observed that NAD+ is rapidly consumed, leading to the inhibition of GAPDH. The NAD+ depletion is likely related to a strong poly(ADP-ribose) polymerase (PARP) activation, as evidenced by the rapid appearance of poly(ADPribosylated) proteins (PAR) following the exposure to ascorbate/menadione combination (Fig. (**4**)). PARP activation is likely provoked by the DNA damage occurring within ascorbate/menadione-treated cells, as indicated by the phosphorylation of the histone H2AX (the phosphorylated form being known as γ-H2AX), a marker of DNA strand breaks (Fig. (**4**)) [107]. Due to the glycolysis arrest, the ATP depletion will follow, culminating in cell death.
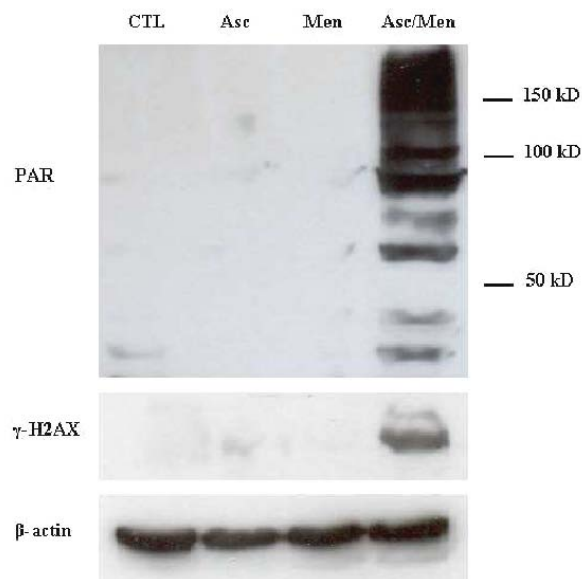

Fig. (4). Effect of ascorbate/menadione on protein poly(ADP)ribosylation in K562 cells. K562 cells were incubated in the absence (control) or in the presence of 2 mM sodium ascorbate, 10 mM menadione or a mixture of both compounds. Cells were harvested after 90 minutes and immunoblotting was performed using antibodies against poly(ADP-ribose) (PAR), ß-actin and γ-H2AX.

Although the activation of PARP is obvious, it does not entirely explain the cytotoxicity triggered by the ascorbate/menadione combination since PARP inhibitors only decrease cell death by 60% [106]. This indicates that other critical events are likely occurring upstream in the cascade leading to cell death, as evidenced by the suppressive effect of vanadate. These results prompted us to suggest that PARP activation is a major but probably not the only intracellular event related to cell death. The way by which PARP is activated is also a matter of discussion. PARP activation occurs in response to DNA damage, but the way by which this latter event occurs remains to be discovered. One might expect that DNA damage induced by the ascorbate/menadione combination is due to transition metal ions (iron and/or copper) which catalyze the formation of hydroxyl radicals in the vicinity of DNA. Since the addition of mannitol (an hydroxyl radical scavenger) failed to protect against the ascorbate/menadione cytotoxicity, this latter pathway is of interest but remains to be demonstrated [95].

A Particular Necrotic Cell Death

Previous reports of ascorbate/menadione driven cancer cell death postulated the occurrence of a new kind of cell death, namely the autoschizis [108]. Indeed, most of ascorbate/menadione-treated cancer cells are characterized by a particular morphology, as nicely reviewed by Jamison *et al.* [109]. These cells exhibit exaggerated membrane damage that drives progressive loss of organelle-free pieces of cytoplasm through a series of self-excisions. Based on this particular morphology, such a cell death was called autoschizis by Gilloteaux *et al.* [108]. During the process, the nucleus becomes smaller and most of organelles relocate around it. At the extreme, cells can be reduced to a perikaryon that consists of an apparently intact nucleus surrounded by a thin rim of cytoplasm containing damaged organelles. At this step, the final cell volume is decreased by at least one-third to one-half the original cell size and karyolysis occurs. Contrary to apoptosis, such a process is accompanied by the release of intracellular constituents, thus provoking *in vivo* inflammatory response [109]. Although this cell death was essentially described from a morphological point of view, several biochemical markers were also used in order to characterize the cell death pathways induced by the ascorbate/menadione combination [110-112].

The absence of caspase activity was reported at any dosage and in different cell lines. This was further confirmed by both the use of caspase inhibitors (Fig. (**5**)) and the analysis of typical caspase substrates [95,96,105]. Such a lack of activity is likely the reflection of the absence of activation since no pro-caspase processing was observed [96, 105]. Interestingly, a weak release of cytochrome c was generally observed upon ascorbate/menadione exposure, coupled to a loss of mitochondrial membrane potential [96, 113]. Since cytochrome c was released from mitochondria without caspase activation, we postulated that apoptotic signals, if any, are being blocked upstream in the caspase activation
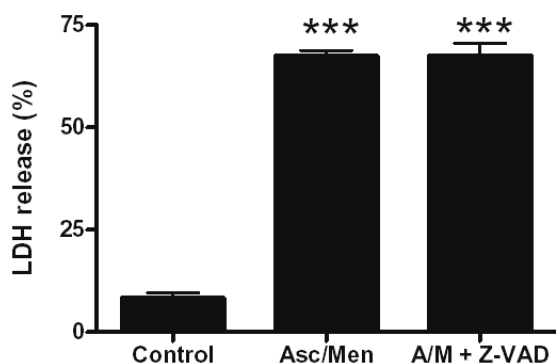

Fig. (5). K562 cells were incubated for 24 hours either in the presence or the absence of the ascorbate/menadione combination (used at 2 mM and 10 μM, respectively). Z-VAD-FMK was used at 10 μM without preincubation. Cellular viability was estimated by measuring the activity of lactate dehydrogenase (LDH), both in the culture medium and in the cell pellet obtained after centrifugation. The results are expressed as a ratio of released activity to the total activity. The results represent the mean ± S.E.M of at least three independent experiments.

pathway. Actually, the absence of apoptosis in ATP-depleted cells (such as in the case of ascorbate/menadione-treated cells) can be explained by the ATP-dependence of some apoptotic steps such as the apoptosome formation [114-116].

The exposure of cancer cells to the ascorbate/menadione combination was reported to induce DNA strand breaks [96]. This damage did not correspond to an oligosomal DNA fragmentation since the electrophoretic profile exhibited a smear pattern [105, 117-122]. Taken together, the absence of caspase activity and the DNA fragmentation profile suggest a necrotic outcome for cancer cells treated by the ascorbate/menadione association. This profile was further confirmed by flow cytometry (double staining with Annexin-V and propidium iodide) [96, 108, 121]. Other experiments of flow cytometry performed on human bladder tumour cells exposed to ascorbate/menadione also revealed a growth arrested population and a population undergoing cell death. Cells in G1 arrested in G1 while those in S phase progressed through S phase and arrested in G2/M [121].

In conclusion, based on the biochemical features of autoschizis as well as on its particular morphology, we suggest that this cell demise induced by ascorbate/menadione must be considered as a subtype of necrotic cell death, according to the recommendation of the Nomenclature Committee on Cell Death (NCDD) [123]. The analysis of the cytoskeletal modifications that occur following the exposure to ascorbate/menadione (cleavage of cytoskeletal proteins, actin polymerisation, etc), as well as the study of other parameters (intracellular Ca^{2+} level, involvement of lysosomal proteases) could be helpful in the future to better define this concept of autoschizis.

Additional Mechanisms to Explain the Antitumoural Effect

In vitro, the oxidative stress is the main mechanism by which ascorbate/menadione exerts its cytolytic action. Nevertheless, we cannot exclude the involvement of other pathways in the *in vivo* activity of this combination. In that sense, several studies were performed, including studies on a putative effect on the immune response, the destabilization of oncogenic proteins and the influence of the association on the angiogenic and metastatic processes. The rationale for studies on the immune response arises from the fact that an association of ascorbate with a benzoquinone has been reported to enhance the immune function [124]. Thus, we explored a putative stimulation of the immune system by the ascorbate/menadione combination on the cytolytic T lymphocyte (CTL) response of mice immunized with tumour-specific peptides model, a model described by Silla *et al.* in 1999 [125]. However, no influence of this combination was noted, suggesting that stimulation of the immune system is unlikely [100].

Another pathway that could explain the antitumoural effect exhibited by ascorbate/menadione involves the degradation of key oncogenic proteins. Indeed, the exposure of K562 cells (a human leukaemia cell line) to this association leads to the degradation of Bcr-Abl, a critical protein for K562 cell survival (unpublished data). Actually, we postulate that the ATP depletion caused by oxidative stress provokes the disassembling of stabilizing complexes between some heat shock

proteins (such as Hsp 90) and Bcr-Abl, resulting in the proteolysis of the latter. Since Bcr-Abl is required for cell survival, its degradation could be an interesting antitumoural mechanism.

The last mechanism investigated was a putative anti-angiogenic effect. Indeed, our results showed that culture media in which tumour cells were incubated in the presence of the ascorbate/menadione combination develop a strong inhibitory effect in one model reflecting the angiogenic capacity (tube formation, unpublished data). This inhibition is not linked to a cytotoxic effect on endothelial cells by the combination itself but rather appears to be mediated by the leakage of an anti-angiogenic compound. The identity of this latter agent remains to be determined, notably by the use of screening methods such as protein arrays. A putative anti-angiogenic effect is of major importance since it could par-

ticipate in the antitumoural effect exhibited *in vivo* by this association, as well as in the inhibition of metastasis described by Taper *et al.* [89].

As our knowledge of the ascorbate/menadione combination growths, it is likely that multiple mechanisms explain its antitumoural activity *in vivo* (Fig. (**6**)). In that sense, many of these pathways are still to be discovered and the involvement of those described *in vitro* remains to be confirmed *in vivo*.

THE FIRST STEPS TOWARDS A CLINICAL APPLICATION?

The ultimate question for every compound expected to possess an antitumoural activity is that of its clinical relevance, taking into account its efficacy as well as its safety. This is usually done through a multistep process that follows
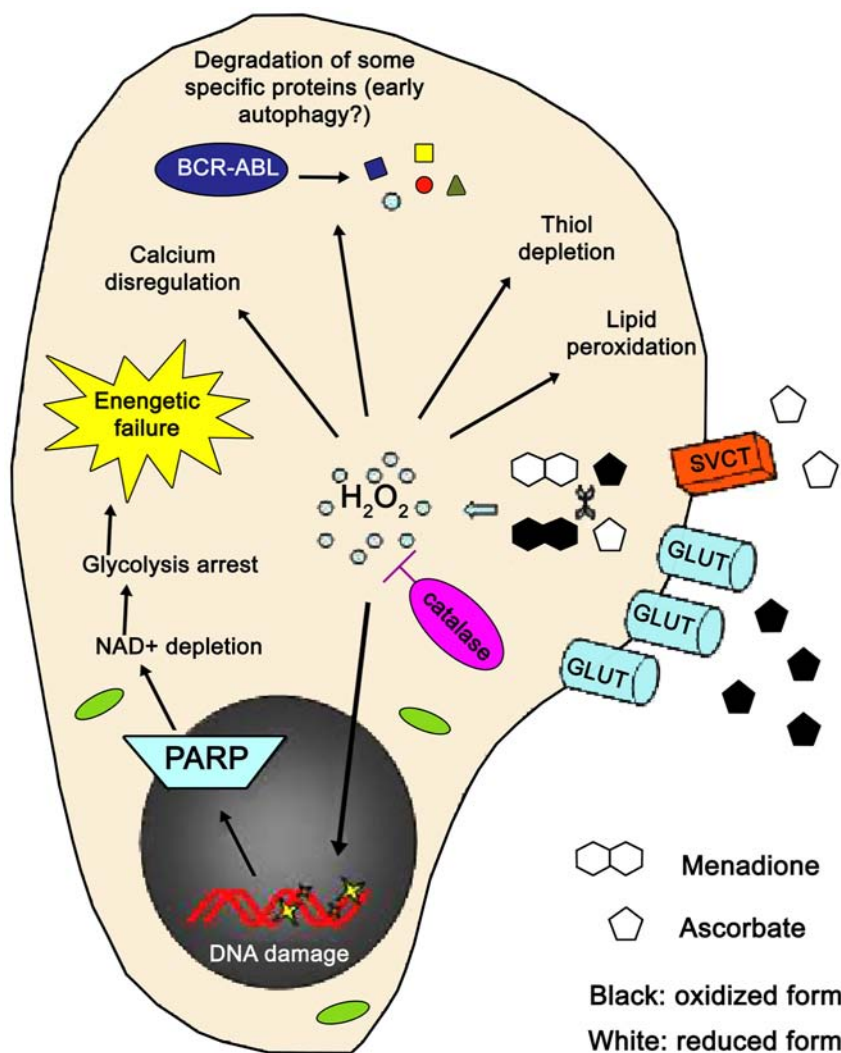

Fig. (6). Schematic illustration of the supposed mechanism of action of the ascorbate/menadione combination. The redox-cyling that occurs between ascorbate and menadione leads to the production of intracellular hydrogen peroxide that triggers a variety of damage. PARP becomes activated in response to DNA damage, leading to NAD+ depletion and subsequent glycolysis arrest. Since cancer cells rely on glycolysis for their ATP production, this provokes an energetic crisis that kills the cell. Dying cells exhibit a necrotic profile reported as autoschizis and characterized by huge membrane deformations. Since cancer cells have a poor antioxidant status and frequently over-express glucose transporters, they are particularly sensitive to the action of ascorbate/menadione, thus explaining the efficacy of the combination against a broad range of tumours.

the guidelines published by international agencies such as the European Agency for the Evaluation of Medicinal Products (EMEA) or the Food and Drug Administration (FDA). The problem with compounds such as ascorbate and menadione is that they are primarily perceived as vitamins, a term associated with the idea of low-toxic products that therefore do not require any pre-clinical evaluation before use in clinics. Nevertheless, as illustrated by Pauling's studies, this lack of early-phase research leads to a bad evaluation of some critical parameters (doses, route of administration, optimal schedule) resulting in mixed results and controversy [48]. Since ascorbate and menadione were already registered as drugs, many data dealing with their pharmacodynamic, pharmacokinetic and toxicological properties are already available. In addition to these data, a lot of knowledge has been acquired about the combination itself.

The usual objectives of pre-clinical studies are the investigation of the mechanism(s) of action, resistance, schedule dependencies as well as the anti-tumour activity of the products *in vivo*. Concerning the mechanism(s) of action, we have clearly identified the redox-cycling of the ascorbate/menadione combination and the subsequent oxidative stress generated as the explanation for the synergistic antitumoural effect exhibited by this combination. This oxidative stress, mainly observed *in vitro*, is responsible for the strong cytolytic effect of ascorbate/menadione, as observed in several cancer cell lines (for review, see Buc Calderon *et al.*, 2002) [94]. According to this mechanism of action, we can anticipate that cancer cell resistance could arise from the overexpression of antioxidant enzymes. In that sense, tumours such as mesothelioma that contain simultaneous overexpression of several antioxidant enzymes and related proteins with antioxidant capacity could be resistant towards ascorbate/menadione [27].

The results obtained in tumour-bearing mice support the effectiveness of the ascorbate/menadione combination *in vitro* (Fig. (**7**)). Nevertheless, these studies were conducted in a few models (murine hepatoma TLT cells, human erythroleukaemia K562 cells and UMUC-14 urothelial carcinoma cells) and remain to be carried out at a larger scale [83,84,87-89,96]. Indeed, these studies have highlighted the *in vivo* antitumoural activity of the ascorbate/menadione combination but did not provide us with a typical schedule of treatment. Most of the work performed in rodents was using i.p. injections of 1g/kg and 10mg/kg of ascorbate and menadione respectively. Similar doses have already been used in humans with each compound separately, and these appeared to be relatively safe [57, 75]. Nevertheless, since the ascorbate/menadione combination exerts a synergistic effect, this prompts us to recommend a preliminary phase I study in order to determine the maximal tolerated doses for the association itself. This could be achieved by using the classical ratio of 100 to 1 (for ascorbate and menadione, respectively), a ratio that was previously demonstrated as the most effective one [85]. Hence a safe dose will be defined, thus avoiding a misevaluation of the antitumoural effect of the combination.

The route of administration is another critical parameter that remained to be assessed. Although oral administration was effective *in vivo* and appeared to be the easiest, pharma-

cokinetic data suggest that is not appropriate for the administration of high doses of ascorbate in humans [54]. Indeed, complete plasma saturation occurs at the daily oral dose of 1000 mg of ascorbate [55,56]. This dose was associated with a plasma concentration of approximately 100 μM, whereas our *in vitro* conditions use 2 mM ascorbate that is twentyfold higher. Therefore, the only possibility to reach such "pharmacologic" concentrations is the i.v. administration [53,57, 58]. As highlighted in this section, considerable work has already been carried out on the ascorbate/menadione combination but an optimised protocol remains to be discovered (i.e. route of administration, doses, schedule) and the putative clinical applications are still to be explored as well.
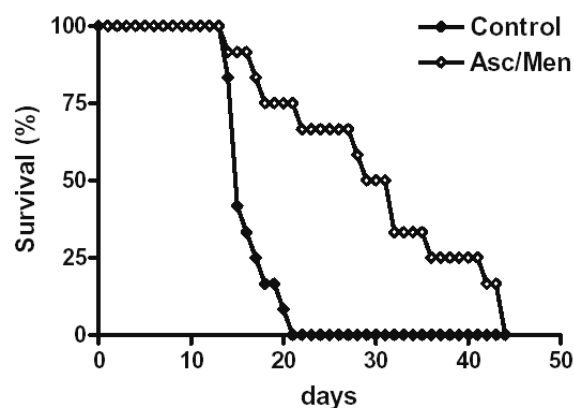

Fig. (7). Effect of ascorbate/menadione on the survival of mice bearing the ascitic TLT tumour. Approximately 10^6 of Transplantable Liver Tumor (TLT) cells were intraperitoneally (i.p.) injected into male NMRI mice. Forty-eight hours after implantation, ascorbate/menadione-treated group was injected i.p. with a mixture of sodium ascorbate and menadione sodium bisulfite (1 g/kg and 10 mg/kg, respectively). The mean survival time was of 15 days for the control group whereas it reached 29 days for the group treated by ascorbate/menadione (p<0.001, using Student's t-test).

CONCLUSION

The combination of ascorbate and menadione could be of particular interest in cancer therapy for several reasons. First, since many tumour cells exhibit defects in the apoptotic signalling pathways, there is a need for additional death mechanisms. Second, since cancer cells are often deficient in antioxidant defenses and accumulate ascorbate through the overexpression of glucose transporters, they are expected to be more sensitive towards an oxidative stress induced by the ascorbate/menadione combination than healthy cells. Third, due to the high energetic dependence of cancer cells on glycolysis, the impairment of such an essential pathway may explain the effectiveness of this combination on many tumour cells. Taken together, our data suggest that the association of ascorbate/menadione could represent a new interesting auxiliary cancer therapy. Since both compounds are lowtoxic and cheap, they could be, indeed after a correct preclinical evaluation, easily included in the classical anticancer protocols without any supplementary risk for the patients.

ACKNOWLEDGEMENTS

ABBREVIATIONS

CTL   =   Cytolytic T lymphocyte

DHA   =   Dehydroascorbate

HK   =   Hexokinase

hsp   =   Heat shock protein

GAPDH   =   Glyceraldehyde-3-phosphate dehydrogenase

GLUT   =   Glucose transporter

GSH   =   Glutathione

LDH   =   Lactate dehydrogenase.

NAC   =   N-acetylcystein

PAR   =   Poly(ADP-ribosylated) proteins

PARP   =   Poly(ADP-ribose) polymerase

PET   =   Positron-emission tomography

PSA   =   Prostate-specific antigen

ROS   =   Reactive oxygen species

SOD   =   Superoxide dismutase

TLT   =   Transplantable liver tumour

Trx   =   Thioredoxin

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