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## REVIEW

# The antimutagenic and cytoprotective effects of amifostine: the role of p53

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**Summary**
Radiotherapy and chemotherapy are the basic approaches in cancer treatment, but these procedures are often associated with a number of undesirable side effects worsening the quality of life of the patient. In recent years a number of protective compounds capable of reducing or eliminating these side effects have been extensively investigated (for example: dexrazoxan, mesna, glutathion or N-acetylcystein). One of these compounds is amifostine (WR-2721), a broad-spectrum cytoprotective drug, selectively protecting normal tissues from the toxic effects of therapy, while the malignant tissues are subject to the anti-tumour effects of the treatment. In addition, several studies have revealed certain antimutagenic activities of amifostine making this agent potentially useful in the prevention of therapy-induced secondary malignancies. This article summarizes the information available on the antimutagenic and cytoprotective effects of amifostine and its effects also on the activity of the p53 tumour suppressor.

*Keywords:* amifostine – tumour suppressor p53 – cytoprotective and antimutagenic compounds – WR-2721 – WR-1065

## HISTORY OF AMIFOSTINE DEVELOPMENT AND ITS CLINICAL USAGE

Amifostine (S-2[3-aminopropylamino-] ethyl phosphorothioic acid; Ethyol®), previously known as WR(Walter-Reed)2721, was originally developed as a radioprotective reagent during the Cold War in the 1950s by the Walter Reed Institute as part of the Anti-radiation Drug Development Programme of the US Army. The goal of this program was to identify compounds that were capable of protecting the military and civil persons from the adverse effects of radiation in the event of a nuclear war. More than 4400 sulphydryle-containing substances with radioprotective properties were tested and amifostine was found to exhibit the most effective radioprotection and the lowest toxicity profile as evidenced by its MTD (Maximum tolerated dose). Consequently, it was investigated for its potential role in radiotherapy and chemotherapy by alkylating agents, organoplatine derivates and anthracyclines (Santini

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and Giles 1999). In 1969 Yuhas and Storer (1969) demonstrated that amifostine-pretreated normal tissues were protected from the non-specific toxicity of therapeutic radiation while tumour tissues were not. During the 1980s clinical studies of phase I and II accumulated evidence that amifostine could protect normal tissues not only from irradiation but also from chemotherapy. In 1996 amifostine was registered by the Food and Drug Administration (FDA) as a cytoprotective agent suitable for application during cisplatin-based chemotherapy of ovarian cancer (Andreassen et al. 2003). Nowadays, amifostine is considered as a broad-spectrum radio- and chemoprotective drug, and it has been clinically used as a reducer of the toxic side effects induced by a number of anticancer agents in patients suffering from various types of tumours (their list is presented at "Ethyol monograph" and on the web pages http://www.drugs.com)

Amifostine is generally well-tolerated and its application is associated only with transient side-effects including nausea, vomiting, sneezing, sleepiness and occasional allergic reactions (Hensley et al. 1999). With daily administration schedules hypocalcaemia may occur, probably resulting from the inhibition of parathyroid hormone activity (Wadler et al 1993). The most clinically significant toxic effect induced by amifostine is hypotension (Andreassen et al. 2003).

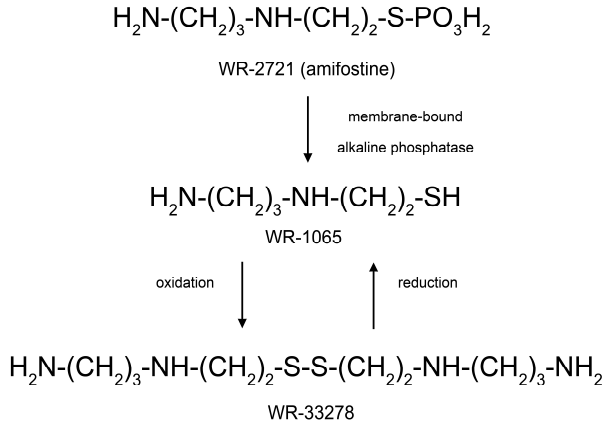


Fig.1. **Metabolism of amifostine** (adapted according to Andreassen et al. 2003)

## METABOLISM OF AMIFOSTINE

WR-2721 is rapidly dephosphorylated *in vivo* either spontaneously or due to the activity of alkaline phosphatase, a membrane-bound enzyme. The resulting free thiol, WR-1065, represents its active form (Shaw et al. 1999) and is the main metabolite of amifostine. It is responsible for the cytoprotective effects that can easily penetrate cells. Amifostine itself cannot mediate cytoprotection (Hensley et al. 1999). WR-1065 can be consequently oxidized to a symmetrical disulfide WR-33278 or a mixture of disulfides with endogenous thiols and thiol-containing proteins (van der Vijgh and Korst 1996) (Fig. 1).

## AMIFOSTINE PREFERENTIALLY PROTECTS NON-NEOPLASTIC TISSUE

The mechanism by which amifostine exerts its selective protection of normal tissues is based on the ability of WR-1065 to reach higher concentration in cells of normal tissues than in cells of tumour tissues. This could be caused by a combination of several biological features. The concentration of alkaline phosphatase is higher in normal cells than in most types of tumour cells. Therefore conversion of WR-2721 to its active form is more effective in non-neoplastic tissue (Capizzi 1999). The efficiency of the uptake of WR-1065 varies markedly in different tissues. Organs with an extensive uptake of WR-1065 include kidneys, salivary glands, bone marrow, liver, heart, lungs, and intestinal mucosa, whereas a low concentration of amifostine has been detected in the brain and spinal cord (Santini and Giles 1999).

Another reason for amifostine´s selective protection effects is the poor vascularisation of most tumours in comparison with normal tissues, resulting in a significantly reduced supply of drugs to tumours (Culy and Spencer 2001). The difference in pH between normal and tumour tissue also plays a role. A relatively high pH in normal tissues is optimal for the metabolism and uptake of active WR-1065 (Calabro-Jones et al. 1988). In addition, amifostine is not dephosphorylated by acidic phosphatase, therefore the acidic pH

associated with most tumours reduces the formation and uptake of WR-1065.

Most preclinical studies show that amifostine did not reduce, and in specific instances rather enhanced the cytotoxic effect of radio- or chemotherapy of tumour tissue (Culy and Spencer 2001). Nevertheless, there are some experimental studies indicating that amifostine may demonstrate tumour-protective effects, but to a much less extent than with normal tissues. No evidence of tumour protection has been reported in clinical trials (Zhang et al. 1992, Koukourakis 2003).

## THE ANTIMUTAGENIC AND CYTOPROTECTIVE EFFECTS OF ACTIVE THIOL WR-1065

As mentioned earlier, the WR-1065 metabolite is responsible for the cytoprotective effect of amifostine (Capizzi 1999). The mechanism is highly complex and involves the induction of the cascade of the intracellular molecular processes. During cancer therapy, free radicals are formed which can cause damage to, or death of cells by affecting the biomolecular structures of lipids, proteins and DNA (Camhi et al. 1995). Scavenging of these reactive species using the SH group at the end of the molecule is considered to be the main mechanism of the WR-1065 cytoprotective action (Kataoka et al. 2000). WR-1065 can also directly bind to the active derivates of alkylating and platine compounds, inactivate them and thus prevent DNA damage (DeNeve et al. 1988).

Recent studies have also suggested other ways in which amifostine´s derivates can affect cell response to cytotoxic stress: e.g. inhibition of apoptosis (Ramakrishnan and Catravas 1992, Romano et al. 1999, Provinciali et al. 1999), alteration of gene expression (Woloschak et al. 1995, Liu et al. 1997, North et al. 2000), and modification of enzyme activity (Murley and Grdina 1995). For example, the active form WR-1065 can activate the DNA-binding activity of the redox-sensitive transcription factor NF-κB (Murley et al. 2001), alter the expression of a number of genes including thymidin kinase (Woloschak et al. 1995), *c-myc* (Liu et al. 1997), or manganese superoxide dismutase (Murley et al. 2001), and affect the activity of topoisomerase-IIα which is involved in DNA synthesis (Murley et al. 1997). Modulations of the p53 tumour suppressor-controlled- signal pathways are also involved in the mechanism of WR-1065's cytoprotective effects (Pluquet et al. 2003b). Another metabolite of amifostine, WR33278, is structurally similar to natural polyamines, and it can also affect the processes of DNA synthesis, repair, gene expression, and cell cycle (Savoye et al. 1997). These properties especially are responsible for the antimutagenic effects of amifostine´s derivates, and for limiting the risk of the therapy-related incidence of secondary malignities (Grdina et al. 2002a). In addition to the acute cytoprotective action, where WR-1065 must be present at the time of the irradiation or anti-cancer drug treatment, antimutagenic properties are also evidenced after the action of the therapeutic agent has finished. Classical cytoprotection requires administration of amifostine in high concentrations. These heavy doses of amifostine must be applied intravenously. On the other hand, the antimutagenic effects of WR-1065 can be induced in much lower doses (10-20 times lower) than for cytoprotection. For prevention of therapy-induced mutagenesis oral administration of amifostine is possible (e.g. with liposomes) (Grdina et al. 2002b).

## THE ROLE OF THE p53 PROTEIN IN THE AMIFOSTINE-INDUCED CELLULAR ACTION

*Activation of the p53 pathway by the amifostine metabolite WR-1065*

The p53 tumour suppressor is a sequence specific transcription factor that activates or suppresses expression of several target genes in response to stress signals. This affects several important cellular processes such as apoptosis and cell growth. The choice of the genes that are activated or repressed by p53 in response to a specific stress signal depends on many factors including the character and intensity of the stress, the ensuing modifications of the p53 protein, interactions of p53 with other cellular proteins, and the physiological and genetic background of the cells expressing p53 (Vousden 2002).

Target genes transactivated by p53 include regulators of the cell cycle in $G_1$ and $G_2$ phases (*p21*$^{Waf1}$, *GADD45, 14-3-3-σ*), regulators of apoptosis (*Bax-1*, *Aip-1*, *APO-1/Fas, Apaf-1*), and genes involved in the control of intracellular redox metabolism (*PIG-3*, *COX-2, NOS-2*). The p53 protein also regulates DNA replication, transcription, and repair through mechanisms which involve the direct formation of complexes with several other cellular proteins (Vousden and Lu 2002).

Several *in vitro* studies indicate that activation of the p53 pathway can play an important role in the mechanism of the cytoprotective and antimutagenic action of amifostine´s metabolites (North et al. 2000, Lee et al. 2003). The exposure of cultured cells to WR-2721 or WR-1065 leads to p53 protein accumulation, activation, and subsequent induction of the p53 target genes resulting in cell cycle arrest (North et al. 2000). The

cells treated with WR-1065 clearly induce an expression of the gene coding for the cell cycle regulators *p21$^{Waf1}$* and *GADD45* and slightly of the gene coding for MDM2. No induction of transcription of the pro-apoptotic genes, such as *Bax-1* or *PIG-3* was detected. Transcription of the *p21$^{Waf1}$* gene coding for an inhibitor of cyclin-dependent kinases causes cell cycle arrest in the $G_1$ phase (North et al. 2000, Shen et al. 2001, Mann and Hainaut 2005). Therefore, WR-1065 induces the $G_1$-phase cell cycle arrest rather than the pro-apoptotic p53 pathway, thus allowing repair of damaged DNA before replication, and the prevention of fatal incorporation of mutations into the genomes of non-malignant cells (North et al. 2002). However, malignant cells often contain a nonfunctional mutant p53, no p53 or an impaired p53 pathway. When treated with WR-1065, these cells cannot undergo $G_1$ arrest (Sherr et al. 2000). The effectiveness of chemo- and radiotherapy is not altered when this drug is included in the treatment regime. Therefore, the functional status of p53 is one of the factors that are responsible for the selective cytoprotective effect of WR-1065 on non-malignant tissue (Mann and Hainaut 2005).

*WR-1065 activates p53 through JNK*

Under normal conditions, p53 is constitutively repressed by two proteins: MDM2 ("**m**urine **d**ouble **m**inute **2**"), and the inactive form of JNK ("c-**J**un **N**-terminal **k**inase"). These proteins mediate p53 degradation by the proteasome. In response to stress, the p53 protein is post-translationally modified on its N- and C- terminal domains which leads to the protein stabilization (p53 is accumulated in a nucleus), and conversion from its latent to its active form. This active form of p53 can bind the specific DNA sequences of target genes with high affinity.

Transduction of signals in response to DNA-damage is mediated by kinases of the PI3-kinase superfamily (ATM, ATR and DNA-PK), the cell cycle kinases Chk1 and Chk2, and kinases of the MAPK/SAPK family (p38 and JNK). Members of the PI3K and Chk families phosphorylate Ser-15 and Ser-20 in the N-terminus of p53, and in the region of MDM2-binding. These phosphorylations prevent the MDM2-mediated degradation of p53. Regulation of the p53 stability by JNK is MDM2-independent. Binding of the inactive JNK to residues 97-116 marks the p53 for proteosomal degradation. The activation of JNK by biological or chemical stress leads to dissociation of the p53-JNK complex, and active JNK may further play a role in p53 activation by phosphorylation at Thr-81. Activation of JNK is mediated by genotoxic as well as non-genotoxic stress, e.g. heat or osmotic shock, and antioxidative reagents (Prives and Hall 1999, Appella and Anderson 2001, Toledo and Wahl 2006).

The exact mechanism of p53 activation by amifostine and its metabolites is not yet well known. WR-1065 induces the accumulation and stabilization of p53 through alternative stress signal pathways, which are different from those activated by DNA-damage factors (Pluquet et al. 2003b). WR-1065 selectively activates JNK in response to the antioxidant stress induced by WR-1065 treatment. This leads to the phosphorylation of p53 at Thr-81 and the reduction of more than 50% of p53-JNK complexes (Pluquet et al. 2003a). There is a two-fold increase in the level of reduced glutathione in cell line MCF-7 upon treatment with WR-1065, and this supports the existence of anti-oxidant stress induced by WR-1065 (Minet et al. 2001).

Another possible mechanism of p53 activation by the polyamine analog WR-1065 could include disruption of polyamine intracellular metabolism (Mitchell et al. 1998). Kramer et al. (1999) showed that many polyamine antagonists, such as their analogues, may disrupt polyamine metabolism, thus causing activation of the p53-p21-pRb pathway, and inducing the $G_1$-phase arrest in cells with wild type p53. Also in this case, p53 activation is mediated by JNK which is activated in response to either drug induced overexpression or repression of S-adenosylmethionin decarboxylase, the main regulatory enzyme in the biosynthesis of higher polyamines. Furthermore, the inhibition of S-adenosylmethionin decarboxylase by various drugs has been shown to possess antiproliferative and antitumour activity (Paasinen-Sohns et al. 2000).

*WR-1065 directly stimulates the DNA-binding activity of p53 in vitro*

It was shown by Hainaut and Milner (1993) that the p53 protein is sensitive to oxidation-reduction *in vitro*. The p53 protein contains several critical cystein residues located at the DNA-binding surface, and the reduction of these cysteins is important for the sequence-specific DNA-binding capacity. Reduction produces an active, DNA-binding form of the p53 protein while oxidation disrupts functional p53 conformation and inhibits sequence-specific DNA binding.

WR-1065 directly interacts with p53 and modifies its cystein residues by the free sulfhydryl group. This results in increased binding of the p53 protein to the consensus target DNA sequences and increased transactivation of specific target genes. The phosphorylated form of the drug WR-2721 cannnot stimulate DNA-binding *in vitro*, indicating that the free thiol group is essential for this effect (Pluquet et al. 2003b). Similarly, two other redox-dependent transcription factors, NF-κB and AP-1, are activated by WR-1065 *in vitro*, indicating that the redox effect is not specific only for the p53 protein (Shen et al. 2001). Since the p53 protein level increases upon treatment with WR-1065, it is

likely that both mechanisms, i.e. alteration of p53 redox state and protein stabilization by WR-1065, are involved in the DNA-binding activation of p53.

Another hypothesis suggests that the polyamine moiety of WR2721 plays an essential role in modulation of the p53-DNA interaction, as polyamines are heavily charged, and cationic molecules were shown to interact with nucleic acids as well as proteins to stabilize macromolecular complexes. They facilitate oligomerization of nucleosomes *in vitro* and may stabilize the highly condensed state of chromosomal fibers *in vivo* (Pollard et al. 1999). Polyamines have an impact on p53-DNA interactions. They can directly interact with the p53 protein thus stabilizing its active, standard conformation. They can also upregulate the DNA-binding- and transactivation activities of the p53 mutants with a retained capacity for weak interaction with DNA (Maurici et al. 2001).

Using a yeast expression system Maurici et al. (2001) showed that amifostine can partially restore the transactivation function of some flexible p53 mutants. Mutations in the *p53* gene can be found in more than 50% of common forms of human cancers. Most of these mutations are missense mutations disrupting the structure of the p53 DNA-binding domain and thus affecting its ability to interact with p53 responsive elements (p53RE). The majority of mutations completely disrupt the DNA-binding of p53 by affecting the architecture of the DNA-binding domain or by the substitution of amino acid residues essential for direct contact of the protein with DNA. However, a number of mutants still retain some activity towards all or just some certain responsive elements. Most of these mutants selectively bind to "high affinity" p53REs, e.g. to the promoter of the $p21^{Waf1}$ gene ("**w**ild-type **a**ctivator-**1**"), but they fail to bind to "low affinity" p53REs, e.g. to the promoter of the *bax-1* gene (Flaman et al. 1998). These mutations preferentially affect the amino acids of flexible loops between the "β-sandwich scaffold" of the DNA-binding domain, and the amino acids directly interacting with DNA (Maurici et al. 2001). Such mutants often possess a temperature-dependent DNA-binding activity that occurs at the permissive temperature 32 °C. The temperature-dependency correlates with the structural flexibility of the DNA-binding domain. Many temperature-dependent p53 mutants display conformational changes related to changes in biochemical properties, intracellular localization and function (Milner 1995).

North et al. (2002) showed that active WR-1065 has a direct effect on conformational changes of the p53 protein. In non-permissive conditions the conformation of the temperature sensitive mutant V272M is restored upon treatment with WR-1065. In human tumour cell line TE-1 derived from cells of a spinocellular carcinoma that contain one copy of the *p53* gene with V272M mutation, WR-1065 activates the transcription of several p53 target genes and induces growth arrest in the $G_1$ phase. At the same time, the transcription of $p21^{Waf1}$ and *GADD45* is strongly induced, transcription of the *MDM2* gene is induced only weakly, and expression of the *PIG3* gene is not induced at all. These results support the hypothesis that WR-1065 stabilizes the structure of the p53 protein that allows binding of "high affinity"- but not "low affinity" p53REs (North et al. 2002).

*Dependence of amifostine and its metabolites on the p53 status*

The dependence of the effects of amifostine and its metabolites on the status of p53 remains controversial and varies in different cell models and according to the conditions of the amifostine treatment. The ambiguity of results is demonstrated by studies by Lee et al. (2003) and Mann and Hainaut (2005) and. Both studies dealt with the mechanisms of the action of amifostine and the p53 status in colon cancer cell line HCT116. Both studies provide evidence that the mechanism of amifostine action depends on the presence of functional p53 protein in HCT116 cells, but the resulting biological effects are different. In the study by Mann and Hainaut (2005) induction of the expression of the genes involved in the regulation of the cell cycle was detected in cells with functional p53, upon WR-1065 treatment. Expression of most of the proapoptotic genes was not activated; even after the prolonged exposure of cells to WR-1065. In contrast, Lee et al. (2003) observed the induction of apoptosis in cells treated with amifostine, although this effect was again dependent on the p53 status. Cells with functional p53 protein actually displayed partial resistance to amifostine-induced apoptosis, whereas the frequency of apoptotic cells increased upon treatment with amifostine if the cells lacked p53. Mann and Hainaut (2005) explain these controversial effects of amifostine on apoptosis, by the high concentration of amifostine (3.8 mM) used by the Lee group (Lee et al., 2003). In conditions of high amifostine concentration, the intracellular concentration of WR-1065 can reach the cytotoxic level 1.5 mM. In a number of systems, WR-1065 exerts its antimutagenic and cytoprotective effects in concentrations ranging from 100 μM to 1 mM (North et al. 2000). The level of cytotoxic products ($H_2O_2$, acrolein and cysteamine) rises also upon WR-1065 degradation by Cu-dependent amine oxidases present in the serum (Meier and Issels 1995).

Other studies also show the dependence of amifostine on the p53 status. For example, in a human breast carcinoma cell line MCF-7 and mouse 3T3 fibroblasts, amifostine activates the standard form of the p53 protein causing induction of the $p21^{Waf1}$ expression and growth arrest in the

$G_1$/S phase. In contrast, in the MCF-7-derived cell line MDD2 lacking functional p53 protein, no induction of $p21^{Waf1}$ occurred (North et al. 2000). In the p53-null human lung cancer cell line H1299 expressing exogenous functional p53, amifostine enhanced cell sensitivity to the cytotoxic effects of amifostine, and increased the rate of apoptosis when compared with controls H1299 lacking p53 (Pataer et al. 2006). Shen et al. (2001) showed the p53-dependent protection of mouse embryonic fibroblasts treated with WR-1065 from cell death induced by paclitaxel.

Kataoka et al. (2000) studied the dependence of the cytoprotective effects of WR-1065 on the p53 status in four cell lines derived from cells of glioma differing in the p53 status: they produced either the standard p53 protein or the mutant one having missense mutations in the *p53* gene. WR-1065 exhibited the cytoprotective effects in all four cell lines tested that did not depend on the p53 status. The results of this study suggested that not only normal tissue but also tumour cells can be protected from radiation when exposed to sufficiently high doses of WR-1065. Similar results were obtained by Acosta el al. (2003) in experiments with human myeloid leukemia K562 and NB4 cells transfected with the *p53* gene containing the temperature sensitive mutation. In these cells, amifostine impaired the p53-dependent apoptosis by silencing the apoptosis-related genes. This leaves opened the possibility that amifostine could reduce the effectiveness of anti-tumour therapies if dependent on active p53. There are several factors that can reduce these adverse effects of amifostine and its metabolites *in vivo*. First, conversion of amifostine by alkaline phosphatase to its active metabolite WR-1065 is an essential step in the cytoprotective action of amifostine. Differences in activity of this enzyme in malignant and non-malignant cells have an impact on the formation of WR-1065. Second, the cytoprotective effect of WR-1065 is also directly dependent on its final concentration in a tissue. In a tumour tissue, the concentration of WR-1065 is relatively low due to the lower content of alkaline phosphatase and the relatively poor functional vasculature of solid tumours as compared to normal tissues. At such low concentrations that do not exceed 0.1 mM, WR-1065 fails to protect cells from radiation-induced cell death. For cytoprotection, the minimum threshold level of amifostine must be exceeded (Hensley et al. 1999).

## CONCLUSION

Amifostine is a cytoprotective and antimutagenic compound which selectively protects normal tissues from the toxic effects of radio- and chemotherapy. Although the main mechanism of cytoprotection by this agent is presumably based on scavenging free radicals, the active metabolite of amifostine WR-1065 exhibits a pleiotropic effect on several signal pathways involved in cytoprotection. One of the proteins affected by WR-1065 is the p53 tumour suppressor which plays a central role in the response of the cell to various types of stress. WR-1065 induces accumulation and activation of the p53 protein, thus inducing expression of the p53 target genes, preferential regulators of the cell cycle. These findings indicate that it is the ability to modulate the p53-driven pathways that is responsible for the cytoprotective function of amifostine and its metabolite WR-1065.

There are also studies describing the opposite effects of amifostine: protection of cancer cells and partial suppression of anti-cancer therapy depending on the p53 status. Hence, it is necessary to exactly elucidate the molecular mechanisms responsible for the effects of amifostine and its derivates on cells as well the role the p53 protein plays in these processes.

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