The effect of Amifostine, in chemotherapy, radiotherapy, as potential Cytoprotectant, and Immunomodulatory, in Cancer and Autoimmunity Treatment and Prevention

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Abstract: It was investigated for its potential role in radiotherapy and chemotherapy by alkylating agents, organoplatinederivates and anthracyclines(1),(2), 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(3).

Certain cancers with proximal tumor spread (such as Hodgkin's disease and lung cancer) require large-field radiation therapy, which increases the potential for injury to normal tissue.

Previous experimental studies provided preliminary evidence that modulation of the radiation response of the CNS in vivo by systemic administration of amifostine is possible and feasible.

Several studies have revealed certain anti-mutagenic activities of amifostine making this agent potentially useful in the prevention of therapy-induced secondary malignancies.

Initial preclinical studies demonstrated that amifostine could protect treated mice from lethal doses of radiation, and this protection did not extend to transplanted mammary tumor cells (2).

In this article, I discuss Amifostine, the Cytoprotectants effect against chemo- and radiotherapy induced cytotoxicities, Chemistry of amifostine, Pharmacodynamic properties of amifostine,Protection against cytotoxic chemotherapy, Protection against radiotherapy, cytotoxic effects of radiotherapy as well as the role of p53 Protein in the Amifostine Induced Cellular Action

Key Word: Amifostine, chemotherapy, radiotherapy, Cytoprotectant, Immunomodulatory, Cancer, and Autoimmunity

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1. Introduction

Amifostine is a naturally occurring thiol that canprotect cells from damage by scavenging oxygen-derived freeradicals(4).Preclinical studies showed that this agent wasable to selectively protect normal cells from cytotoxicity withoutaffecting tumor cells(5),(6),(7).Amifostine is a prodrug that isdephosphorylated to the active agent WR-1065 by membranealkaline phosphatase. Amifostine is able to exert its cytoprotectiveeffect by the disparity in uptake in normal cells comparedto tumor cells. There is a much lower uptake in tumorcells. Tumor cells have low membrane alkaline phosphataseactivity and a low pH(8).This results in decreased uptake andreduced metabolism to the active agent culminating in an upto a 100-fold difference in concentration between normal andtumor cells(9).The maximum tolerated dose is not knownand was not reached in the phase I studies. The currently re-commended dose range is from 740-910 mg/m2(10).Amifostineis well tolerated with the main toxicities being nausea, sneezing, allergic reactions, metallic taste and hypotension (5),(11),(12),(13),(14).Transient hypocalcaemia has also been reportedand is on the basis of an inhibition of parathyroid hormone(1),(15).Cisplatin is associated with a dose-dependant reversiblenephrotoxicity that may be reduced by amifostine. In atrial involving 242 patients with advanced ovarian cancer,patients were randomized to receive cyclophosphamide andcisplatin (100 mg/m2) with or without amifostine (910 mg/m2).There was a statistically significant difference in the reductionin glomerular filtration rate in the patients not receiving amifostine(33 versus 10% had a >40% reduction). There wasalso a significant difference in the number of patients able toreceive treatment as scheduled after the 5th cycle. This studyalso demonstrated significant reductions in the incidence offebrile neutropenia, days in hospital and grade 2 or 3 neurotoxicity.There was no difference in response rate or survival.This trial was designed to detect toxicity differences and maybe too small to detect small differences in efficacy or survival(14).This study has had little impact on current practice, asthere is evidence demonstrating equivalence of carboplatinand cisplatin. Carboplatin is much better tolerated than cisplatinand has much less nephrotoxicity. It is important to beaware of a potentially significant drug interaction with carboplatinand amifostine. Amifostine increases the area

underthe curve of carboplatin, which may be due to the transientreduction in GFR associated with the transient hypotension(16).Although amifostine does not appear to have an impacton survival caution is advised in its use in potentially curativemalignancies such as germ cell tumors as there is a theoreticalrisk of inhibition of antitumor effect. It has been suggestedthat amifostine may increase the potential for dose escalationof cisplatin. There is however no data at this stage showinga clear and significant benefit for increasing dose intensityin ovarian cancer.

2. Metabolism of Amifostine

WR-2721 is rapidly dephosphorylated *in vivo* eitherspontaneously or due to the activity of alkaline phosphatase, a membrane-bound enzyme. Theresulting free thiol, WR-1065, represents its active form(17),and is the main metaboliteof amifostine. It is responsible for thecytoprotective effects that can easily penetrate cells.Amifostine itself cannot mediate cytoprotection(4). WR-1065 can beconsequently oxidized to a symmetrical disulfideWR-33278 or a mixture of disulfides withendogenous thiols and thiol-containing proteins(16).

3. Biochemical properties, pharmacokinetics and metabolism

Following administration, amifostine becomes widelydistributed throughout the body and accumulates in highestconcentrations in the kidney, salivary glands, intestinalmucosa, liver and lung(18),(19). It does not cross the blood–brain barrier and has low accumulation in skeletal muscle.Approximately 4% is bound to plasma protein. In tissueamifostine is dephosphorylated to its active form, WR-1065,by alkaline phosphatase(20).The lower pH in tumor andcomparative abundance of functional alkaline phosphatasein normal tissues versus tumor partially accounts for theselective uptake of WR-1065 and protection of normaltissue(20).Active transport of WR-1065 into normal tissues,compared with passive or reduced active transport in tumorcells(9),and poor or altered blood flow in tumor capillariesmay also contribute to selective accumulation of WR-1065in normal tissue versus tumor cells. WR-1065 affectsexpression of genes involved in apoptosis, cell cycle regulationand DNA repair(21),and can induce expression of MnSOD(manganese superoxide dismutase), contributing to delayedradioprotective effects(22). In addition to WR-1065,amifostine has an inactive disulfide metabolite, WR-33278.WR-2721 is rapidly metabolized and taken up in tissue(half-life, 8 min), with a terminal half-life of WR-1065 of~□7 h. The prodrug and its metabolites are excreted renally.

4. Cytoprotectants against chemo- and radiotherapy induced cytotoxicities

4.1. Cytoprotectants

Dose-limiting toxicity secondary to antineoplastic chemotherapy and/or radiotherapy isdue to the inability of cytotoxic drugs to differentiate between normal and malignantcells. The consequences of this may include impairment of patient quality of life becauseof toxicity, and reduced tumor control because of the inability to deliver adequate doseintensive therapy against the cancer. Specific examples of toxicity against normal tissuesinclude cisplatin-related neurotoxicity and nephrotoxicity, myelotoxicity secondary totreatment with alkylating agents and carboplatin, oxazaphosphorine-inducedhaemorrhagic cystitis, and cumulative dose-

related cardiac toxicity secondary toanthracycline treatment. The concept of site-specific inactivation of chemotherapy drugs and/or highly reactiveelectrophilic intermediates induced by radiation with cytoprotective agents has beenextensively explored in both preclinical and clinical studies. The aim of cytoprotectiveagents is to improve the therapeutic ratio of the cytotoxic drug by reducing potentialdose-limiting toxicity to normal tissue. By definition, cytoprotectants must notcompromise the antitumor efficacy of the chemotherapy agent and radiation therapy, andthey should not be associated with additional toxicity that might otherwise interfere withthe delivery of adequate chemo- and radiotherapy. Consequently, the "ideal" chemo/radioprotector should have the following properties:(23).

- Act selectively in normal tissues as opposed to tumor
- Be nontoxic
- Access normal tissues in adequate concentrations to elicit radiation modificationor chemotherapy protection
- Make a radiation/chemotherapy dose less effective to normal tissues by:

1. Decreasing radiation-induced damage
2. Scavenging free radicals
3. Chemically "repairing" radicals induced by radiation/chemotherapy
4. Enhancing enzymatic repair pathways
5. Other mechanisms

Take into account the appropriate timing of drug delivery andradiation/chemotherapy treatment for maximal protection. In principal, the ideal protector allows for a larger anticancer dose to be delivered to thetumor. It is important to remember that in many tumors, response to radiation orchemotherapy is dose-dependent; therefore, increasing the dose delivered to the tumorwill increase the likelihood of tumor cure.The first cytoprotectant to be used was folinic acid (calcium folinate; leucovorin),designed to overcome methotrexate-induced toxicity. Since that, several cytoprotectivecompounds have been extensively investigated, including dexrazoxane, glutathione, ORG2766, mesna and amifostine.[11] Among these compounds, the most noteworthy aredexrazoxane, mesna and amifostine because they have not only been approved by theFDA, but have also been routinely used, worldwide, in a clinical setting.The anthracycline antibiotics, including doxorubicin (adriamycin), daunorubicin andepirubicin, are among the most active anticancer agents against a wide range of solid andhaemopoietic malignancies. However, anthracycline-induced cardiac toxicity, whichappears to be associated with the generation of reactive oxygen species involving theformation of an anthracycline-iron complex, can limit effective clinical use of the abovecompounds. With this recognition, two promising metal-chelating agents havedemonstrated a cardioprotective effect during acute and chronic treatment withdoxorubicin and daunorubicin. One of them is razoxane (ICRF-159). Dexrazoxane(ICRF-187) is the more water soluble (+)-enantiomer of razoxane, which can beadministered parenterally. The current FDA approval for dexrazoxane use is restricted towomen with breast cancer who have already received 6 cycles of doxorubicin-basedchemotherapy.The oxazaphosphorine-based alkylating agents, including ifosfamide andcyclophosphamide, undergo metabolic activation by the hepatic microsomal enzymesystem to form phosphoramide mustard and acrolein. Acrolein and other urotoxicmetabolites are subsequently excreted intact into the urinary bladder to producehaemorrhagic cystitis. In the absence of a chemoprotective agent, ifosfamide andcyclophosphamide are associated with dose-limiting urothelial toxicity. Mesna (sodium-2-mercapto-ethane sulfonate) has been developed as a specific chemoprotectivecompound against acrolein-induced bladder toxicity.Dexrazoxane

and mesna have a relatively limited spectra of toxicity protection (i.e.,cardiac and urothelial, respectively), whereas amifostine appears to be a broad-spectrumselective cytoprotective agent that has a broader potential tissue-protection spectrum. Abroad-spectrum selective cytoprotective agent can be defined as one that protectsmultiple normal organs from the toxicity of cytotoxic antineoplastic therapies withoutprotecting the tumor. Amifostine was originally developed during the height of the coldwar by the Walter Reed Army Institute of Research (WRAIR) as part of a United StatesArmy classified research project to identify an agent that could be used to protect militarypersonnel and the population against atomic radiation in the event of nuclear warfare. Of 4400 chemicals screened for this purpose, amifostine was selected as having the mosteffective radioprotective properties and a relative safety profile(24).Further laboratory andclinical studies have shown that amifostine can protect a broad range of normal tissuesand organs (e.g., bone marrow, peripheral nerve, heart, kidney, salivary gland and otherswith the exception of central nervous system) against the cytotoxic effects of alkylatingagents, platinum compounds, anthracyclines, taxanes and irradiation withoutcompromising antitumor cytotoxicity. To date, it is the broad-spectrum cytoprotectiveagent with the largest preclinical and clinical database. Based on both laboratory andclinical evidence, amifostine may be the most promising radioprotector for the liver(25).

5. Chemistry of Amifostine

Amifostine (Ethiofos, WR-2721) is a low molecular weight (MW 214.2) thiophosphateester prodrug. Amifostine is highly water soluble, with the solubility of the trihydratebeing more than 9 g per 100 ml at room temperature. The compound has four ionizablegroups, two of which are associated with the phosphate function and two with the amino function. At physiological pH, the drug exists as a double zwitterion with an isoelectric point of about 6.6. Amifostine is also very polar, with an octanol/water partition coefficient smaller than 0.01, indicating minimal octanol partitioning. Its free thiol active metabolite (WR-1065) has an octanol/water partition coefficient of 0.037(26). Because passage of drugs through lipid membranes and interaction with macromolecules at receptor sites sometimes correlate well with the octanol/water partition coefficient of the drug, both amifostine and WR-1065 are orders of magnitude away from the lipoidalpartitioning associated with good membrane permeability. When administered intravenously, amifostine has shown to have good protection against radiation. However, after oral administration of the compound, a significant amount of the radioprotective activity of the compound is quickly lost(24),perhaps due to an acidcatalyzedhydrolysis of the ester bond in the stomach prior to absorption. The resulting WR-1065 is presumably further metabolized to inactive compounds resulting in a loss of radioprotective activity. Further detailed studies have indicated that amifostine is unstable at the gastric pH. The stomach pH ranges from 1 to 3, and amifostine is hydrolyzed to WR-1065 under acidic conditions. The hydrolysis reaction of amifostine, which appears to be pH- and temperature- dependent but nonenzymatic, proceeds by cleavage of the P-S bond to yield a thiol (WR-1065) and inorganic phosphate(27).(i.e., $H2N(CH2)3NH(CH2)2SPO3H2 + H2O \rightarrow H2N(CH2)3NH(CH2)2SH + H3PO4$). The maximal rate of hydrolysis occurs at pH 3.0. However, at neutral pH, no detectable hydrolysis occurs over 4 hours at room temperature. The half-life for hydrolysis of amifostine at the low pH of stomach juice at physiologic temperature is about 30.5 minutes. The hydrolysis reaction is also strongly temperature dependent. Cooling amifostine sample to 0°C minimizes the hydrolysis rate of amifostine to an acceptable rate even in highly acidic conditions. In the 1 M perchloric acid solution (pH<1) that is used to

deproteinize blood samples containing amifostine and WR-1065, the rate of conversion is 0.6%/hr at 0°C. However, at room temperature the rate of hydrolysis in the perchloric acid mixture is 74 times greater. In studies with cells in culture, no detectable drug uptake or radioprotection can be found when cells were exposed to amifostine in medium alone, but when alkaline phosphatase was added to the medium efficient uptake leading to appreciable cellular levels of WR-1065 and radioprotection were observed.(28). Additionally, amifostinedephosphorylation in mouse alkaline phosphatase enzyme preparations was inhibited by vandate, an alkaline phosphatase competitive inhibitor. All these results support the hypothesis that alkaline phosphatase is the catalyst responsible for the hydrolysis of amifostine and subsequent uptake of WR-1065 by cells in vivo. Alkaline phosphatase is located in the plasma membrane surface of cells and is particularly rich in the endothelial cells of arterioles in various tissues, in the epithelial cells of the proximal tubule of the kidney, and in the microvilli of the small intestine. Studies of standard alkaline phosphatase substrate in rat jejunum and colon specimens localize alkaline phosphatase to the rim of the jejunal brush border and show negligible activity in the colon(29).Human and mouse isoforms of alkaline phosphatase have a pH optimum between 8 and 9 with amifostine as the substrate.

6. Detection of amifostine and WR-1065

Several serious obstacles have hampered the development of bioanalytical methods for amifostine. The compound is acid-labile, has no convenient chromophore, and has essentially no solubility in organic solvents because it is extremely polar. Lack of solubility in organic solvents precludes its extraction from biological fluids and its polarity places limitations on the types of chromatographic systems that might be used to separate it from endogenous materials. Detection of the free thiol (WR-1065) in biological matrixes presents a challenge since the thiolcan rapidly be oxidized to form either symmetrical or non-symmetrical (with other endogenous thiols) disulfides. In spite of these difficulties, direct and reliable measurements of amifostine and WR- 1065 in biological matrixes have been achieved in several laboratories using electrochemical detection coupled to liquid chromatography (30),(31).An amperometricelectrochemical detector equipped with a thin film Hg/Au working electrode was used, and selectivity was enhanced by the use of a low electrode potential +0.15 V versus Ag/AgCl reference electrode. In liquid chromatography with electrochemical detection (LCEC), analyte eluting from the analytical LC column undergoes electrolysis by passing over a planar electrode held at a fixed potential. If the potential is greater than that required for the electrolysis of the analyte, a measurable charge passes from electrode to analyte (or vice versa). The resulting current is directly proportional to the concentration of solute passing through the electrode. In terms of thiol detection with amalgamated gold electrode, the mechanism is presented by the following electrochemical oxidation reaction: $2RSH + Hg \rightarrow Hg(SR)2 + 2H+ + 2e-$. The symmetrical disulfide (WR-33278) can be detected by using a dual electrode thin-layer cell in a series arrangement. The upstream electrode, held at –1.0 V vs Ag/AgCl, reduces the disulfides to the thiols which are detected downstream at +0.15 V vs Ag/AgCl. Non-symmetrical disulfides are difficult to detect since they are formed by a variety of protein and/or other compounds that are available physiologically (example: cysteine, glutathione). Alternatively, the total amount of WR-1065-related species could be measured by reducing all disulfides bonds and then measuring the levels of WR-1065. Other published analytical methods for measuring levels of amifostine and WR-1065, especially for amifostine, are not direct and either use fluorescent derivatization(fluorescamine, monobromobimane) with the

detection by fluorescence(32), [20] or utilize the coulometric detector(33), [21] which is believed to be more efficient than a traditional amperometric one. Unfortunately, analytical methods that involve conversion of amifostine to WR-1065 prior to analysis are indirect measures of amifostine, resulting in a more complicated validation process and perhaps less accuracy.

7. Pharmacodynamic properties of amifostine

7.1. Mechanism of action

Radiotherapy and chemotherapy are two main approaches in cancer patient treatment. Clinically, irradiation is derived from gamma (usually a 60Co or 137 Cs source), x-ray or neutron emissions in most situations, whereas an electron beam or beta irradiation is used sometimes. The much more energetic gamma rays and x-rays, like ultra-violet rays, can interact directly with the DNA molecule. However, they cause most of their damage by ionizing the molecules, especially water, surrounding the DNA and/or DNA itself. This forms free radicals, i.e., chemical substances with an unpaired electron. These free radicals, especially those containing oxygen, are extremely reactive and immediately attack neighboring molecules. When such a free radical attacks a DNA molecule, it can change a base, but it frequently causes a single- or double-stranded breakage. Singlestrandedbreaks are ordinarily not serious because they are easily repaired by rejoining the ends of the severed strand. However, double-stranded breaks are very difficult to repair properly, so they frequently cause a lasting mutation. Additionally, chemotherapy drugs such as alkylating agents or platinum agents (cisplatin, carboplatin) can activate the formation of DNA-DNA interstrand crosslinks or platinum-DNA and platinum-protein adducts. Amifostine is a prodrug that is dephosphorylated by the membrane-bound enzyme alkaline phosphatase, to form the free thiol metabolite, WR-0165. WR-1065 is the main metabolite responsible for the cytoprotective effects of amifostine and is the metabolite most readily taken up into cells. Once inside cells, WR-1065 provides protection from radiotherapy and/or chemotherapy through several mechanisms. They include the following:

- WR-1065 can lower intracellular oxygen concentrations by competing with oxygen to prevent oxygen interactions with DNA radicals, which can generate potentially harmful hydroperoxides, resulting in fixation of damage and an increase in the risk of cell death(34).
- The oxygen-independent mechanism appears to involve radical scavenging, such as those derived from radiation therapy or specific drugs (e.g., doxorubicinderivedsuperoxide anions), and/or hydrogen donation reactions(35).
- WR-1065 can not only bind directly, and thus detoxify the active species of alkylating agents(36). or platinum agents(37), in normal tissues, but also partially reduce DNA platination by the cytotoxic agent (formation of cispaltin-DNA adducts).
- Some evidence indicates that both WR-1065 and amifostine can form complexes with cisplatin active species and detoxify them(38).
- WR-1065 exerts cytoprotective effects, in part, via a catalytic inhibition of the enzyme DNA topoisomerase II alpha, leading to the subsequent accumulation of cells in G2 phase and to prolong the cell cycle, thus providing more time for DNA repair(39).
- The symmetric disulfide WR-33278, which is a metabolite of WR-1065, has cytoprotective properties as well. Those cytoprotective properties are explained by the structural similarities of the symmetrical disulfide to the polyamine speramine. WR-33278 binds more avidly to DNA

than does speramine andenhances the relaxation of supercoiled DNA mediated by topoisomerase I(40).

• Post-treatment of irradiated cells with WR-1065 has been shown to markedly attenuate radiation-induced apoptosis. WR-1065 has also reduced apoptosis caused by several chemicals(41).The protective effects of amifostine are largely limited to normal, and not tumor, tissue. This selective protection is based on the ability of WR-1065 to be taken up in higher concentration in normal organs than in tumor tissues. This preferential uptake is due to a combination of several biological features. First, drug delivery is significantly impaired in tumor as compared with normal tissue due to the poor vascularisation of most tumors. Furthermore, alkaline phosphatase, the membrane-bound enzyme responsible for the dephosphorylation of amifostine to WR-1065, is largely distributed in capillaries and arterioles of normal tissues; however, solid tumors are poorly vascularised and tend to contain low levels of alkaline phosphatase. In normal human lung cells, alkaline phosphatase activity was found to be 275-fold higher than in non-small cell lung cancer cells. Consequently, less activation of amifostine to the active metabolite WR-1065 happen in tumor tissues because of lower levels of alkaline phosphatase. Thus, a difference in dephosphorylation ability in healthy versus malignant tissue may explain the selective protection of amifostine against cytotoxic chemotherapy and/or radiotherapy.(42).Another factor which contributes to the selective protective effects of amifostine is the difference in pH between normal and tumor tissues. The relatively high pH of normal tissues is optimal for WR-1065 formation as well as WR-1065 uptake. Amifostine is not dephosphorylated by acidic phosphatase, so the acidic pH associated with many tumors may restrict both the formation and uptake of WR-1065. It was shown that a decrease of 0.3 units in pH caused a two-fold reduction in the cell uptake rate of WR-1065. This means that even if WR-1065 does become available to tumor tissues it will not be absorbed by the cells at rate comparable to that of normal tissue.(43),(44).Finally, amifostine may be actively absorbed by normal tissue cells and only passively absorbed by tumor cells. As to the regional delivery of amifostine to the liver, which may offer more protection to the liver than systemic administration, it has been documented that the major source of blood flow to macroscopic hepatic cancers is by way of the hepatic artery. In contrast, the delivery of nutrients to normal tissues is primarily a function of the portal circulation. Thus, amifostine selectivity in liver may not only be enhanced by differences between normal tissue and tumor in alkaline phosphatase activity as described above, but also by differences in the drug's regional route of delivery (i.e., portal vein is favored) (45).

8. Protection against cytotoxic chemotherapy

The protective activity of amifostine against the tissue damaging effects of cytotoxic agents (e.g., carboplatin, carmustine, chlormethine, cisplatin, cyclophosphamide, fluorouracil, lomustine, melphalan or oxidopamine) has been assessed in animal models and in patients with cancer. In a preclinical study with mice,(46), amifostine has been shown to reduce cisplatininducednephrotoxicity without interfering with the cisplatin antitumor effect. This study suggests that the protection offered by amifostine allowed a 2.2-fold increase in cisplatindose to 19 mg/kg before the occurrence of nephrotoxicity, which resulted in an increased antitumor effect of cisplatin. Clinical trials(47),(48),(49),(14),ofamifostine in combination with cisplatin also demonstrated a significant protection from the nephrotoxicity

and neurological toxicities associated with the use of cisplatin, without compromising its antitumor efficacy. In conclusion, amifostine may be considered for the prevention of nephrotoxicity in patients receiving cisplatin-based chemotherapy. Neutropenia, consisting primarily of leukopenia, is the principal dose-limiting toxic effect of cyclophosphamide. An expert panel from the American Society of Clinical Oncology (ASCO) recommends that amifostine be considered for the reduction of neutropenia-associated events in patients who receive alkylating-agent chemotherapy(50).Carboplatin toxicity differs significantly from that of cisplatin. The usual dose-limiting toxic effect of carboplatin is bone marrow suppression, particularly thrombocytopenia. Pretreatment with amifostine increased carboplatin maximum tolerated dose from 400 to 500 mg/m2, without compromising its antitumor activity(51).Pretreatment with amifostinehas shown significant protection of bone marrow, immune system and intestinal crypt cells from the toxicity induced by a broad range of antineoplastic agents in a number of studies (52),(53),(54),(55).Amifostine does not appear to affect tumor response to, or antitumor activities of, chemotherapy. Although amifostine may demonstrate tumor-protective effects under certain experimental conditions in a small number of early preclinical studies, the protection of tumor cells was typically low and variable, being dependent on dose, tumor type and size, and administration time. No evidence of tumor protection has been reported in clinical trials(56).Amifostine is generally well tolerated and is associated with transient side effects, including nausea, vomiting, a warm or flushed feeling and occasional allergic reactions. The most clinically significant toxicity is hypotension. Based on the recommendation by the American Society of Clinical Oncology (ASCO), the suggested dose of amifostinewith chemotherapy in adults is 910 mg/m2, and is administered IV over 15 minutes, 30 minutes before chemotherapy(50).

9. Protection against radiotherapy

A protective effect of amifostine against radiation has been convincingly demonstrated in mice, dogs and monkeys against x-, γ- and neutron-irradiation. Yuhas(57),demonstrated a dose-modifying factor (DMF; ratio of irradiation doses with and without amifostinerequired to produce a specific effect in 50% of animals at a given time) of 2.7 against 30- day mortality in mice. Protection of dogs with amifostine was demonstrated at 200 mg/kg, a dose producing one death due to drug, but five other animals survived the toxic effects of irradiation. At 150 mg/kg amifostine, a better tolerated dose, 8 of 16 dogs survived the toxic effects of irradiation. Rhesus monkeys were protected by 250 mg/kg of amifostine administered intravenously 30 minutes before irradiation.[12] While some studies in animal models have shown minimal protection of the tumor, only limited preclinical data are available regarding intrahepatic cancers. Studies in rats(58),(59),have demonstrated that systemic administration of amifostine protects hepatocytes with a dose modification factor of 2, and that the liver is protected from fibrosis with a dose modification factor that is greater than 2.

10. Cytotoxic effects of radiotherapy

Symon et al.(60),have recently evaluated whether systemic or portal venous administration of amifostine could protect the normal liver from the effects of ionizing radiation without compromising tumor cell kill in a rat liver tumor model. A micronucleus assay was used in this study and has been shown to be a sensitive measure of hepatocyte radiosensitivity. For instance, Alati et al.(61),have shown that the radiation dose response for the induction of

micronuclei in hepatocytes is linear both in air and under hypoxic conditions. Rats implanted with liver tumors were infused with 200 mg/kg amifostine over 15 min via the femoral or portal vein. After a single 6-Gy fraction irradiation, the frequency of hepatocyte micronuclei after administration of saline, systemic amifostine and portal venous amifostine was 18.7 ± 1%, 6.8 ± 1% and 9.9 ± 2%, respectively, corresponding to a radiation equivalent effect of 6 ± 0.5 Gy, 1.8 ± 0.3 Gy, and 2.5 ± 1.3 Gy, respectively. Both amifostine conditions showed considerably less radiation effect than saline-treated control ($p<0.01$); the two amifostine conditions did not differ significantly ($p=0.3$). The surviving fraction of tumor cells was not affected by amifostine treatment and was 0.03 ± 0.02 and 0.05 ± 0.03 for systemic and portal venous delivery, and 0.06 ± 0.02 for control animals ($p=0.34$). These findings demonstrate both systemic and portal venous administration of amifostineeffectively protect hepatocytes from ionizing radiation without compromising tumor cell kill in a clinically relevant animal model, and amifostine may be a selective normal tissue radioprotectant in liver cancer. The possible benefits of using amifostine in combination with radiation therapy include reducing treatment-related toxicity and escalation of radiation dose in the curative treatment of cancer. Although several randomized clinical trials(62),(63),have been conducted to determine amifostine protection in patients receiving radiation treatment for different cancers, its only approved use in combination with radiotherapy is as a protector against irradiation-induced xerostomia. This approval is based on the data from a large multi-center study in patients undergoing radiation therapy for head and neck cancer. Amifostine-treated patients demonstrated a statistically significant decrease in acute and chronic xerostomia compared to control patients. Another recent randomized trial in patients with lung cancer confirmed these results. Amifostine treatment significantly reduced pneumonitis and esophagitis, without decreasing tumor control. These and other clinical trials give strong support to the use of amifostineas a radioprotector. When given with radiation therapy for head and neck cancer, the recommendedamifostine dose is 200 mg/m2/day given as a slow IV push over 3 minutes, 15 to 30 minutes before each fraction of radiation therapy. The liver appears to be a particularly promising organ for a radioprotective strategy using amifostine. As described previously, even a modest protective effect would permit a clinically meaningful increase in radiation dose to be delivered. In a small-scale study (internal protocol in progress), seven patients with diffuse intrahepatic cancer were treated using whole liver radiation with amifostine pretreatment. Patients received 150 mg/m2 of amifostine prior to each dose of radiation, with a plan of radiation dose escalation. Treatment was delivered with concurrent hepatic arterial FdUrd. No patients developed RILD. There were no episodes of hypotension and no ≥ grade 3 nausea. The median survival of all patients was 10 months. Another preliminary study(64),showed that 19 of 203 patients treated with focal and whole liver radiation with amifostinepretreatment developed RILD without hepatic arterial FdUrd. From this study, patients with primary hepatobiliary cancers had a significantly greater risk of complication than those with colorectal cancer metastatic to the liver. These data suggest that amifostineadministered systemically may protect the liver in patients with intrahepatic cancer who are undergoing whole liver radiation. In summary, amifostine has the promise of being an effective radioprotector that could improve patient treatment outcomes and the quality of life. However, amifostine'sradioprotective potential has materialized only in the treatment of head and neck cancer. Carefully designed preclinical and clinical trials may help to broaden the use of amifostine, for example, in liver cancer patients.

11. 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 (65). 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(66).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(67).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(28).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(67). 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 tumourprotection has been reported in clinical trials(68),(69).

12. The role of p53 Protein in theAmifostineInduced Cellular Action

12.1. 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 (70).Target genes transactivated by p53 include regulators of the cell cycle in G1 and G2 phases (*p21Waf1*, *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 withseveral other cellular proteins(70).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 (71),(72).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(71). Thecells treated with WR-1065 clearly induce an expression of the gene coding for the cell cycle regulators p21Waf1 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 p21Waf1 gene coding for an inhibitor of cyclindependent kinases causes cell cycle arrest in the G1 phase(71), (73),(74).Therefore, WR-1065 induces the G1-phase cell cycle arrest rather than the proapoptoticp53 pathway, thus allowing repair of damaged DNA before replication, and the prevention of fatal incorporation of mutations into the genomes of non-malignant cells(75).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 G1 arrest(76).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 nonmalignant tissue(77).

13. 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-translationallymodified 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 DNAdamageis 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 phosphorylationsprevent 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 proteosomaldegradation. 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(78),(79),(80).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 activatedby DNA-damage factors(81).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(82).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 antioxidant stress induced by WR-1065(83).Another possible mechanism of p53 activation by the polyamine analog WR-1065 could include disruption of polyamine intracellular metabolism(84),(85),showedthat many polyamine antagonists, such as theiranalogues, may disrupt polyamine metabolism, thus causing activation of the p53-p21-pRb pathway, and inducing the G1-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-adenosyl-methionin decarboxylase, the main regulatory enzyme in the biosynthesis of higher polyamines. Furthermore, the inhibition of Sadenosylmethionindecarboxylase by various drugs has been shown to possess antiproliferative and antitumour activity(86).

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

It was shown by(74).that the p53 protein is sensitive to oxidation-reduction *invitro*. 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, DNAbindingform of the p53 protein while oxidationdisrupts functional p53 conformation and inhibits sequence-specific DNA binding. WR-1065 directly interacts with p53 and modifies its cysteinresidues by the free sulfhydryl group. This resultsin 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(81).Similarly, two other redoxdependenttranscription 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(73).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*(87). 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 upregulatethe DNA-binding- and transactivation activities of the p53 mutants with a retained capacity for weak interaction with DNA(88).Using a yeast expression system(88),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 DNAbindingdomain and thus affecting its ability to interact with p53 responsive elements (p53RE). The majority of mutations completely disrupt the DNAbindingof 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 *p21Waf1* 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(89).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(88).Such mutants often possess a temperature-dependent DNA-binding activity that occurs at the permissive temperature 32 °C. Thetemperature-dependency correlates with thestructural flexibility of the DNA-binding domain. Many temperature-dependent p53 mutants display conformational changes related to changes in biochemical properties, intracellular localization and function(90),(75).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 spinocellularcarcinoma 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 G1 phase. At the same time, the transcription of *p21Waf1* 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-1065stabilizes the structure of the p53 protein that allows binding of "high affinity"- but not "low affinity" p53Res(75).

15. 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 amifostinetreatment. The ambiguity of results is demonstrated by studies by(72),(77),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(77),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,(72).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 (77),explain these controversial effects of amifostine on apoptosis, by the high concentration of amifostine (3.8 mM) used by the Lee group(72). 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(71).The level of cytotoxic products (H2O2, acroleinand cysteamine) rises also upon WR-1065 degradation by Cu-dependent amine oxidases present in the serum(91).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 *p21Waf1* expression and growth arrest in the G1/S phase. In contrast, in the MCF-7-derived cell line MDD2 lacking functional p53 protein, no induction of *p21Waf1* occurred (71). In the p53-null human lung cancer cell line H1299 expressing exogenous functional p53, amifostineenhanced cell sensitivity to the cytotoxic effects of amifostine, and increased the rate of apoptosis when compared with controls H1299 lacking p53(92),(73),showed the p53-dependent protection of mouse embryonicfibroblasts treated with WR-1065 from cell deathinduced by paclitaxel. (93),studied the dependence of the cytoprotective effects of WR-1065 on the p53 status in four cell lines derived from cells of gliomadiffering 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 (94), experiments with human myeloid leukemia K562 and NB4 cells transfected with the *p53* gene containing the temperature sensitive mutation. In these cells, amifostineimpaired 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 amifostineby alkaline phosphatase to its active metabolite WR-1065 is an essential step in the cytoprotectiveaction 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(4).

16. Conclusion

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. 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.Toxicity may also result in treatment delay or cessation, which may adversely affect outcomes. Chemo-protective therapies have been developed in an attempt to reduce these toxicities and improve the therapeutic window of cytotoxic agents. The ideal chemo-protective agent should be easy to administer, non-toxic, not alter the pharmacokinetics of the cytotoxic agent and should not inhibit or reduce the antitumor activity.Additional studies are warranted to investigate the protective effect of amifostine with differing regimens of administration, more clinically relevant fractionation regimens and longer follow-up, as well as, further study is needed to better define the benefits of amifostine in modern radiation oncology practice.

17. Reference

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