Role of superoxide dismutase system in the radiation response of tumor cells

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Urszula Florczak

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Dekan:	Prof. Dr. rer. nat. H. A. Mallot
1. Berichterstatter:	Prof. Dr. rer. nat. H. P. Rodemann
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LIST OF ABREVIATIONS

2-ME	2-Methoxyestradiol
APS	Amuniumpersulfate
AREG	Amphiregulin
ATM	Ataxia telangiectasia mutataed
ATP	Adenosintriphosphat
BSA	Bovine serum albumin
CuZnSOD	Copper zinc superoxide dismutase
DAPI	4',6-diamidino-2-phenylindole
DDC	Diethyldithiocarbamate
DMEM	Dublecco´s modified eagle medium
DMSO	Dimethylsolfoxide
DNA	Deoxyribonuccleic acid
DNA-PK	DNA-dependent protein kinase
DSBs	Double strand breaks
DTT	Dithiothreitol
ECL	Enhanced chemilominescence
ECSOD	Extracellular superoxide dismutase
EDTA	Ethylendinitrilotetraessigsäure
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ErbB	Erythroblastic Leukemia Viral Oncogene Homolog
EtOH	Ethanol
FCS	Fetal calf serum
G	Gram
Gy	Gray
H	Hour
H_2Odd	Twice-destilled deionised water
HEPES	N-(2-Hydroxyethyl)-piperaszin-n-N‹-2-Ethansulfonic acid
HER	Human EGF-related
HRP	Horseradish Peroxidase
IGF-1	Insuline-like growth factor-1

IGF-1R	Insuline-like growth factor-1 receptor
IP	Immunoprecipitation
IR	Ionizing radiation
kDa	Kilodalton
L	Litre
M	Molar
mA	Milliampere
min	Minute
ml	Millilitre
mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
MnSOD	Manganese superoxide dismutase
MOI	Multiplicity of infection
MTCO1	Mitochondrially encoded cytochrome c oxidase I
NBT	Nitro blue tetrazolium
NF-κB	Nuclear factor κB
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
NP-40	Nonidet P-40
NSCLC	Non-small cell lung cancer
OD	Optical density
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase-B
PVDF	Polyvinylidene fluride
rAAV2	Recombinant adeno-associated viruses 2
rpm	Revolutions per minute
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RT	Room temperature
RTK	Receptor tyrosine kinase
SD	Standard deviation

SDS	Nantriumdodecylsulfate
SDS-PAGE	SDS polyacrilamide gel electrophoresis
SEM	Standard error
SF	Surviving fraction
siRNA	Small interference RNA
SOD	Superoxide dismutase
TBST	Tris-Buffered SalineTween 20
TEMED	N,N,N´,N´- Tetarmethylethylenediamine
TGFα	Transforming growth factor alpha
UV	Ultraviolet
VEGFR	Vascular endothelial growth factor receptor
WB	Western blotting
XOD	Xanthine oxidase

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SUMMARY

Superoxide dismutase (SOD) system is known to be overexpressed in tumor cells as compared to normal cells. Exposure to ionizing radiation (IR) is able to induce MnSOD and CuZnSOD function which as a consequence may protect tumor cells against radiation toxicity during radiotherapy. Previously it has been shown that receptor tyrosine kinases of the epidermal growth factor receptor family members ErbB1 and ErbB2 are involved in cellular radiation responses and in case of ErbB1 also potentially relevant for the activation of SOD activity. In the present study the interaction of ErbB1 and ErbB2 signaling and SOD protein expression and enzyme activity was investigated.

Experiments were performed by using the lung carcinoma cell lines A549, H460 and H661. Radiation-induced SOD activity was analyzed by a standardized superoxide dismutase activity assay. Applying Western blotting, the pattern of SOD expression and activation of AKT as well as DNA-PKcs was analyzed. Colony formation assay and γH2AX foci assay were applied to measure cellular radiosensitization and repair of DNA-double strand break (DNA-dsb). To downregulate SOD expression small interfering RNA (siRNA) was used.

It could be shown that clinically relevant doses of irradiation stimulate both enzyme activity and protein expression in a dose dependent manner. Differential analyses applying targeting strategies (siRNA and inhibitors) indicated that CuZnSOD is primarily regulated in response to radiation exposure by ErbB2 and not ErbB1 mediated downstream signaling. IR induced SOD activity was demonstrated to be clearly dependent on PI3K/AKT activity. To investigate the effect of SOD expression on post irradiation survival a previously described SOD inhibitor, 2-ME was used. Combination of 2-ME with radiation doses of 1-5 Gy enhanced radiation sensitivity, however, the radiosensitizing effect of 2-ME could be demonstrated to be independent of inhibition of SOD enzyme activity but dependent on a marked impairment of IR-induced AKT/DNA-PKcs activity mediated by 2-ME.

The data presented indicate for the first time that the expression of CuZnSOD induced by ionizing radiation depends on radiation-stimulated ErbB2-signaling. Moreover, targeting of SOD by siRNA does not lead to radiosensitization of tumor cells. In contrast to the theoretically expected result, treatment with 2-ME which inhibits SOD enzyme activity radiosensitizes cells rather through inhibition of PI3K-AKT-DNA-PKcs signaling than through blockage of SOD enzyme function.

ZUSAMMENFASSUNG

Es ist bekannt, dass das Superoxid-Dismutase (SOD) System in Tumorzellen, verglichen mit normalen Zellen, überexprimiert wird. Ionisierende Strahlung (IR) kann die MnSOD- und CuZnSOD-Funktionen induzieren, welche die Tumorzellen vor Strahlungsvergiftung während einer Strahlentherapie schützen. In vorherigen Untersuchungen wurde gezeigt, dass ErbB1 und ErbB2, Rezeptortyrosinkinasen der epidermalen Wachstumsfaktor-Rezeptoren-Familie, in die zelluläre Strahlungsantwort involviert und im Fall von ErbB1 möglicherweise relevant für die Aktivierung der SOD-Aktivität sind. In der vorliegenden Arbeit wurde nun untersucht, in wiefern die ErbB1- und ErbB2-Signalwege auf die SOD-Proteinexpression und -Enzymaktivität Einfluss nehmen.

Die Experimente wurden mit den Lungenkrebs-Zelllinien A549, H460 und H661 durchgeführt. Die strahlungsinduzierte SOD-Aktivität wurde mit einem standardisiertem Superoxid-Dismutase Aktivitätsassay analysiert. Mit Westernblotting wurde das SOD-Expressionsmuster und die Aktivierung von AKT und DNA-PKcs untersucht. Colony Formation Assay und γH2AX Foci Assay wurden benutzt, um die zelluläre Strahlungsempfindlichkeit und die Reparatur von DNA-Doppelstrangbrüchen (DNA-dsb) zu messen. Um die SOD-Expression herunterzuregulieren wurde small interfering RNA (siRNA) eingesetzt.

Es konnte gezeigt werden, dass klinisch relevante Bestrahlungsdosen sowohl die Enzymaktivität als auch die Proteinexpression dosisabhänig stimulieren können. Analysen mit unterschiedlichen Zielstrategien (siRNA und Inhibitoren) zeigten, dass CuZnSOD als Antwort auf Bestrahlung primär durch ErbB2- und nicht ErbB1-vermittelte, nachgeschaltete Signalwege reguliert wird. Es wurde demonstriert, dass die strahlungsinduzierte SOD-Aktivität klar abhängig von der PI3K/AKT-Aktivität ist. Um die Effekte der SOD-Expression auf das Überleben nach Bestrahlung zu untersuchen, wurde ein früher beschriebener SOD-Inhibitor, 2-ME, benutzt. Eine Kombination von 2-ME mit Bestrahlungsdosen von 1-5 Gy verbesserte die Strahlenempfindlichkeit. Jedoch ist der Effekt von 2-ME auf die Strahlungsempfindlichkeit unabhängig von der Inhibition der SOD-Enzymaktivität, sondern abhängig von einer deutlichen Verringerung der strahlungsinduzierten AKT/DNA-PKcs-Aktivität vermittelt durch 2-ME.

Die vorliegenden Daten zeigen zum ersten Mal, dass die Expression von CuZnSOD, induziert durch ionisierende Strahlung, vom strahlungsinduzierten ErbB2-Signalweg abhängt. Ferner führt das Targeting von SOD mit siRNA nicht zu einer Strahlenempfindlichkeit der untersuchten Tumorzellen. Im Gegensatz zum theoretisch erwarteten Ergebnis, d.h. dass 2-

ME die SOD-Enzymfuntion blockiert, konnte gezeigt werden, dass 2-ME eine Strahlensensitivität von Tumorzellen durch Blockade des PI3K/AKT/DNA-PKcs-Signalwegs induziert.

1. INTRODUCTION

World Health Organisation (WHO) defined cancer as a large group of diseases that can affect any part of the body. Cancer causes death worldwide. Based on WHO report in 2007 almost 8 million people died because of cancer (around 13% of all deaths). In 2030 the number of deaths from cancer will be probably increased to 12 million. The main types of cancer leading to overall cancer mortality each year are: lung, stomach, colorectal, liver and breast cancers. One of the methods of cancer treatment is radiation therapy (RT). It can be combined with surgery, chemotherapy or hormonal therapy. The aims of cancer treatment are cure, prolongation of life, and improvement of the quality of life for patients. RT is often applied as postoperative treatment, alone or in combination with chemotherapeutics. RT is a successful therapeutic approach. The response of cancer to radiation treatment is different and depends on its intrinsic radiosensitivity. There are highly radiosensitive cancer cells which can be rapidly killed by small doses of radiation. These include leukaemias, lymphomas and germ cell tumours. The more radioresistance epithelial cancers require markedly higher doses of radiation. The radiosensitivity of the cancer can be increased by certain drugs which are given during the radiotherapy. Examples of radiosensiting drugs include: cisplatin.

At the cellular level the most important effect of radiation therapy is the damage of macromolecules (DNA, proteins and lipids) in cancer cells. It is possible through either direct or indirect effect (Goodhead 1994; Nikjoo *et al.* 1997). The most important macromolecule which can be damaged in cancer cells through ionizing radiation is DNA. DNA damages can be repaired by cellular DNA repair systems (Wood *et al.* 2001, Friedberg 2003, Rothkamm and Löbrich 2003, Preston 2005). The most dangerous DNA lesions induced by ionizing radiation are double strand breaks (dsb). Incomplete or defective DNA – dsb repair results in mutations and genomic instability (Vamvakas *et al.* 1997, Richardson and Jasin 2000; Deckbar *et al.* 2007). Several different DNA repair pathways are involved in DNA - dsb repair but the most important is nonhomologous end – joining (NHEJ) (Pardo *et al.* 2009). One of the most important proteins involved in NHEJ DNA repair is DNA protein kinase (DNA-PK) (Mahaney *et al.* 2009, Pardo *et al.* 2009). DNA-PK is a nuclear serine/threonine kinase which consists of a catalytic subunit and regulatory subunit which contains the Ku70/80 subunits (Jeggo *et al.* 1995). DNA-PK is a member of the phosphatidylinositol-3-kinase (PI3-K) superfamily (Collis *et al.* 2005).

The majority of the biological effect of ionizing radiation (IR) is due to its indirect effect (Kamat *et al.* 2000). This effect is caused mainly by the radiolysis of water molecules leading to the formation of highly aggressive free radicals. It has been shown that approximately two-

thirds of the biological damage by radiation is due to free radical–mediated indirect action (Sun *et al.* 2007). A free radical is an atom or molecule that contains one or more unpaired electrons (Halliwell 1991). Among many types of radicals those of most concern in biological systems are derived from oxygen and known collectively as reactive oxygen species (ROS) of which the superoxide radical (O_2^-) is the most important one (Murphy 2009). ROS are continuously produced in cells during normal aerobic life (Murphy 2009). The sources of ROS are mitochondria, where they are formed in the respiratory chain, via cyclo-oxygenase pathway and by few enzymes, such as xanthine oxidase, NADPH oxidase and cytochrome P450 oxidase (Janssen *et al.* 1993, Murphy 2009). Furthermore, ROS formation can be also induced by exogenous agents. Hiperoxia (Fehrenbach and Northoff 2001), mineral dusts (van Klaveren and Nemery 1999), heat (Fehrenbach and Northoff 2001), UV (Svobodova *et al.* 2006), and ionizing radiation (Sun *et al.* 2007) can induce ROS production in cells. ROS play an important role in physiology of cells. They can regulate signal transduction and phagocytose (Milligan *et al.* 1998, Bast *et al.* 1991, Hancock *et al.* 2001). However, under stress conditions the level of ROS is increased and can cause oxidative damage to cellular components such as DNA, proteins and lipids (Zablocka and Janusz 2008). Cells can protect themselves against IR-induced ROS through production of superoxide dismutases which can catalyze the reaction of disproportionation of superoxide anion to hydrogen peroxide and oxygen (McCord and Fridovich 1969, Halliwell 1991).

1.1 Molecular targeted therapy

Traditional cancer therapy can not distiguish normal cells from cancer cells and the cytotoxic effect has a negative influence not only to cancer cells but also normal cells. Molecular targeted cancer therapy was developed based on the biological differences existing between normal and malignant cells. The expression and activity of some proteins involved in tumor growth and progression are changed in cancer cells. Identification of these proteins and developing drugs interfering with them allows to make cancer therapy more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells. Molecular targeted therapy can also be combined with cytotoxic therapy to make malignant cells more sensitive to radio- or chemotherapy (Isobe *et al.* 2005, Becker *et al.* 2006). There are many tagets of molecular targeted therapy described, i.e. RTK and its downstream pathways.

1.1.1 Receptor tyrosine kinase targeting

Receptor tyrosine kinases (RTK) play important role in cellular signalling and are often mutated or dysregulated in cancer cells. This results in resistance of cancer cells to radio- and chemotherapy (Schmidt-Ullrich *et al.* 2003). Thus, tyrosine kinase receptors are important targets in anti-cancer therapy.

1.1.1.1 EGFR family

One prominent family of tyrosine kinase receptors family is the ErbB (Erythroblastic Leukemia Viral Oncogene Homolog) receptor family or EGF (Epidermal Growth Factor) receptor family of transmembrane RTKs (Receptor Tyrosine Kinases). It consists of four members, EGFR (EGF Receptor)/ErbB1/Her1, ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4 (Hynes and Horsch 2001). EGFR family receptors act as proproliferative and cytoproctive (Valerie *et al.* 2006). ErbB1 and ErbB2 are involved in the development of many types of human cancer. ErbB overexpression is associated with tumorigenesis of the breast, ovaries, brain, and prostate gland (Holbro *et al.* 2003). All ErbBs have in common an extracellular ligand-binding domain, a single membrane-spanning region, and a cytoplasmic protein tyrosine kinase domain (Holbro *et al.* 2003). Recent data indicated that phosphoryation of ErbB1 and ErbB2 can be also mediated ligand independably by ionizing radiation (Harari and Huang 2002, Bowers *et al.* 2001).

The best strategies for EGFR pathway targeting are monoclonal antibodies against EGFR (Sato *et al.* 1983, El-Rayes *et al.* 2004) and small-molecule RTK inhibitors (Lichtner *et al.* 2001, El-Rayes *et al.* 2004). Both types of targeting downregulate signal transduction pathways of EGFR (Bruns *et al.* 2000, Albanell *et al.* 2001, El-Rayes *et al.* 2004). Monoclonal antibodies downregulate expression of EGFR while inhibitors prevent only receptor autophosphorylation without affecting EGFR expression (El-Rayes *et al.* 2004, Ciardiello *et al.* 2004). After binding to EGFR, monoclonal antibodies block growth factors binding to the receptor and activate receptor dimerization and downregulation (Ciardiello *et al*. 2004). EGFR targeting monoclonal antibodies are cetuximab, a chimeric human-mouse IgG1 (Ciardiello *et al*. 2004), panitumamb and matzumab (Valerie *et al.* 2006). RTK inhibitors block EGFR-TK activity by competition with ATP for binding to the intracellular catalytic domain of the receptor (Valerie *et al.* 2006). Many RTK inhibitors have been designed for radiation therapy: erlotinib, a quinazoline derivate (Ciardiello *et al.* 2004, Valerie *et al.* 2006), AG1478, lapatinib and canertinib (Valerie *et al*. 2006).

Lapatinib and canertinib are dual inhibitors and can block not only EGFR but also ErbB2-TK activity (Valerie *et al.* 2006). ErbB2 acts as heterodomerization partner for ErbB family members. ErbB2 dimerization with EGFR, ErbB3 and ErbB4 can be blocked by humanized monoclonal antibody to ErbB2 pertuzumab (Swanton *et al.* 2006). The widely used ErbB2 monoclonal antibody is trastuzumab (Herceptin) (De Laurentiis *et al.* 2005).

1.1.1.2 VEGFR

The other promising target in anticancer therapy is vascular endothelial growth factor (VEGF) and its receptor (VEGFR). VEGF family play a important role in angiogenesis (Bansal *et al.* 2009). It binds to two tyrosine kinase receptors VEGFR-1 and VEGFR-2 (Reinmuth *et al.* 2006). To target this receptor few strategies has been developed. The humanized monoclonal anti-VEGF antibody bevacizumab or sorafenib and a multitargeted kinase inhibitor have been recently studied (Bansal *et al.* 2009).

1.1.1.3 IGF-1R

Another important RTK in onclology is the insuline-like growth factor-1 receptor (IGF-1R) (Teillet *et al.* 2008). It is involved in the process of transformation of normal cells to a cancer cells (Pollak *et al.* 2004, Karamouzis *et al.* 2006). IGF-1R activation promotes tumor survival and growth (West 2009, Karamouzis *et al.* 2006). To target this receptor few strategies have been developed, like monoclonal antibodies figitumumab, cixutumumab or small-molecule inhibitor OSI-906 (West 2009). There are evidences that IGF-1R activation can be responsible for resistance to EGFR inhibitors in anticancer therapy (Jones *et al.* 2006). Therefore, possibilities of combination of EGFR and IGF-1R inhibitors have been investigated (West 2009).

1.1.2 PI3K / AKT pathway targeting

Phosphoinositide 3-kinase (PI3K), downstream protein of RTK, is a heterodimer protein, which consists of a 110 kDa catalytic subunit and an 85 kDa regulatory subunit. PI3K participates in cellular processes, including cell growth, transformation, migration and differentiation (Franke 2008, Engelman 2009). The PI3K pathway has been shown to be an essential survival mechanism in a number of cell types (Engelman 2009). A main component of the PI3K signalling pathway is AKT which is an essential serine/threonine kinase (Franke

2008). It has been shown that AKT plays a role in immune activation, cell proliferation, apoptosis and cell survival (Schmidt-Ullrich *et al.* 2000, LaVallee *et al*. 2002, Shukla *et al.* 2007). Therefore, PI3K / AKT pathway is an interesting target of anticancer therapy (Le Tourneau and Siu 2008).

Another attractive target in cancer therapy is the serine/threonine kinase AKT (Le Tourneau and Siu 2008). Several AKT inhibitors have been developed. Kozikowski *et al.* described a new novel class of phosphatidylinositol (PI) analogues that selectively block AKT activation and selected downstream substrates without affecting of activation of upstream kinases. It blocks AKT phosphorylation without affecting the total AKT. An AKT inhibitor currently tested in phase 2 clinical trials is perifosine which targets the pleckstrin homology domain of AKT and prevents its translocation to the plasma membrane (Gills *et al.* 2009).

The AKT inhibitor API-59CJ-OMe (Jin *et al.* 2004) inhibits AKT kinase activity and induces apoptosis in cell lines with high AKT activity, but has little effect on cells without AKT activity. Therefore, API-59CJ-OMe may have therapeutic potential for those cancers that harbour PTEN mutations and AKT activation. Derivative of API-59CJ-OMe inhibitor API-59-CJ-OH has been described to block AKT phosphorylation and sensitize tumor cells to radiation treatment (Toulany *et al*. 2008). Treatment with this inhibitor reduced clonogenic survival and enhanced radiation sensitivity in lung carcinoma cell lines A549 and H460 (Toulany *et al*. 2008). Moreover, API treatment induced apoptosis only in apoptosis-sensitive H460 but not in apoptosis-resistant A549 cells.

1.1.3 Superoxide dismutase (SOD) and cancer

1.1.3.1 Cellular antioxidant defense system

As reviewed by Schmidt-Ullrich *et al*. (2000) ROS play an important role in cell death and signal transduction by ionizing radiation. To counterbalance the action of free radicals, cells are equipped with antioxidants, which are able to scavange ROS, prevent their formation or repair the damages, which they cause (Halliwell 1991). Antioxidant system in cells includes: antioxidant enzymes, ancillary enzymes, glutathione, metal-binding proteins, flavonoids and urate (Sies 1991, Halliwell 1994). Antioxidant enzymes include: superoxide dismutases (SOD), catalase and glutathione peroxidase (Zablocka and Janusz 2008).

Superoxide dismutases which are ubiquitous components of cellular antioxidant system can catalyze the reaction of disproportionation of superoxide anion to hydrogen peroxide and oxygen (McCord and Fridovich 1969):

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide is removed by catalase and glutathione peroxidase (Rhee SG *et al.* 2005) (Fig. 1.1).

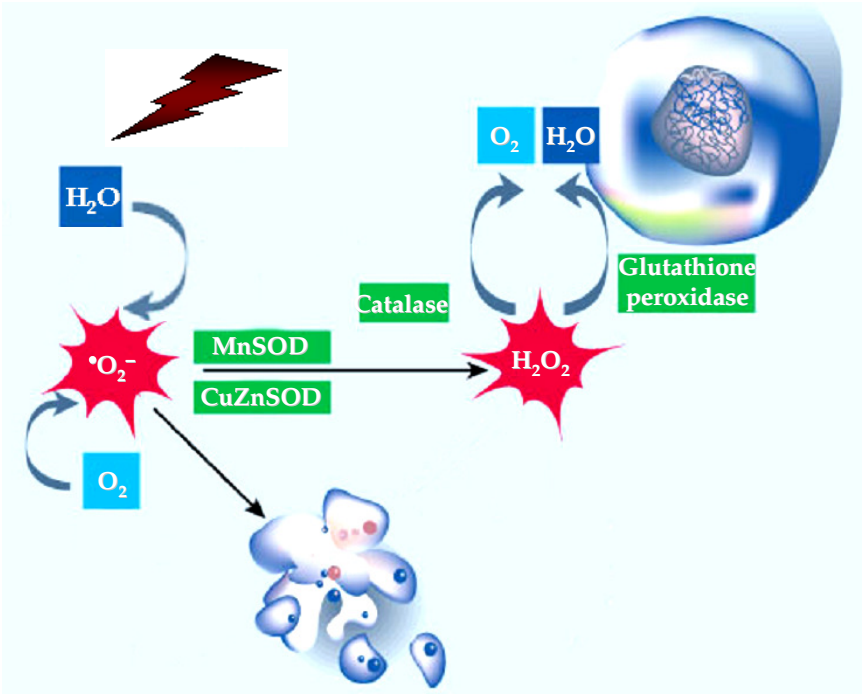


Fig.1.1: Schematic illustration of cellular antioxidant system. Based on Cleveland *et al.* 2000.

In mammals, there are three types of SOD: cytoplasmic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2) and extracellular SOD (SOD3) (Hirose *et al.* 1995). There is no similarity in sequence and structure between MnSOD and CuZnSOD (Culotta *et al.* 2006). SOD is involved in a fast and efficient detoxification of superoxide radicals after exposure of cells to ionizing radiation (IR) (Motoori *et al.* 2001). Thus, treatment with SOD inhibitors may be an effective approach to enhance cell killing by ionizing radiation.

MnSOD, discovered by Fridovich *et al.*, is composed of tetramers of 21 kDa subunits. Each subunit contains a single manganese atom which is bound at the active site (Fridovich *et al.*). MnSOD is localised in mitochondria matrix (Hirose *et al.* 1995). The enzyme respiratory chain in mitochondria is a main source of superoxide anions (Murphy 2009). A high level of superoxide dismutases containing manganese helps to remove high toxic reactive oxygen species (Zablocka and Janusz 2008).

CuZnSOD is mainly localized in cytosol (Chang *et al.* 1988). A small amount of CuZnSOD has been also found in the intermembrane space of mitochondria (Field *et al.* 2003, Lindenau *et al.* 2000, Okado-Matsumoto and Fridovich 2002, Sturtz *et al.* 2001, Weisiger and Fridovich 1973), in nuclei, lysosomes and peroxisomes (Chang *et al.* 1988). However, the presence of CuZnSOD in mitochondria is controversial (Vijayvergiya *et al*. 2005). In mammals high expression of CuZnSOD has been found in the liver, where xanthine oxidase is highly expressed in kidney (Asayama and Burr 1985). CuZnSOD is composed of two subunits which have an immunoglobulin – like fold with an active site for one copper and one zinc ion (Culotta *et al.* 2006) Moreover, CuZn containing superoxide dismutase without new protein synthesis is activated by copper (Brown *et al.* 2004, Schmidt *et al.* 2000, Bartnikas and Gitlin 2003).

Superoxide dismutases by their antioxidant activity play an important role in cancer. Numerous studies show that there is a correlation between tumor progression and SOD activity and expression. Initially studies have reported that SOD can suppress tumor development (Kwee *et al.* 1991, Safford *et al.* 1994). However, recent findings showed contradictory results. Increase of SOD expression results in higher aggressiveness and invasion of some cancers (Connor *et al*. 2007, Nelson *et al*. 2003). SOD overexpression has been found in aggressive human solid tumors (Kinnula and Crapo 2004) and associated with poor outcome and with resistance to cytotoxic drugs and radiation (Kim *et al.* 2003, Kinnula and Crapo 2004). Thus, SOD can be assumed as a potential target of anticancer therapy (Yokoe *et al.* 2009).

Manganese superoxide dismutase is one of the downstream target genes of the nuclear factor-κB (NF-κB) (Saccani *et al.* 2001, Murley *et al.* 2004, Sun *et al.* 2007). The *MnSOD* gene has NF-κB-binding sites in its promoter and enhancer (Xu *et al*.1999, Kiningham *et al*. 2001, Dhar *et al*., 2004). NF-κB is involved in radioresistance of tumor cells (Fan *et al.* 2007). In response to radiation, NF-κB activates the expression of MnSOD leading to tumor cell protection (Guo *et al*. 2003, Murley *et al*. 2004). NF-κB signalling pathway activates expression of numerous genes involved in immune response, inflammation, cell survival, and tumorigenesis (Sun *et al.* 2007). The NF-κB family consists of RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100) (Criswell *et al*. 2003). NF-κB is normally inactivated in the cytoplasm by its inhibitor IκB family members (Sun *et al.* 2007). NF-κB can be activated by radiation-induced reactive oxygen species (Criswell *et al*. 2003). There are two major NF-κB pathways: the p50:RelA dimer-mediated classic pathway and alternative pathway mediated by the p52:RelB dimmer (Sun *et al.* 2007). During activation of

classical pathway the IκB kinase complex, which consists of IKKα, IKKβ, and IKKγ is activated that results in IκBα phosphorylation, ubiquitination, and degradation by the 26S proteasome. The p50:RelA dimer is released and translocated into the nucleus (Sun *et al.* 2007). After activation of the alternative pathway the IKKα homodimer is activated that leads to the partial degradation of p100 and activation of the p52:RelB dimer (Sun *et al.* 2007). Both pathways are induced by radiation treatment (Russell and Tofilon 2002, Sun *et al.* 2007).

Flavonoids are a class of plant secondary metabolites which posses antioxidant activity (Yu *et al.* 2005). Flavonoids have a polyphenolic structure. It has been shown that flavonoids act as free radical scavengers and modulate enzymatic activities (Duthie and Crozier *et al.* 2000, Yu *et al.* 2005). Flavonoids showed strong radical scavenging activities. Among flavonoids the most potent radical scavenger is rutin which showed the highest radical scavenging activity (Yu *et al.* 2005).

1.1.4 SOD inhibitors

Diethyldithiocarbamate (DDC) is a copper-chelating agent which inactivates SOD (Hong *et al.* 1998). It was observed that DDC administered to tumors four hour before ionizig radiation exposure exerts a radiosensitizing effect (Kent *et al.* 1991). Another SOD inhibitor TRIEN was described as inhibitor which enhanced the amount of DNA breaks after gamma irradiation in healthy donors as well as in patients with hereditary diseases with defective DNA damage processing, like Bloom and Marfan syndrome. TRIEN increased also cellular radiosensitivity (Zasukhina *et al.* 2003).

1.1.4.1 2-methoxyestradiol as SOD inhibitor

2-methoxyestradiol (2-ME), physiological metabolite of natural and synthetic estrogen (Ray *et al.* 2006, Zhu *et al.* 1998), generated from hydroxylation at the 2-C position of 17ß-estradiol by cytochrome P450 and from methylation at the same position by catechol-O-methyl transferase (COMT) (Fig. 1.2) (Ray *et al.* 2006, Zhu *et al.* 1998). 2-ME is a promising anticancer agent. A wide range of anti-tumor effects of 2-ME have been reported (Ray *et al.* 2006, Lakhani, Figg 2003). The exact mechanism of action of 2-ME is still not completely clear. Several possible mechanisms have been suggested, including antiproliferative effect - 2-ME selectively inhibits proliferating cells (Kamat *et al.* 2000, Ray *et al.* 2006, Zhu *et al.* 1998), anti-angiogenic effect (Lin *et al.* 2007, Ray *et al.* 2006, Pribluda *et al.* 2000) and

apoptosis (LaVallee *et al.* 2002) which can be induced through several mechanisms e.g. p53 upregulation, death receptor, free radical generations, activation of c-Jun N-terminal-activated kinase (JNK), Bcl-xL and Bcl-2 phosphorylation, increased expression of FAS or mitochondrial release of cytochrome c (Zhu *et al.* 1998). Moreover, 2-ME enhances radiation sensitivity in various cancer cell types (Huober *et al.* 2007, Zou *et al.* 2006, Zou *et al.* 2007). Therapeutic doses of 2-me show minimal or no toxicity (Ray *et al.* 2006, Lakhani *et al.* 2003, Pribluda *et al.* 2000).

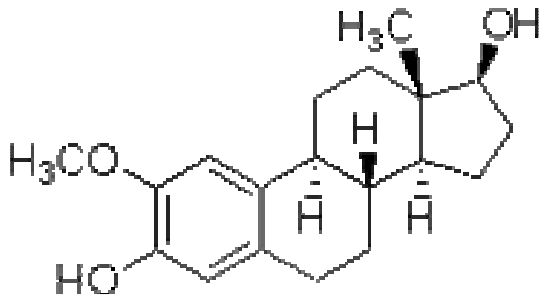


Fig.1.2: Chemical structure of 2-methoxyestradiol

It has been identified that 2-ME is an inhibitor of both Mn and CuZnSOD activity and preferentially kills human leukemia cells (Huang *et al*. 2000). On the other hand Kachadourian *et al*. reported that 2-ME does not inhibit superoxide dismutases but increase superoxide production in the cells.

It has been reported that 2-ME induced also apoptosis proceeds through reactive oxygen species and blockage of AKT activity in human leukemia cells (Gao *et al.* 2005). It has been also published that 2-ME down-regulated AKT phosphorylation in K562 cells (Zhang *et al.* 2009), in human acute myeloid leukaemia cells (Chow *et al.* 2008), in gastric cancer cells (Lin *et al.* 2007) and PC3 prostate cancer cells (Shimada *et al.* 2004). 2-ME can block 17ß-estradiol –induced AKT phosphorylation in human breast cancer cells (Vijayanathan *et al.* 2006). In combination with bis(ethyl)norspermine (BE-3-3-3) 2-ME altered AKT activity. Inhibition of AKT phosphorylation by 2-ME indicates that PI3K/AKT survival pathway is blocked by this inhibitor (Lin *et al.* 2007, Barchiesi *et al.* 2006).

1.2 Aim of the study

Ionizing radiation (IR) induces superoxide dismutases (SOD) which may protect cells against radiation effect. With respect to tumor biology this of special interest as it is known that cancers cells are also characterized by enhanced levels of SOD. Furthermore, IR induces activation of membrane bound receptor tyrosine kinases (RTK) i.e. EGFR which has been proposed to be involved in tumor repopulation during conventional radiotherapy. Moreover, in recent studies have been shown that ionizing radiation (IR) activates the PI3K/AKT survival pathway which plays important role in post-irradiation survival through regulation of DNA-double strand break repair. Likewise, the PI3K/AKT pathway and NF-κB activity have been shown to regulate SOD expression.

Therefore, the present investigation was focused on the following objectives:

1. The effect of ionizing radiation on superoxide dismutase expression and activity in lung carcinoma cell lines.
2. The role of radiation induced SOD activity and expression in radioprotection of lung cancer cells *in vitro*.
3. The role of RTK and its downstream pathways in SOD regulation following ionizing radiation exposure.
4. The mechanism of 2-ME mediated radiosensitization in lung carcinoma cell lines.
5. NF-κB dependent MnSOD nuclear translocation.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory chemicals

Acetic acid	Merk
Acrylamide	Roth
APS	Aldrich
β-Glycerol phosphate	Sigma Aldrich
β-Mercaptoethanol	Sigma Aldrich
β-Propanol	Merk
Bromphenol blue	Pharmacia Biotech
BSA	Roth
Coomassie® Brilliant Blue G 250	Serva
Crystal Violet	Sigma Aldrich
DAPI	Serva
DMSO	Sigma Aldrich
DTT	Sigma Aldrich
EDTA	Sigma Aldrich
Ethanol	Merk
Formaldehyde	Merk
HCL	Roth
HEPES	Sigma Aldrich
KCl	Merk
Lipofectamine™ 2000	Invitrogen
Mowiol	Sigma Aldrich
$NaHCO_3$	Biochrom
Nitro blue tetrazolium (NBT)	Sigma Aldrich
Nonidet P-40 (NP-40)	Sigma Aldrich
Penicilin-Streptomycin	Gibco
Ponceau S	Sigma Aldrich
Potassium phosphate buffer	Sigma Aldrich
Protein A Sepharose	Amersham Bioscience
Riboflavin	Sigma Aldrich

SDS	Serva
Sodium chloride	Merk
Sodium fluoride	Merk
Sodium orthovanadate	Sigma Aldrich
TEMED	Sigma Aldrich
Triton X 100	Sigma Aldrich
TRIZMA-Base	Sigma Aldrich
TRIZMA-HCL	Sigma Aldrich
Trypsin	Serva
Tween 20	Roth

2.1.2 Kits and other materials

96-wells plates	Nunc, Germany
Cell culture materials	Falcon, UK/ Greiner, Solingen, Germany
ECL detection kit	Amersham Pharmacia Biotech, UK
Phosphatase inhibitor cocktail 1	Sigma Aldrich, Taufkirchen, Germany
Protease inhibitor	Roche, Manheim, Germany
Protein A sepharose	Amersham Biosciences, Freiburg, Germany
PVDF 0.20 µm membrane	Schleicher & Schüll, Dassel, Germany
SOD activity assay kit	Chemicon, Schwalbach, Germany
Sterile filter 0.2 µM	Sartorious, Hannover, Germany
Sterile filtertips	Greiner BioOne, Germany
X-ray film	Agfa-Gevaert, Belgium
Whatman paper	Schleicher & Schüll, Dassel, Germany

2.1.3 Instruments

β-counter	Wallac, Freiburg, Germany
Centrifuge	Hettich, Germany
Electrophoresis Units	Hoefer, USA
ELISA reader	Anthos Labtec, Salzberg, Austria
Microscopes	Zeiss, Germany
X-ray machine (RS-225)	Gulmay, England

2.1.4 Buffers

Blotting buffer (anode)	3.1 g	Boric acid
	4 ml	SDS 10 %
	200 ml	Methanol
	ad l Lit	H2Odd
	pH 9.0 (NaOH)	
Blotting buffer (cathode)	3.1 g	Boric acid
	4 ml	SDS 10 %
	50 ml	Methanol
	ad l Lit	H_2Odd
	pH 9.0 (NaOH)	
Lysis buffer	50 mM	Tris-HCL pH 7.5
	50 mM	Glycerophosphate
	150 mM	NaCl
	1 mM	NaF
	1 mM	DTT
	1 mM	NaVO4
	10 %	Glycerol
	1 %	Tween 20
	1 %	Phosphatase inhibitor
	1 Tab/10ml	Protease inhibitor
	5 g	SDS
	ad 1 lit.	H_2Odd
	pH ≈ 8,6	
Lysis buffer (SOD activity assay)	10 mM	Tris HCl
	pH 7.5	
	150 mM	NaCl
	0.1 mM	EDTA
	0.5 %	Triton X-100

Lysis buffer A	100 µl	HEPES pH 7.9
(nucleus – cytoplasmic preparation)	40 µl	2.5 M KCl
	2 µl	0.5 M EDTA
	10 µl	0.1 M EGTA
	10 µl	1 M DTT
	1 tablet	Protease inhibitor
	1 %	Phosphatase inhibitor
	ad 10 ml	H_2Odd
Lysis buffer C	50 µl	HEPES pH 7.9
(nucleus – cytoplasmic preparation)	400 µl	2.5 M KCl
	5 µl	0.5 M EDTA
	25 µl	0.1 M EGTA
	10 %	Glycerin
	2.5 µl	1 M DTT
	0.25 tablet	Protease inhibitor
	1 %	Phosphatase inhibitor
	ad 2.5 ml	H_2Odd
PBS	13.7 mM	NaCl
	2.7 mM	KCl
	80.9 mM	Na2HPO4
	1.5 mM	KH2PO4
Protein loading buffer (sample buffer)	2.5 ml	4x Stacking gel buffer
	2.0 ml	SDS (10 %)
	2.0 ml	Glycerin
	0.5 ml	β-Mercaptoethanol
	0.25 mg	Bromphenol blue
	ad 10 ml	H_2Odd
Running buffer (5x)	72.05 g	Glycin
	15.15 g	Tris-Base
	5 g	SDS

	ad 1 lit	H_2O
Separating gel buffer (4x)	18.17 g	Tris-Base
	4 ml	10 % SDS
	ad 100 ml	H_2Odd
	pH 8.8 (12N HCL)	
Stacking gel buffer (4x)	6.06 g	Tris-Base
	4 ml	10 % SDS
	ad 100 ml	H_2Odd
	pH 6.8 (12N HCL)	
Stripping buffer	4.5 g	Glycin
	0.3 g	SDS
	3 ml	Tween 20
	ad 300 ml	H_2Odd
	pH 2.2 (12N HCL)	
TBST	10 mM	Tris-HCL
	pH 7.5 (NaOH)	
	100 mM	NaCl
	0.1 %	Tween 20

2.1.5 Ligands, inhibitors and radical scavengers

Ligands:

AREG	Sigma Aldrich, Taufkirchen, Germany
EGF	Sigma Aldrich, Taufkirchen, Germany
IGF-1	Sigma Aldrich, Taufkirchen, Germany
TGFα	Sigma Aldrich, Taufkirchen, Germany

Inhibitors:

2-ME - 2-methoxyestradiol	Sigma Aldrich Munich, Germany
API-59CJ-OH – AKT pathway inhibitor	Jin *et al.* 2004
ATM inhibitor (KU55933)	Calbiochem, Schwalbach, Germany
BIBX1382BS - EGFR TK inhibitor	Boehringer Ingelhein, Austria
DNA-PKcs inhibitor (NU7026)	Calbiochem, Schwalbach, Germany
LY294002 - PI3K inhibitor	Calbiochem, Schwalbach, Germany
NF-κB Activation Inhibitor	Calbiochem, Schwalbach, Germany

Radical scavengers:

Rutin trihydrate	Sigma Aldrich Munich, Germany

Recombinant adeno-associated viruses:

rAAV2-MnSOD	Veldwijk *et al.* 2004

2.1.6 Small interfering RNA (siRNA)

AKT siRNA	Dharmacon, Chicago, USA
CuZnSOD siRNA	Dharmacon, Chicago, USA
MnSOD siRNA	Dharmacon, Chicago, USA
Non target siRNA	Dharmacon, Chicago, USA
P85 siRNA	Dharmacon, Chicago, USA

2.1.7 Antibodies

Primary antibodies

Actin	Rabbit, monoclonal	Sigma Aldrich
AKT1	Mouse, monoclonal	BD, Transduction Laboratories
CuZnSOD	Rabbit, antiserum	Upstate
DNA-PKcs	Mouse, monoclonal	BD, Transduction Laboratories
EGFR	Mouse, monoclonal	BD, Transduction Laboratories

IGF-IRß	Rabbit, polyclonal	Santa Cruz Biotechnology
Lamin B	Mouse, monoclonal	Abcam
MnSOD	Rabbit, polyclonal	Upstate
MTCO1	Mouse, monoclonal	Biozol
NF-κB	Rabbit, polyclonal	Santa Cruz Biotechnology
PI3K p85	Rabbit, polyclonal	Cell Signaling Technology
P-AKT (Ser-472/3)	Mouse, monoclonal	BD Pharmingen
P-AKT (Ser-473)	Rabbit, polyclonal	Cell signalling Technology
P-AKT (Thr-308)	Rabbit, polyclonal	Cell signalling Technology
P-ATM (Ser-1981)	Mouse, monoclonal	Upstate
P-DNA-PKcs (Ser-2056)	Rabbit, polyclonal	Abcam
P- DNA-PKcs (Thr-2609)	Rabbit, polyclonal	Abcam
P-H2AX (Ser-139)	Mouse, monoclonal	Upstate
P-Tyr (PY20)	Mouse, monoclonal	BD, Transduction Laboratories

Secondary antibodies

Donkey anti-Rabbit	Amersham Pharmacia Biotech
Sheep anti-mouse	Amersham Pharmacia Biotech

2.1.8 Cell culture media

Dulbecco,s Modified Eagle Medium (DMEM) containing 4.5 mg/ml glucose supplemented with 44.04 mM NaHCO3 - Gibco

RPMI-1640 containing L-glutamine, supplemented with 26.8 mM NaHCO3 – Gibco

2.1.9 Cell lines

The following established human cell lines were used:

A549	Human non-small cell carcinoma of lung	ATCC, CCL-185
H460	Human non-small cell carcinoma of lung	ATCC, HTB-177
H661	Human non-small cell carcinoma of lung	ATCC, HTB183

2.2 Methods

2.2.1 Cell culture

Cell lines were incubated in a humidified atmosphere of 93% air / 7% CO_2 at 37°C and routinely assayed for mycoplasma contamination using DAPI staining. A549 cells were cultured in DMEM, H460 and H661 in RPMI. Media were supplemented with 10% FCS and 1% penicillin/streptomycin.

2.2.2 Inhibitors treatment

AKT pathway inhibitor (API-59CJ-OH), 2-methoxyestradiol (2-ME), ATM inhibitor (KU55933) and DNA-PKcs inhibitor (NU7026) were made at 10 mM concentration in dimethyl-sulfoxide (DMSO) and stored at -70°C. For treatment, stock solutions were diluted in culture medium containing 10% FCS to the appropriate working concentrations. Controls received medium containing the corresponding concentration of DMSO.

2.2.3 SiRNA transfection

Cells were transfected with specific siRNA against *AKT1, PI3K* (subunit *P85*)*, MnSOD, CuZnSOD* or *control* siRNA using Lipofectamine™ 2000 according to the manufacturer´s protocol (Toulany *et al*. 2007). In brief, cells were splitted to six-well plates and 24 h later were transfected with negative control or target siRNAs at a final concentration of 50 nM. Suppression of protein was analyzed at day three or four after transfection using Western blot analysis.

2.2.4 Transduction

Cells were seeded in either 6-well or 48-well plates one day before transduction (Veldwijk *et al.* 2004). Cells were transduced with MnSOD containing recombinant adeno-associated virus 2 (rAAV2-MnSOD) at a multiplicity of infection (MOI) 150. For clonogenic survival assay cells were irradiated with doses 2, 3 and 4 Gy 3 days after transduction. 6 h following radiation treatment cells were seeded into clonogenic formation assay and cultured for 10 days. To study the protein level of MnSOD after transduction using Western blotting protein was isolated three days after transduction.

2.2.5 Irradiation

Cells were X-ray-irradiated at 37°C using a Gulmay RS-225 X-ray generator operating at 200 kV and 15 mA.

2.2.6 Clonogenic assay

Clonogenic cell survival following inhibitor treatment or siRNA transfection was analyzed by means of colony formation assay (Toulany *et al.* 2008). After indicated treatment in each experiment, cells were trypsinized, plated in 6-well plates at a constant cell density (250 cells per well). 24 h later cells were irradiated with 1-5 Gy. In experiments using ATM and DNA-PKcs inhibitors cells were treated in 6-well plates for one hour and irradiated. In case of siRNA transfection 48 h after transfection cells were trypsinized, plated in 6-well plates and 24 h later were irradiated. In each of this experiments cells were incubated for 10 days to allow for colony growth. Thereafter culture dishes were stained with Coomasie or crystal violet and colonies with 50 cells or more were scored as survivors. Surviving fraction following irradiation was calculated based on plating efficiency of non-irradiated controls.

2.2.7 SOD activity gel

At indicated time points after treatment cells were washed two times with ice-cold PBS, lysed with lysis buffer and collected in tubes. After sonication lysates were centrifuged at 14000 rpm for 15 min at 4°C. Protein quantification was performed and 0.1 – 1 mg protein was loaded to 12 % native polyacrylamide gel. After electrophoresis gel staining was performed. SOD activity was measured based on the inhibition of the reduction of nitroblue tetrazolium by SOD as described by Beauchamp and Fridovich (1971). Gel was soaked in NBT, washed with water, soaked in dark in potassium phosphate buffer (pH 7.0) containing TEMED and riboflavin, washed with water and illuminated on light box to initiate reaction. The achromatic bands showed the presence of SOD activity.

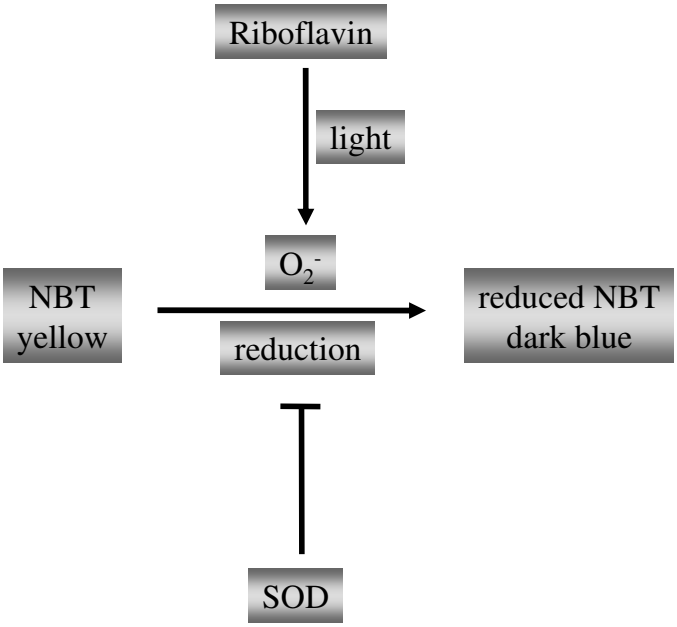


Fig. 2.1: SOD activity gel stainig principle.

2.2.8 Superoxide dismutase enzyme activity assay

SOD enzyme activity was performed from cell lysates according to the supplier's instruction. In this assay xanthine/xanthine oxidase (XOD) system was used to generate superoxide anions ($\cdot O_2^-$) Superoxide anions produced by this system was reduced by SOD expression and resulted in reduction of colorimetric signal. Solutions provided by supplier including xanthine, chromagen, assay buffer and xanthine oxidase were added to the 400 - 500 µg protein samples. Following 2 h incubation in 37^o C optical density (OD) was measured by an ELISA reader at 450 nm (Fridovich 1997, Forman and Fridovich 1973).

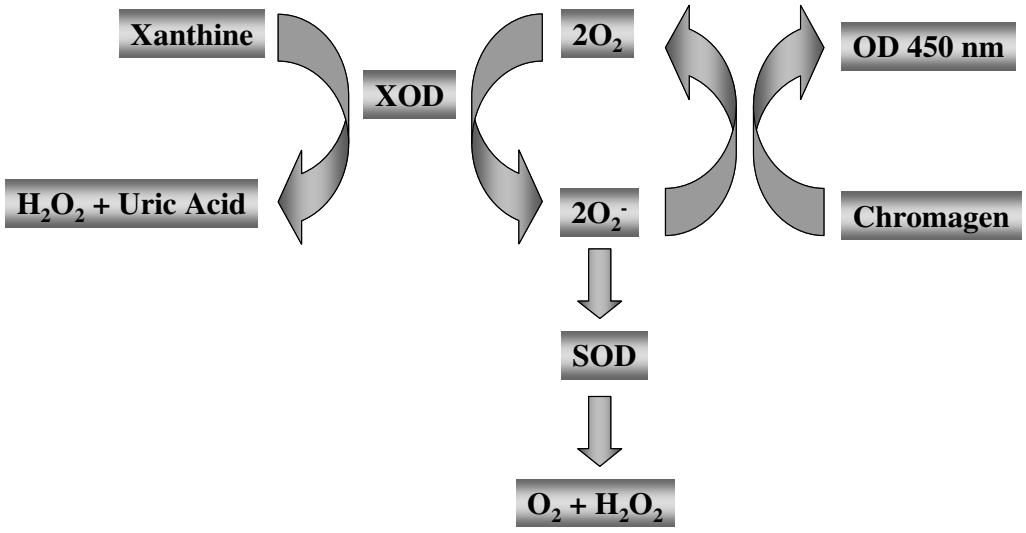


Fig. 2.2: SOD enzyme activity assay principle.

2.2.9 Protein analysis

2.2.9.1 Lysis of cells

At indicated time points after treatment cells were washed two times with ice-cold PBS, lysed with lysis buffer and collected in tubes. After sonication lysates were centrifuged at 14000 rpm for 15 min at 4°C.

2.2.9.2 Nucleus – cytoplasmic preparation

At indicated time points after treatment cells were washed two times with ice-cold PBS and collected in 600 µl PBS. Probes were centrifuged with 1300 rpm for 5 min at 4°C and PBS was removed. Cells were resuspended in 400 µl of buffer A and incubated with shaking in ice. After 30 min incubation 10 % NP-40 was added. Samples were vortexed and centrifuged with high speed for 1 min. Supernatant contained cytoplasmic protein. Pellet was washed with buffer A and recentrifuged with high speed for 1min. 200 µl of buffer C was added to the pellet and samples were vortexed and incubated in ice with high speed shaking for 1 h. Every 15 min vortex was done. After incubation samples were centrifuged with high speed for 1 min at 4°C. Supernatant contained nucleus protein.

2.2.9.3 Protein quantification

For protein quantification the Bio-Rad protein assay kit was used according to manufactures protocol. As standard BSA was used.

2.2.9.4 Immunoprecipitation

Whole lysate or nuclear fraction was incubated with antibody against protein of interest and 50 µl of 50% protein A-sepharose overnight at 4 °C. Precipitates were washed three times with 200 µl lysis buffer and sample buffer was added. Protein samples were boiled for 5 min and subjected to SDS-PAGE.

2.2.9.5 SDS – polyacrylamide gel electrophoresis (SDS-PAGE) and transfer of proteins on nitrocellulose membrane

50 -100 µg of whole lysates were directly subjected to SDS-PAGE. SDS-PAGE was conducted. After SDS-PAGE proteins were transferred to nitrocellulose membrane for 2 - 2.5 hours
at 0.8 mA/ cm^2 of gel. Transferred proteins were stained with Ponceau S (0.1% in 5% acetic acid)

2.2.9.6 Immunoblott detection

After blotting the membranes were blocked by 3% BSA or nonfat dry milk for 1 h at RT. Thereafter, membranes were incubated with primary antibody overnight, washed two times with TBST, incubated with secondary antibody and washed again with TBST. Antibody-antigen complexes were identified using Horseradish Peroxidase (HRP) coupled to secondary anti-IgG antibody. To visualize peroxidase activity luminescent substrates were used. To check the loading membranes were stripped for 30 min at RT, blocked and either actin, lamin A+C or whole protein were detected.

2.2.10 γH2AX foci assay

γH2AX foci assay (Toulany *et al.* 2008) was applied for determination of DNA-double strand breaks (DNA-dsb). Briefly, cells were cultured on glass slides and 24 h later were treated with DMSO and 2-ME. After 2 h cultures were irradiated with single dose of 4 Gy and incubated

for 6 h at 37°C. Thereafter, γH2AX foci assay for residual DNA-dsb was performed. Cells were fixed by 2 % formaldehyde in PBS, washed 3 times in PBS and permeabilized by 0.2 % Triton X 100 in PBS / 1 % BSA. After washing in PBS, samples were blocked in PBS / 3 % BSA. Cells were incubated with P-H2AX antibody over night, washed with PBS / 1 % BSA / 0.5 % Tween 20 and incubated with second antibody. After washing cells were incubated with DAPI / antifade. Number of foci were counted using a fluorescence microscope.

2.2.11 Statistics and densitometry

To compare data between two groups Student`s *t*-test was used. Values are expressed as mean ± standard error. P-values lower than 0.05 ($P<0.05$) were considered as statistically significant.

Densitometric quantification of Western blots were performed using Scion Image software (Toulany *et al.* 2006).

3. RESULTS

3.1 Effect of serum and cell density on MnSOD and CuZnSOD expression.

To investigate the influence of serum and cell density on MnSOD and CuZnSOD expression, different number of A549 cells were seeded into 6 cm plates. Cells were either starved or cultured in medium with 10 % FCS. After 48 h protein isolation was done. As shown in Fig. 3.1 in starved cells MnSOD and CuZnSOD expression were markedly higher than in cells cultured in medium with FCS. Density of cells had almost no effect on MnSOD expression in starved cells and only small effect on CuZnSOD expression. In cells cultured in medium with 10 % FCS SOD expression increased parallel with the increasing of cell density.

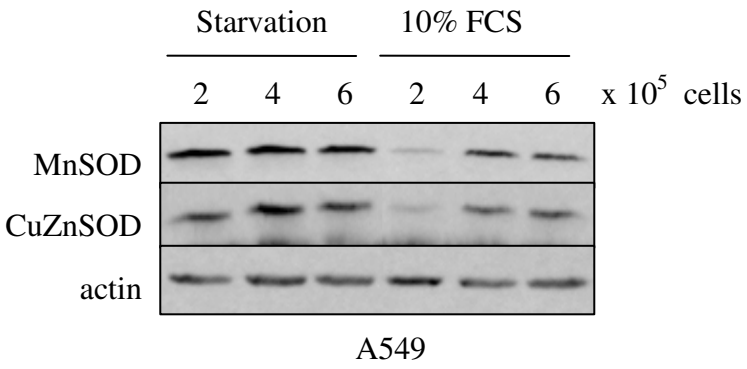


Fig. 3.1: Effect of cell density and serum addition on MnSOD and CuZnSOD basal expression in lung carcinoma cell lines. Cells were grown to reach different status of density. Thereafter cells were lysed, subjected to SDS-PAGE and incubated with antibodies against MnSOD and CuZnSOD. For loading control actin was detected.

3.2 MnSOD and CuZnSOD basal expression in lung carcinoma cell lines.

To compare the basal expression of superoxide dismutases in different lung carcinoma cell lines A549, H460 and H661 cells were seeded into 6 cm plates and were grown to confluent status. Thereafter, the basal level of SOD expression was investigated using Western blot. The basal level of CuZnSOD expression was similar in all investigated cell lines. The expression of MnSOD was almost on the same level in A549 and H661 cells. However, H460 cells express MnSOD at higher level than the other two lung carcinoma cell lines (Fig. 3.2).

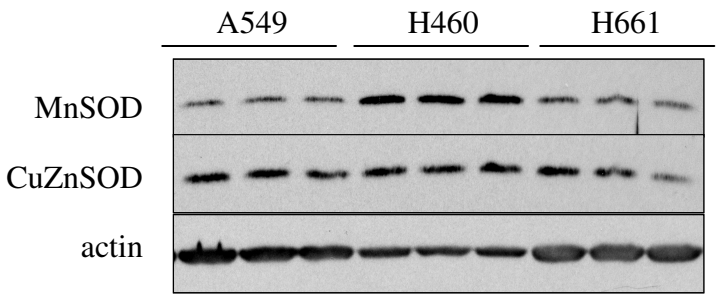


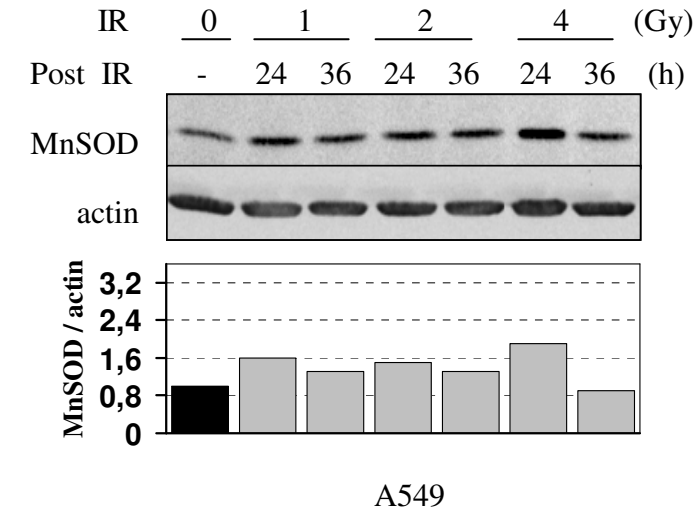
Fig. 3.2: Basal protein expression of MnSOD and CuZnSOD in lung carcinoma cell lines. Confluent cells were lysed, subjected to SDS-PAGE. MnSOD and CuZnSOD expression was analyzed by specific antibodies. Actin was used as a loading control.

3.3 IR-induced MnSOD and CuZnSOD expression in lung carcinoma cell lines.

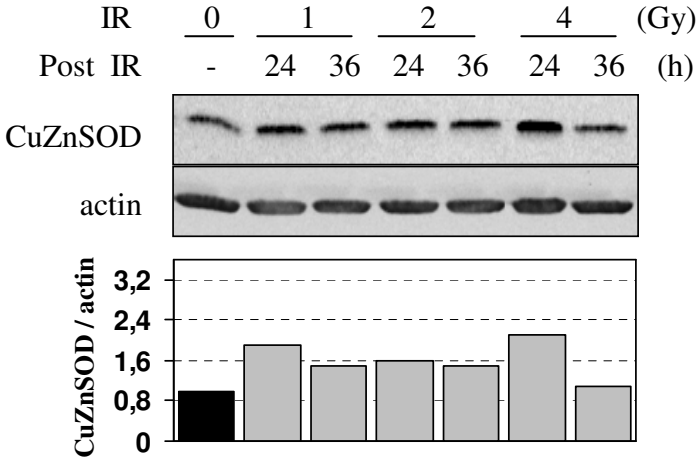
It has been described that ionizing radiation can induce the superoxide dismutases protein level (Kuninaka *et al.* 2000). The effect of irradiation on MnSOD and CuZnSOD expression in lung carcinoma cell lines was investigated. Exposure to single radiation doses (1, 2 and 4 Gy) induced expression of superoxide dismutases in a dose independent manner. As compared to basal levels, 24 h post-ionizing radiation (IR) expression of MnSOD and CuZnSOD was increased by factors of 1.87 ± 0.12 and 1.67 ± 0.13, respectively and decreased to factors of 1.37 ± 0.13 and 1.17 ± 0.13 above basal level 36 h post IR for A549 cells (Fig. 3.3 A). In H460 cells expression of MnSOD was increased by factor of 1.9 ± 0.03 24 h post IR and further increased to factor of 2.6 ± 0.4 36 h post IR. 24 h post IR CuZnSOD expression was increased by factor of 1.7 ± 0.15 and decreased to factor 1.4 ± 0.06 36 h post IR (Fig. 3.3 B).

A549 cells were irradiated 6 x 3 Gy, every 24 h or with single high dose 25 Gy (Fig. 3.4) to compare the effect of high dose and fractionated treatment on MnSOD and CuZnSOD expression. MnSOD and CuZnSOD were only slightly induced by single high radiation dose of 25 Gy. Fractionated treatment increased strongly the level of MnSOD as well as CuZnSOD.

A)



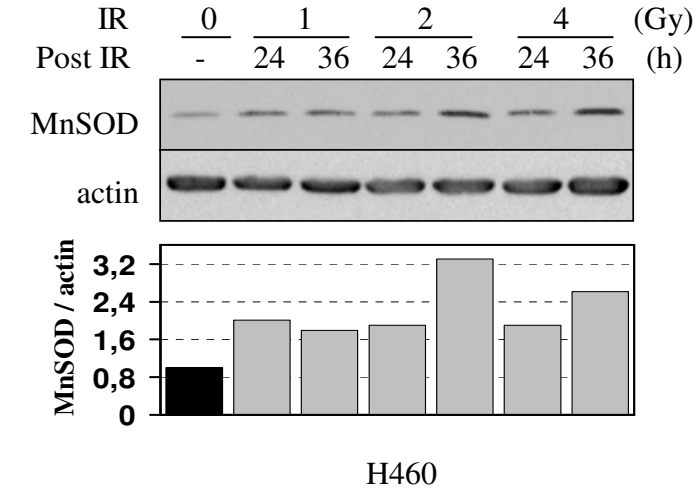
A549



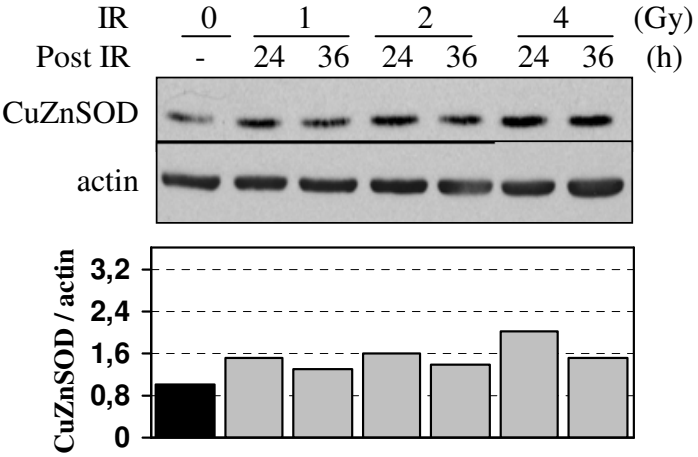
A549

Fig. 3.3 A : IR-induced MnSOD and CuZnSOD protein expression. A549 cells were irradiated with single radiation doses (1, 2 and 4 Gy). At indicated times cells were lysed and MnSOD as well as CuZnSOD and actin were assessed by immunoblotting with specific antibodies. Based on densitometry the relative expression level of MnSOD and CuZnSOD was quantified.

B)



H460



H460

Fig. 3.3 B : IR-induced MnSOD and CuZnSOD protein expression. H460 cells were irradiated with single radiation doses (1, 2 and 4 Gy). At indicated times cells were lysed and MnSOD as well as CuZnSOD and actin were assessed by immunoblotting with specific antibodies. Based on densitometry the relative expression level of MnSOD and CuZnSOD was quantified.

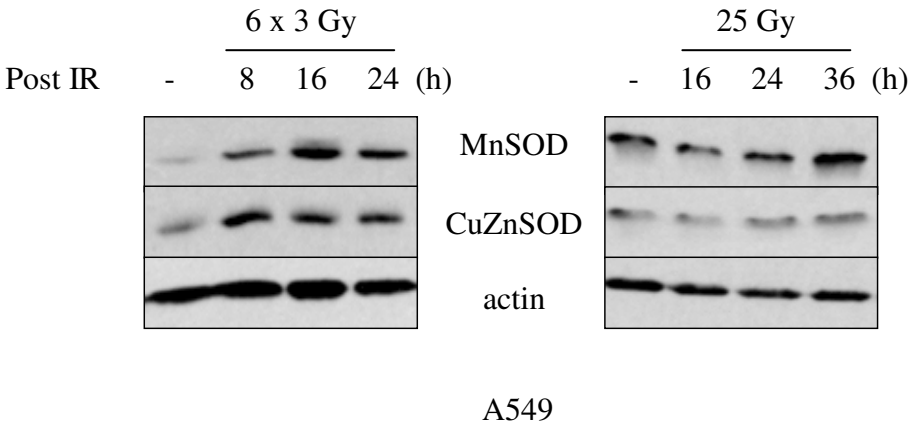


Fig. 3.4: Effect of single dose and fractionated radiation treatment on MnSOD and CuZnSOD expression. A549 cells were irradiated six times with dose of 3 Gy every 24 h. At indicated time points after radiation protein expression of superoxide dismutases was investigated. Actin was used as loading control.

To investigate whether increase of number of radiation doses will further increase expression of superoxide dismutases, A549 cells were irradiated with the same total radiation dose but different number of radiation fraction: 6 x 3 Gy vs. 12 x 1.5 Gy. Based on data shown in Fig. 3.5 it has been observed that both types of fractionation induced SOD protein expression. However, there was not importantly difference between different ways of the radiation treatment.

To investigate whether fractionated radiation has the same effect in H460 cells like in A549, A549 and H460 cells were irradiated 6 x 3Gy and the protein expression level of SOD was compared. In A549 cells MnSOD was strongly and CuZnSOD slightly induced by ionizing radiation. However, fractionated radiation did not increase the level of SOD in H460 (Fig. 3.6).

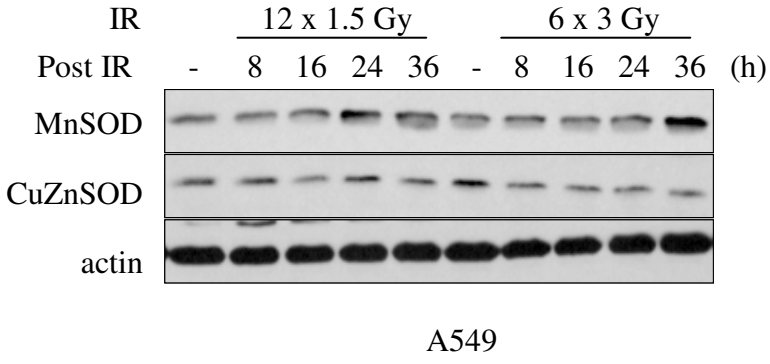


Fig. 3.5: Effect of total radiation dose and number of radiation fraction on MnSOD and CuZnSOD protein expression. A549 cells were irradiated either 12 x 1.5 Gy every 12 h or 6 x 3 Gy every 24 h. At indicated time points after irradiation cells were lysed and subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

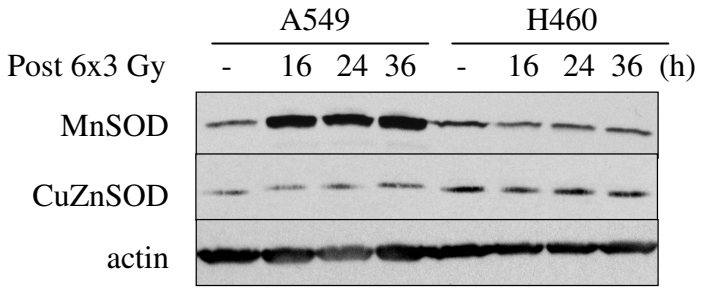
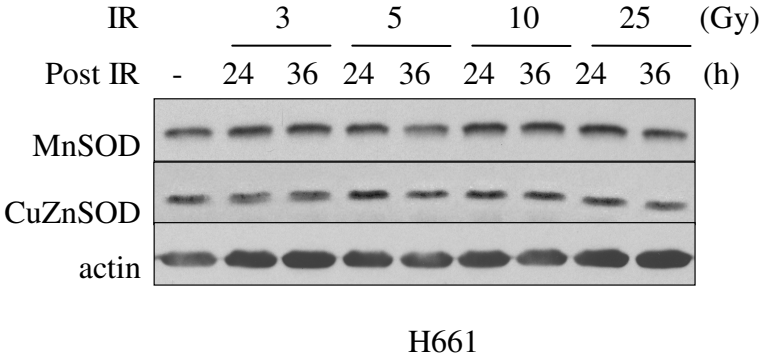


Fig. 3.6: Effect of fractionated radiation on MnSOD and CuZnSOD protein expression in A549 and H460 cells. A549 and H460 cells were irradiated 6 x 3 Gy every 24 h. 16, 24 and 36 h after last radiation fraction protein isolation was performed. Specific antibodies against MnSOD and CuZnSOD were used. Actin was detected as loading control.



H661

Fig. 3.7: IR-induced MnSOD and CuZnSOD protein expression. H661 cells were irradiated with single radiation dose (3 – 25 Gy). Cells were lysed at indicated time points and immunobloting was performed by a MnSOD and CuZnSOD antibodies. As loading control actin was detected.

In H661 cells expression of MnSOD was analyzed following irradiation with doses of 3, 5 10 and 25 Gy. No induction of MnSOD as well as CuZnSOD could be observed (Fig. 3.7).

3.4 IR-induced SOD enzyme activity.

Ionizing radiation induces free radicals what increases the enzyme activity of superoxide dismutases (Durovic *et al.* 2008). Thus, SOD activity assay was performed to analyze the effect of ionizing radiation on SOD enzyme activity in lung carcinoma cell lines. As shown in Fig. 3.8 A SOD enzyme activity in A549 was elevated as early as 1 min after single dose irradiation of 4 Gy and further increased with time. The highest SOD activity has been found 16 h after ionizing radiation. The pattern of IR-induced SOD activity in H460 cells was different. The highest level of SOD activity was observed already one minute after radiation treatment. At the later time points SOD activity was decreased but still induced in comparison

to nonirradiated control (Fig. 3.8 B).

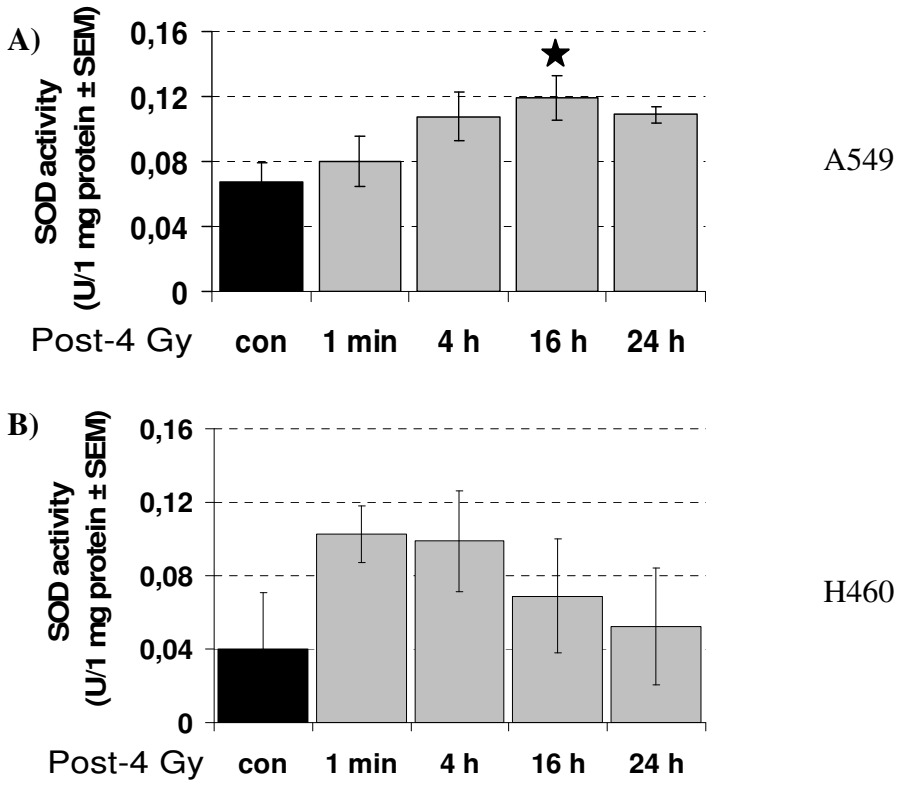
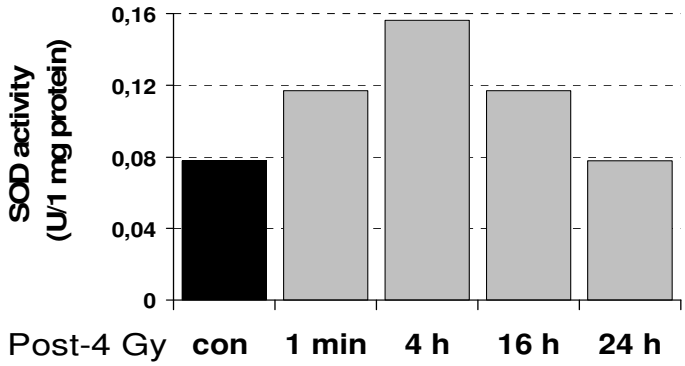


Fig. 3.8: Ionizing radiation induced SOD activity. At indicated times after irradiation protein samples were isolated and SOD activity assay was performed as described in Materials and Methods. Data are mean of three independent experiments. Asterisk indicates significant enhancement of SOD enzyme activity following irradiation in comparison to unirradiated control (Student's *t*-test/$^{*}p<0.05$).

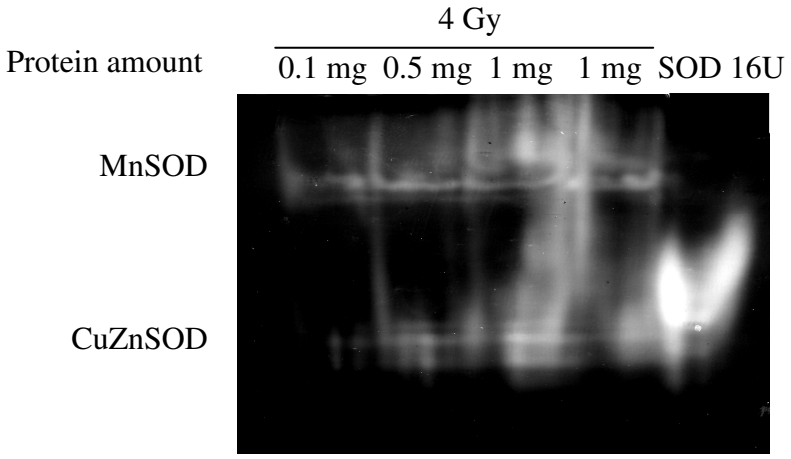
SOD enzyme activity was performed for H661 cells as well. In this cell line ionizing radiation induced SOD was two times higher than in unirradiated control. Likewise, SOD activity was increased already one minute after radiation like in A549 and H460 cells. But the highest level was reached 4 h after irradiation and further decreased (Fig. 3.9).



H661

Fig. 3.9: Ionizing radiation induced SOD activity. Cells were irradiated with 4 Gy, at indicated times after irradiation protein samples were isolated and SOD activity assay was performed as described in Materials and Methods.

To confirm the results a second approach was used. SOD activity in A549 cells was performed using SOD activity gel electrophoresis. Cells were irradiated and different amount of protein was loaded to find best condition for SOD activity study (Fig. 3.10). As positive control 16 U of CuZnSOD has been used.



A549

Fig. 3.10: Ionizing radiation induced MnSOD and CuZnSOD activity. 16 h after irradiation with 4 Gy protein samples were isolated and different amounts of protein were loaded on the native polyacrylamide SOD activity gel as described in Materials and Methods. 16 U of bovine CuZnSOD has been loaded as control. After electrophoresis gel has been stained in NBT.

Thereafter, SOD activity was investigated one minute and 16 h after treatment with ionizing radiation. MnSOD activity was again observed already one minute after radiation and was increased 16 h after IR (Fig. 3.11). No effect of irradiation on CuZnSOD activity was noted.

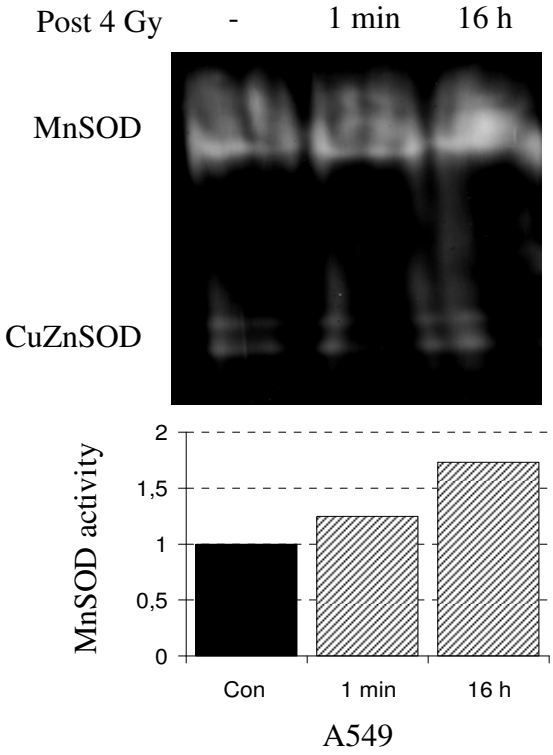


Fig. 3.11: Ionizing radiation induced SOD activity. At indicated times after irradiation with 4 Gy protein samples were isolated and have been loaded on the native polyacrylamide SOD activity gel as described in Materials and Methods. Based on densitometry the level of SOD enzyme activity was quantified.

3.5 Effect of EGFR activity on MnSOD and CuZnSOD expression

Ionizing radiation can induce EGF receptor (EGFR) - signalling in a ligand independent manner (Harari and Huang 2002). Furthermore, treatment of cells with the growth factor EGF results in stimulation of SOD expression (Nishiguchi *et al.* 1994). To investigate the effect of EGFR activity on MnSOD and CuZnSOD expression, A549 cells were treated with different ligands of EGFR. EGFR ligands EGF, TGFα and AREG can stimulate tyrosine phosphorylation of EGF receptor (Toulany *et al.* 2005, Reynolds *et al.* 1981, Marquardt *et al.* 1984, Todaro *et al.* 1990). No effect was found on SOD expression after single treatment with EGF (Fig. 3.12). Thereafter, A549 cells were treated with EGF every 24 h for six days. Likewise, activation of EGFR by ligand treatment did not affect the protein expression of MnSOD and CuZnSOD (Fig. 3.13) .

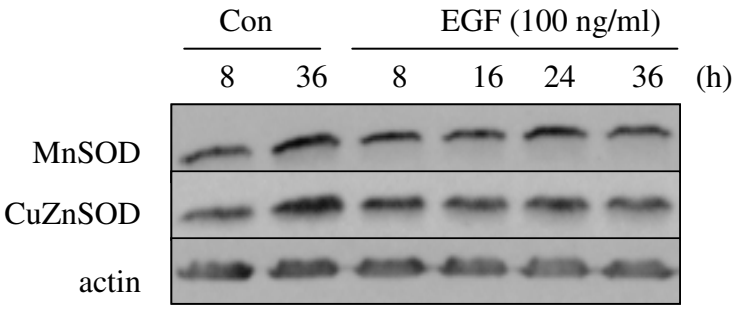


Fig. 3.12: Effect of EGF treatment on MnSOD and CuZnSOD protein expression. At indicated time points following EGF stimulation cells were lysed, subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

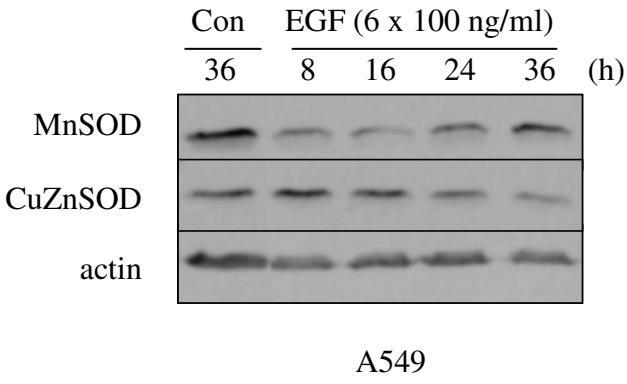


Fig. 3.13: Effect of fractionated treatment with EGF on MnSOD and CuZnSOD protein expression. At indicated time points following fractionated EGF stimulation cells were lysed and subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

For further investigation of the role of EGFR in MnSOD and CuZnSOD expression, single (Fig. 3.14) and fractionated (Fig. 3.15) treatment with other EGFR ligands TGFα and AREG was performed. Likewise, neither TGF α nor AREG induced EGFR activation did affect expression of both superoxide dismutases.

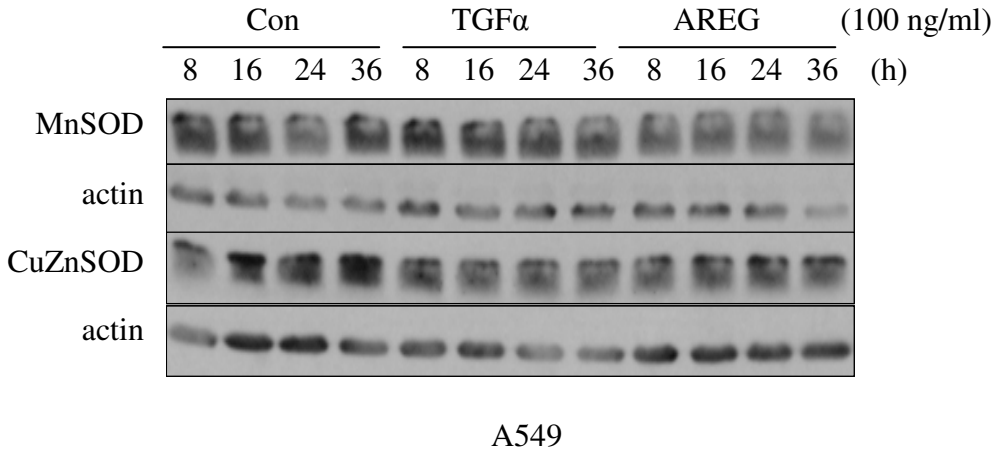


Fig. 3.14: Effect of TGFα and AREG treatment on MnSOD and CuZnSOD protein expression. At indicated time points following either TGFα or AREG stimulation cells were lysed and subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

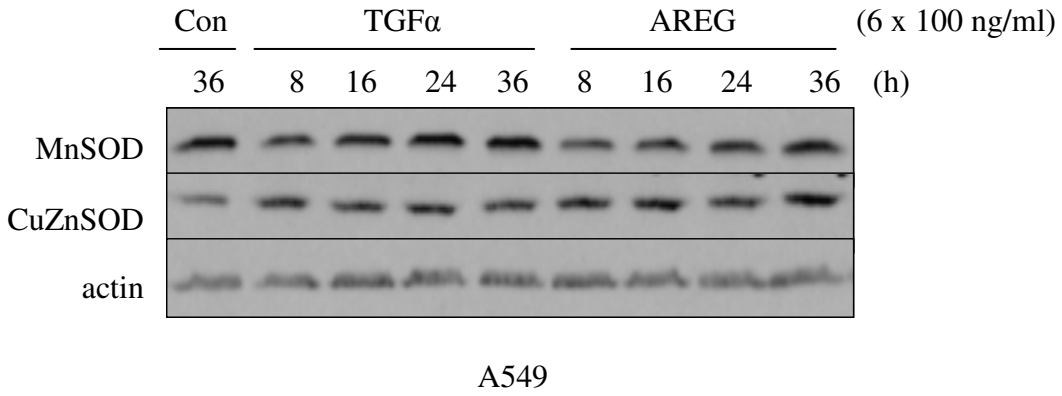


Fig. 3.15: Effect of fractionated treatment with TGFα and AREG on MnSOD and CuZnSOD protein expression. At indicated time points following either TGFα or AREG fractionated stimulation cells were lysed and subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

For studies on the effect of EGF receptor activity on MnSOD and CuZnSOD expression A549 cells were treated with BIBX1382BS inhibitor. BIBX1382BS is a specific inhibitor of the intracellular tyrosine kinase domain of EGFR (Dittrich *et al.* 2002, Nuijen *et al.* 2000). Treatment with inhibitor was performed every 24 h, 1 h before radiation treatment and after radiation inhibitor was removed. Treatment with BIBX1382BS did not affected IR-induced MnSOD and CuZnSOD protein expression (Fig. 3.16).

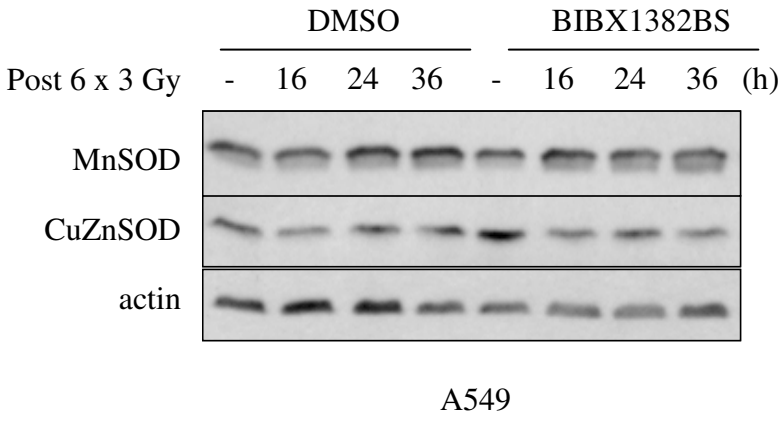


Fig. 3.16: Effect of EGFR inhibition by BIBX on MnSOD and CuZnSOD expression. Cells were treated with 5 µM BIBX 1 h before every fraction. Cells were irradiated 6 x 3 Gy. 1 h after every radiation fraction BIBX1382BS was removed. At indicated time points after the last radiation fraction proteins were isolated, subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

To investigate the effect of EGFR activity on SOD expression a second approach was used. Therefore, A549 cells were transfected with *EGFR* siRNA and expression of superoxide dismutases was investigated. As shown in Fig. 3.17 siRNA against *EGFR* strongly downregulated EGFR level. However, protein expression of MnSOD and CuZnSOD was not affected.

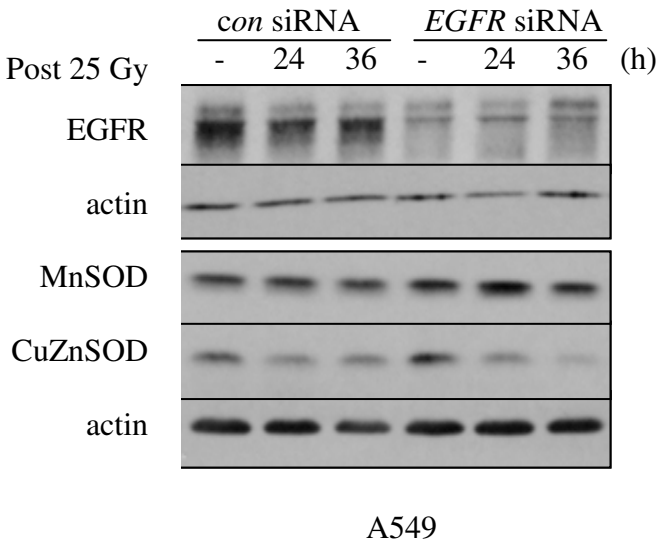


Fig. 3.17: Effect of EGFR expression on MnsOD and CuZnSOD expression. Cells were transfected with *EGFR* siRNA or control siRNA. Three days later cells were irradiated with single dose, lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against EGFR and superoxide dismutases. Actin was used as loading control.

3.6 Effect of ErbB2 activity on MnSOD and CuZnSOD expression

ErbB2 belongs to EGFR family and can be also induced by radiation exposure (Bowers G *et al.* 2001) To study whether IR-induced SOD expression is regulated by ErbB2 kinase activity a selective inhibitor of ErbB2 AG825 (Osherov *et al.* 1993, Levitzki and Gazit 1995) was used. A549 cells were treated with AG825 six times, every 24 h and the effect on SOD expression was analyzed. Radiation induced MnSOD and CuZnSOD could be observed 24 and 36 h after radiation treatment. However, ErbB2 blockage did not affected SOD expression (Fig. 3.18).

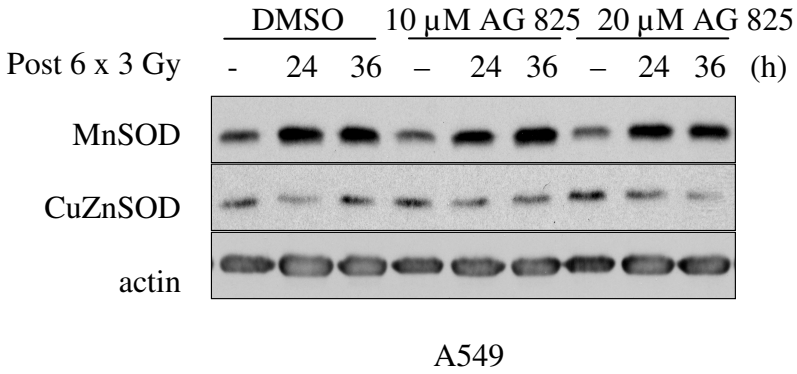


Fig. 3.18: Effect of ErbB2 blockage by AG825 inhibitor on MnSOD and CuZnSOD expression. Cells were incubated with or without AG 825 inhibitor for 1 h before every fraction and irradiated 6 x 3 Gy. Cells were lysed at the times indicated and subjected to SDS-PAGE and incubated with antibodies against MnSOD and CuZnSOD. As loading control actin was detected.

To confirm these results by a second approach A549 cells were transfected with siRNA against *ErbB2* or with control siRNA and expression of SOD was investigated. As shown in Fig. 3.19 *ErbB2* siRNA strongly downregulated ErbB2 receptor. Downregulation of this receptor affected only slightly the protein level of MnSOD but markedly the level of CuZnSOD protein.

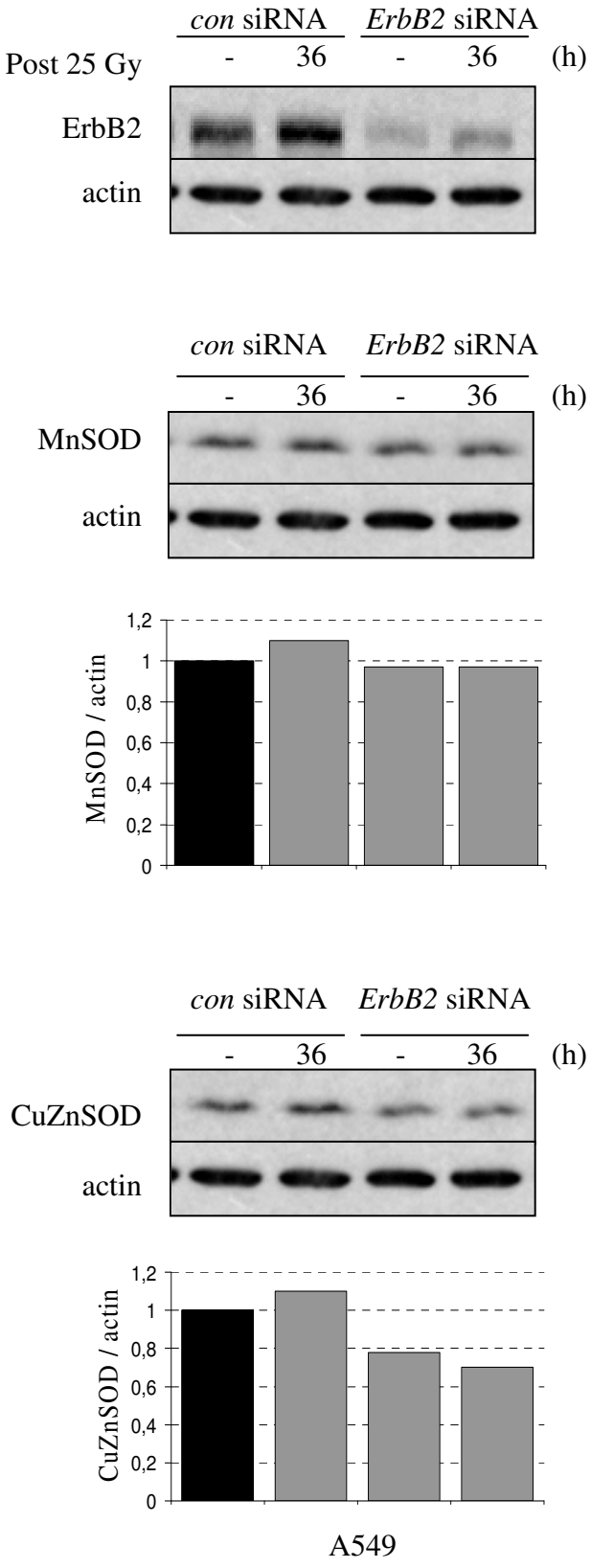


Fig. 3.19: Effect of ErbB2 blockage on MnSOD and CuZnSOD expression. Cells were transfected with *ErbB2* siRNA or control siRNA. Three days later cells were irradiated with single dose, lysed at the times indicated and subjected to SDS-PAGE and incubated with antibodies against ErbB2 and superoxide dismutases. Actin was used as loading control. Based on densitometry the relative expression level of MnSOD and CuZnSOD was quantified.

3.7 Effect of IGF-1R activity on MnSOD and CuZnSOD expression

Ionizing radiation activates several tyrosine kinase receptors which can be involved in SOD activation. Besides EGFR or ErbB2-dependent signaling, other growth factor signaling activated by IR like insulin-like growth factor 1 receptor (IGF-1R) activity (Cosaceanu *et al.* 2007) was investigated. Therefore, A549 cells were irradiated with single dose of 4 Gy and immunoprecipitation with IGF-1R antibody was performed to investigate activation of IGF-1R after ionizing radiation. As demonstrated in Fig. 3.20 IGF-1R activity increased about two-fold after radiation exposure.

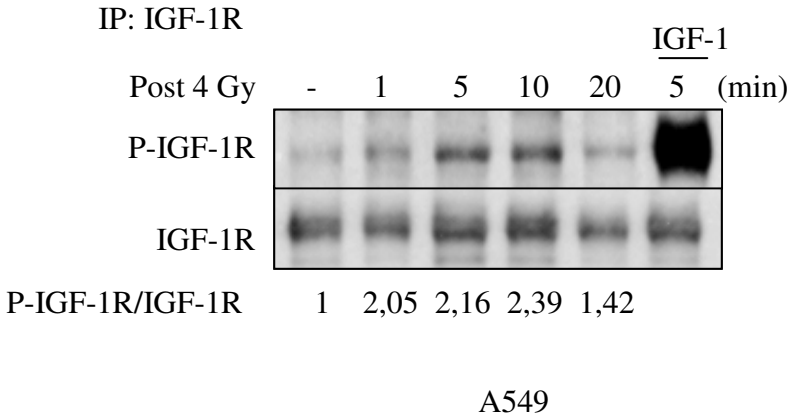


Fig. 3.20: IR-induced IGF-1R phosphorylation. 48 h serum-depleted A549 cells were irradiated with single dose of 4 Gy. Total lysate was immunoprecipitated with IGF-1R antibody and immunoblotted with P-IGF-1R antibody. Blots were stripped and reincubated with antibody against total IGF-1R antibody. IGF-1 ligand was used as positive control.

To study whether IGF-1R activity can regulate MnSOD and CuZnSOD protein expression, A549 cells were treated with IGF-1 ligand. Single and fractionated treatment was performed. Based on data shown in Fig 3.21 no induction of SOD after single treatment with IGF-1 was found. However, fractionated treatment with IGF-1 resulted in activation of CuZnSOD expression but not MnSOD expression (Fig. 3.22).

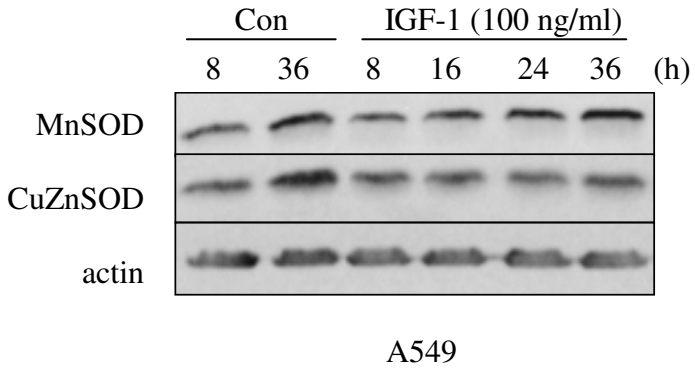


Fig. 3.21: Effect of IGF-1 treatment on MnSOD and CuZnSOD protein expression. At indicated time points following IGF-1 stimulation cells were lysed, subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

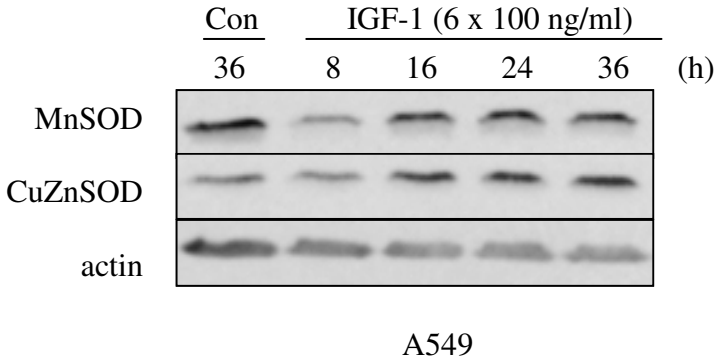


Fig. 3.22: Effect of IGF-1-induced phosphorylation of IGF-1R on MnSOD and CuZnSOD protein expression. At indicated time points following fractionated IGF-1 stimulation cells were lysed, subjected to SDS-PAGE and immunoblotted applying a specific MnSOD and CuZnSOD antibodies. As loading control actin was detected.

3.8 Role of PI3K/AKT pathway on MnSOD and CuZnSOD expression

It has been reported that phosphatidylinositol 3 kinase (PI3K) and its downstream protein kinase effector AKT regulate expression of MnSOD (Banerjee *et al.* 2009) and CuZnSOD (Rojo *et al.* 2004). Thus, the role of PI3K / AKT as downstream pathway of EGFR on the expression of MnSOD and CuZnSOD was investigated. Therefore, PI3K was blocked by LY294002 inhibitor (Vlahos *et al.* 1994), and the effect on SOD expression was analyzed. IR-induced MnSOD and CuZnSOD expression was observed 16, 24 and 36 h after ionizing radiation. As shown in Fig. 3.23 the induction of both SOD was reduced after inhibitor treatment.

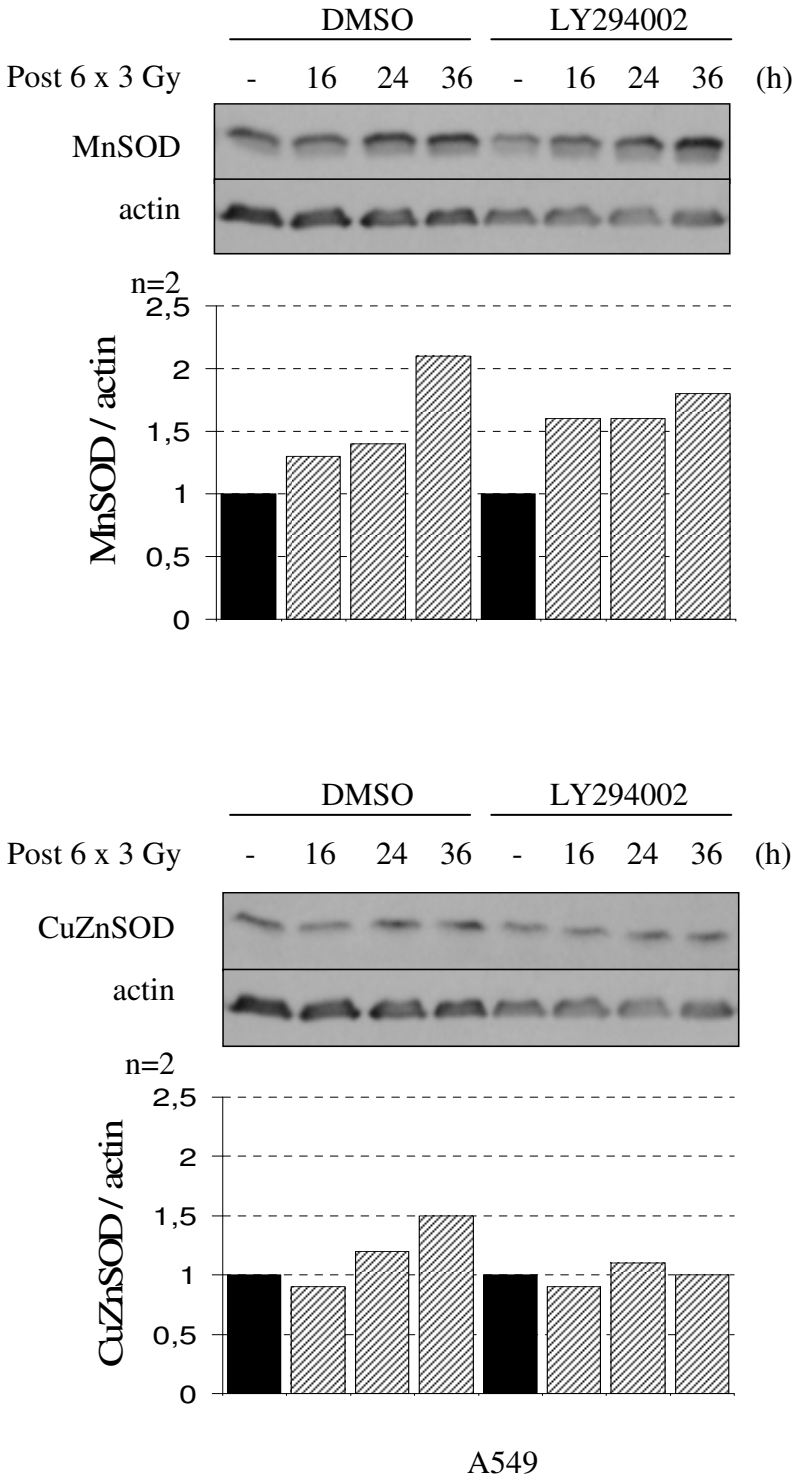


Fig. 3.23: Effect of PI3K inhibitor LY294002 on MnSOD and CuZnSOD protein expression. A549 cells were incubated with PI3K inhibitor 1 h before every radiation fraction of 3 Gy. At indicted time points after last fraction cells were lysed and Western blot has been performed. Blots were incubated with antibodies against MnSOD and CuZnSOD. Actin was detected as loading control. Based on densitometry the relative expression level of MnSOD and CuZnSOD was quantified.

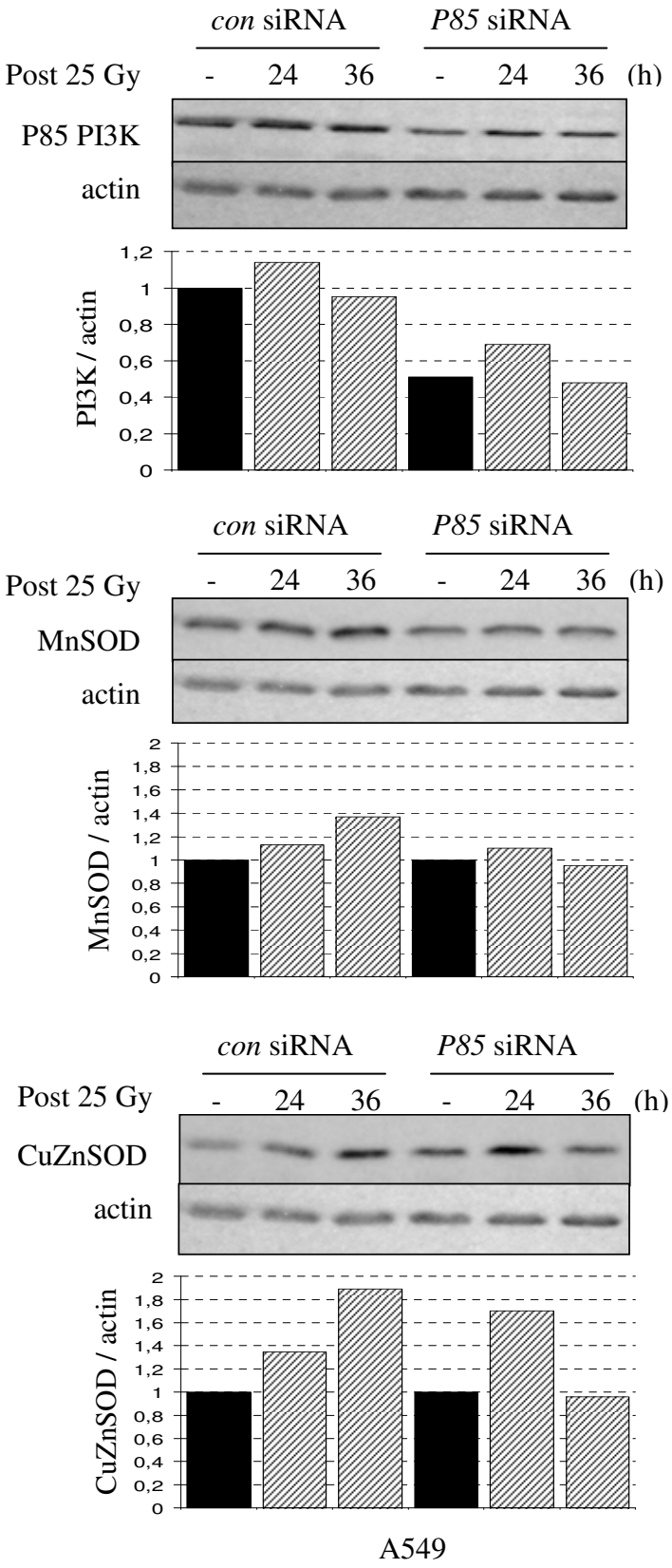


Fig. 3.24: Effect of PI3K blockage on MnSOD and CuZnSOD expression. Cells were transfected with siRNA against *P85* subunit of PI3K or non target siRNA. Three days later cells were irradiated with single dose, lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against P85 and superoxide dismutases. Actin was used as loading control. Based on densitometry the relative expression level of PI3K, MnSOD and CuZnSOD was quantified.

These results were confirmed in second approach using siRNA transfection. A549 cells were transfected with siRNA against subunit of PI3K *P85*. SiRNA transfection did not affect strongly PI3K activity. Nevertheless, based on the results shown in Fig. 3.24 there was a clear effect on MnSOD and CuZnSOD expression. IR-induced MnSOD after transfection with *P85* siRNA was reduced as well as the basal level of MnSOD. Likewise, IR-induced CuZnSOD expression was decreased 36 h after radiation exposure.

To further study the effect of PI3K/ AKT activity on SOD expression AKT kinase activity in A549 cells was blocked by AKT pathway inhibitor API-59CJ-OH (API) (Jin X *et al.* 2004). Induction of MnSOD and CuZnSOD expression was apparent after radiation with single radiation dose of 25 Gy and could be blocked by API (Fig. 3.25).

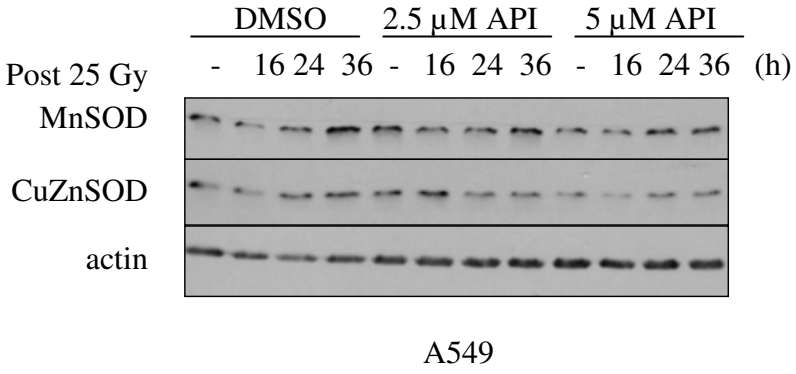


Fig. 3.25: Effect of AKT blockage by AKT pathway inhibitor on MnSOD and CuZnSOD expression. Cells were incubated with or without API for 72 h before radiation with single dose of 25 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against MnSOD and CuZnSOD. As loading control actin was detected.

To confirm the importance of AKT for MnSOD and CuZnSOD expression A549 cells were transfected with siRNA against *AKT1*. As shown in Fig. 3.26 *AKT1* siRNA strongly affected AKT1 protein level and resulted in marked reduction of IR-induced MnSOD protein level and CuZnSOD protein level at least 36 h after radiation treatment.

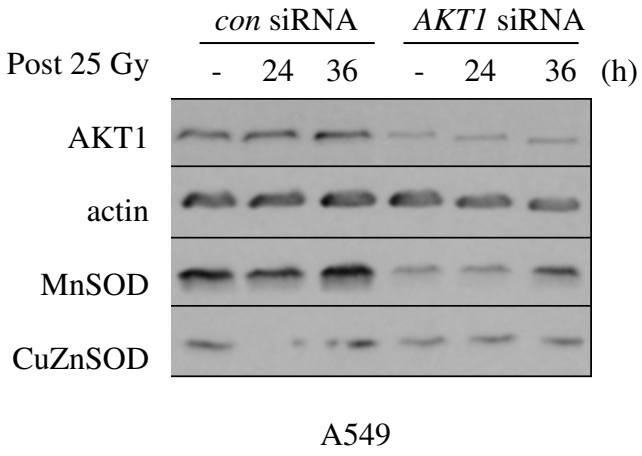


Fig. 3.26: Effect of AKT inhibition on MnSOD and CuZnSOD expression. Cells were transfected with siRNA against *AKT1* or with non target siRNA. Three days later cells were irradiated with single dose, lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against AKT1 and superoxide dismutases. Actin was used as loading control.

Regulation of SOD expression by AKT pathway was studied in H460 cells as well. Cells were treated with AKT pathway inhibitor and irradiated with single radiation dose of 25 Gy. Similarly, reduction of IR-induced SOD has been observed following inhibitor treatment (Fig. 3.27).

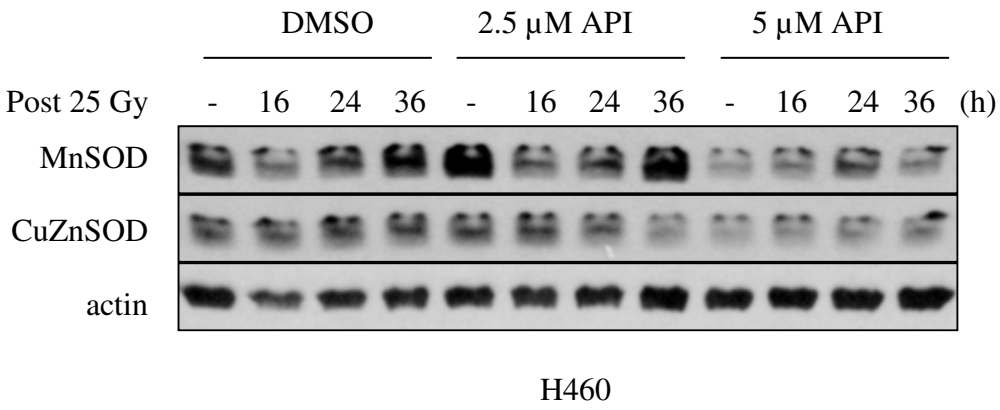


Fig. 3.27: Effect of AKT blockage by AKT pathway inhibitor on MnSOD and CuZnSOD expression. Cells were incubated with or without API for 72 h before radiation with single dose of 25 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against MnSOD and CuZnSOD. As loading control actin was detected.

3.9 IR-induced MnSOD nuclear translocation

It has been described that NF-κB is a transcription factor of SOD (Saccani *et al.* 2001, Sun *et al.* 2007). Therefore, A549 cells were treated with NF-κB activation inhibitor (Tobe *et al.* 2003) and irradiated 6 x 3 Gy. IR-induced MnSOD and CuZnSOD expression was found 8, 16 and 24 h after radiation treatment and it was markedly blocked by inhibitor treatment (Fig. 3.28).

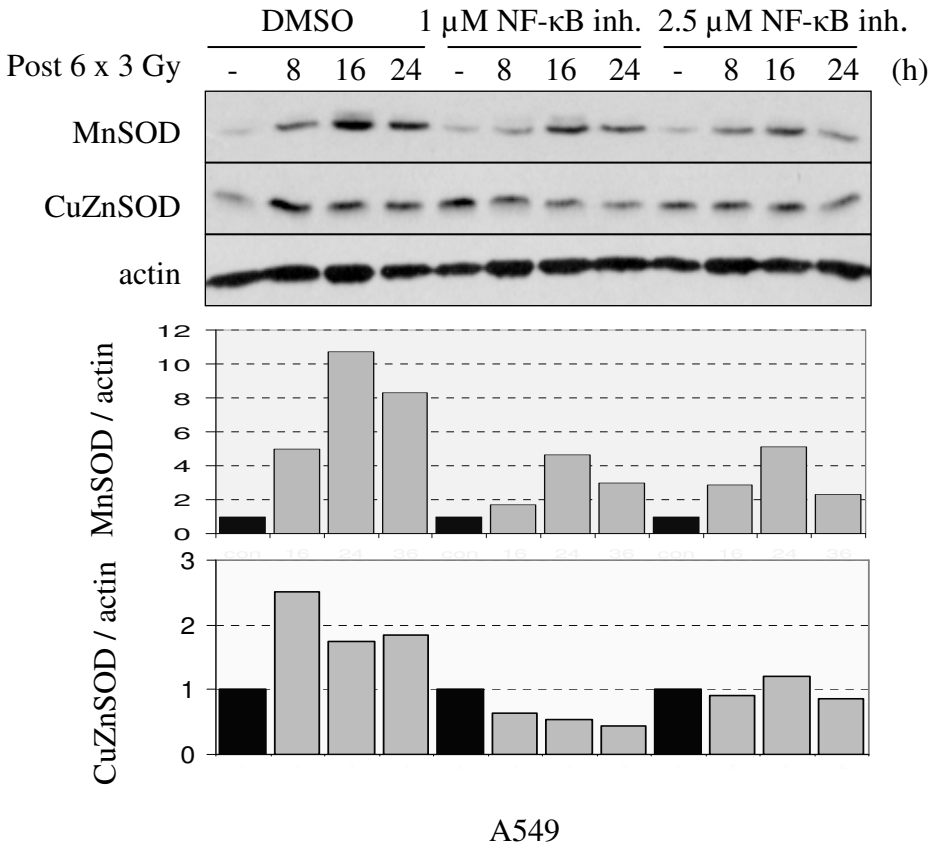


Fig. 3.28: Effect of NF-κB blockage by inhibitor on MnSOD and CuZnSOD expression. Cells were incubated with or without NF-κB inhibitor for 1 h before every radiation fraction of 3 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against MnSOD and CuZnSOD. As loading control actin was detected. Based on densitometry the relative expression level of MnSOD and CuZnSOD was quantified.

To investigate the basal expression of NF-κB and MnSOD in cytoplasnic and nuclear fraction A549 cells were seeded into 15 cm plates and were grown to confluent status. Thereafter, the basal level of NF-κB and MnSOD expression was investigated using Western blot. The basal level of both proteins was found in the cytoplasm as well as in the nucleus (Fig. 3.29).

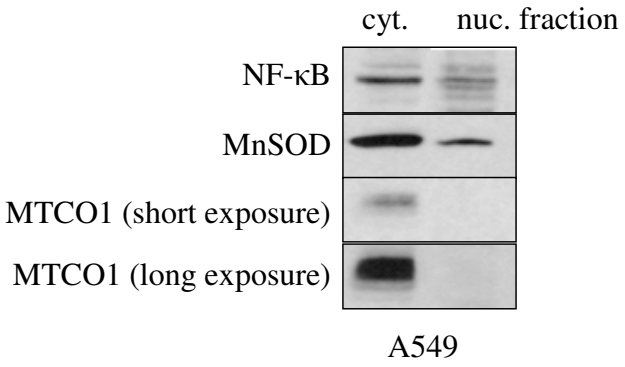


Fig. 3.29: The basal lvevel of NF-κB and MnSOD in cytoplasnic and nuclear fraction. A549 cells were lysed and cytoplasmic and nuclear fraction were isolated. Protein samples were subjected to SDS-PAGE and blots were incubated with antibodies against NF-κB, MnSOD and MTCO1. MTCO1was used as mitochondrial marker.

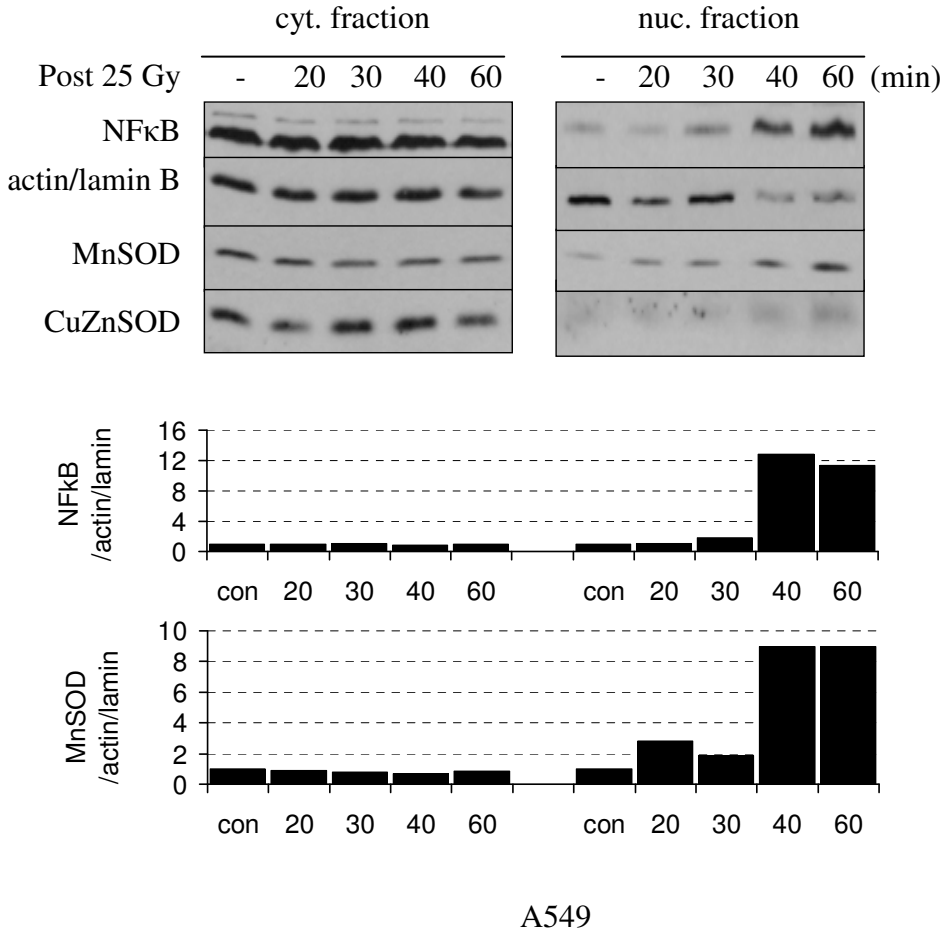


Fig. 3.30: Effect of ionizing radiation on NF-κB and MnSOD nuclear translocation. A549 cells were irradiated with single dose of 25 Gy. At indicated time points after radiation cells were lysed and cytoplasmic and nuclear fraction were isolated. Protein samples were subjected to SDS-PAGE and blots were incubated with antibodies against NF-κB, MnSOD and CuZnSOD. As loading control actin and lamin B were detected. Based on densitometry the relative expression level of MnSOD and NF-κB was quantified.

A549 cells were irradiated with dose of 25 Gy and the level of NF-κB was investigated in cytoplasmic and nuclear fraction. 40 and 60 min after radiation translocation of NF-κB to the nucleus could be observed. A similar pattern in the timing of translocation could be observed for MnSOD but not CuZnSOD (Fig. 3.30).

To investigate the effect of lower radiation dose on IR-induced NF-κB and MnSOD nuclear translocation A549 cells were irradiated with dose of 8 Gy and the level of NF-κB was investigated in cytoplasmic and nuclear fraction. Translocation of NF-κB and MnSOD to the nucleus could be observed. (Fig. 3.31).

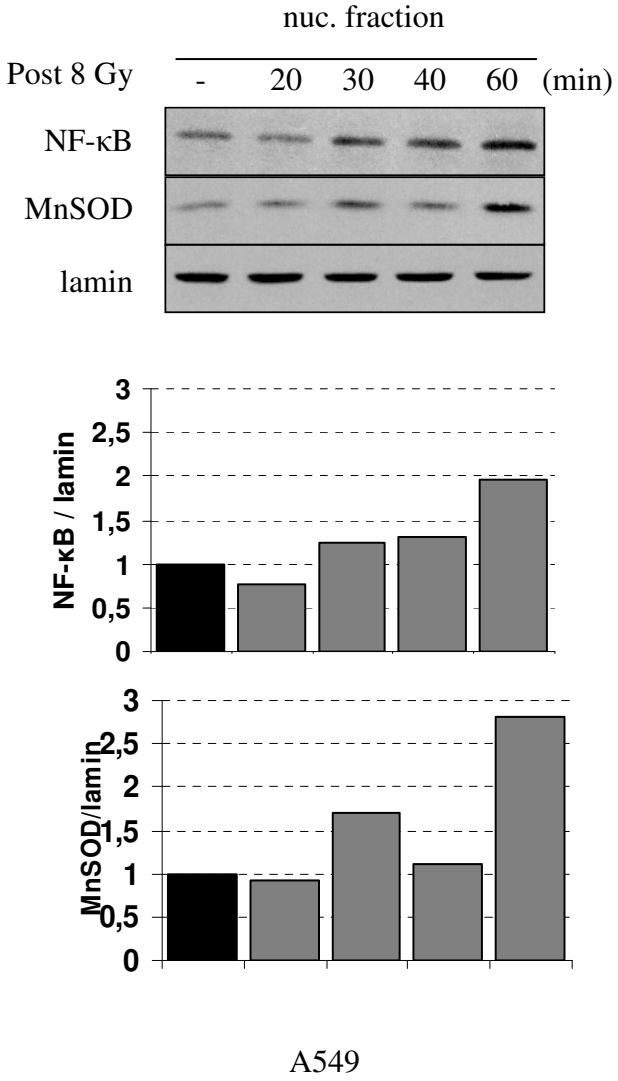


Fig. 3.31: Effect of ionizing radiation on NF-κB and MnSOD nuclear translocation. A549 cells were irradiated with single dose of 8 Gy. At indicated time points after radiation cells were lysed and cytoplasmic and nuclear fraction were isolated. Protein samples were subjected to SDS-PAGE and blots were incubated with antibodies against NF-κB, MnSOD and lamin. Based on densitometry the relative expression level of MnSOD and NF-κB was quantified.

To investigate MnSOD and NF-κB translocation to the nucleus in more detail immunoprecipitation experiment with NF-κB antibody was performed. NF-κB translocation to the nucleus could be observed following ionizing radiation exposure. Moreover, MnSOD in the nuclear fraction was detected as well. Densitometry shown in Fig. 3.32 was prepared based on three experiments. Fig. 3.32 clearly shows similarity of the pattern of NF-κB and MnSOD translocation to the nucleus following radiation treatment. This result indicates that after irradiation MnSOD binds to NF-κB and translocates together with NF-κB to the nucleus.

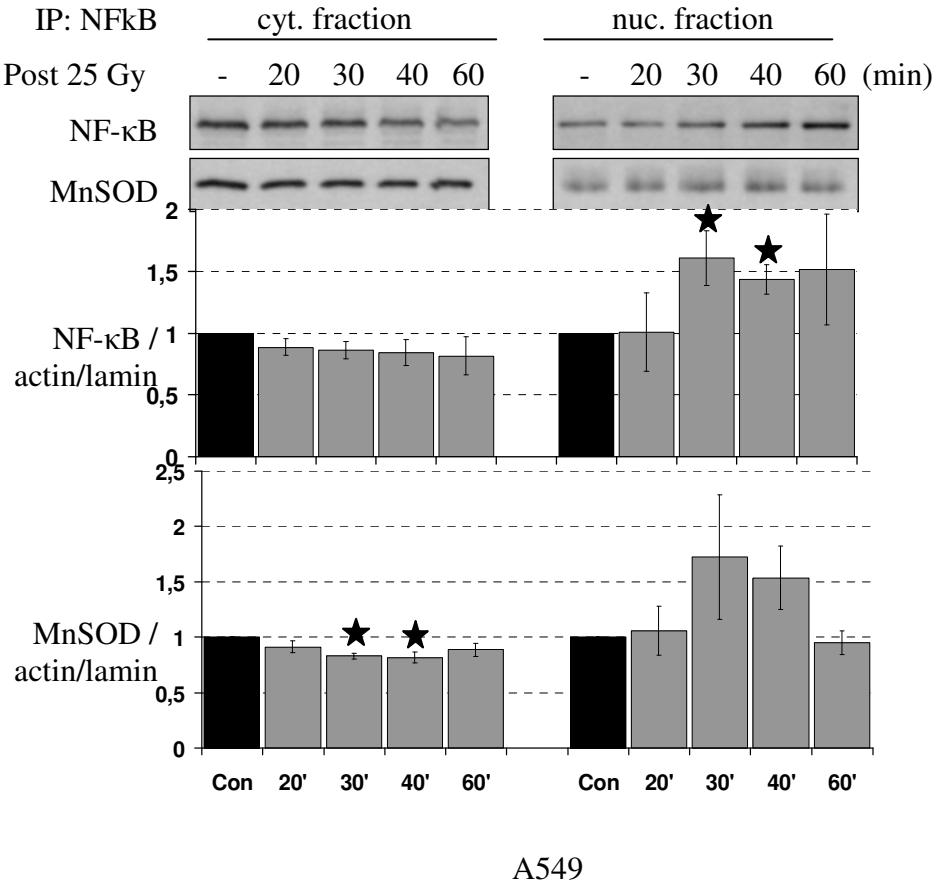


Fig. 3.32: Effect of radiation on NF-κB and MnSOD nuclear translocation. A549 cells were irradiated with single dose of 25 Gy. At indicated time points after radiation cells were lysed and cytoplasmic and nuclear fractions were isolated. Nuclear proteins were immunoprecipitated with NF-κB antibody. Cytoplasmic and nuclear fraction were subjected to SDS-PAGE and blots were incubated with antibodies against NF-κB and MnSOD. Data are a mean of three independent experiments. Based on densitometry the relative expression level of NF-κB and MnSOD was quantified. Asterisks indicate significant change of the NF-κB and MnSOD level following irradiation in comparison to unirradiated control (Student's *t*-test/ $^{*}p<0.05$).

To confirm this result A549 cells were pretreated with NF-κB activation inhibitor (Tobe *et al.* 2003) to block nuclear translocation of NF-κB. As shown in Fig. 3.33 IR-induced nuclear translocation of NF-κB is slightly blocked by NF-κB inhibitor. Interestingly, blockage of NF-κB translocation decreased MnSOD translocation to the nucleus as well what confirming that MnSOD bound to NF-κB is translocated to the nucleus following irradiation.

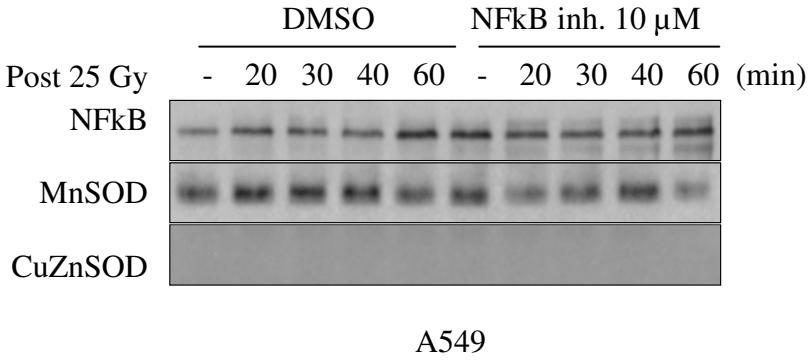
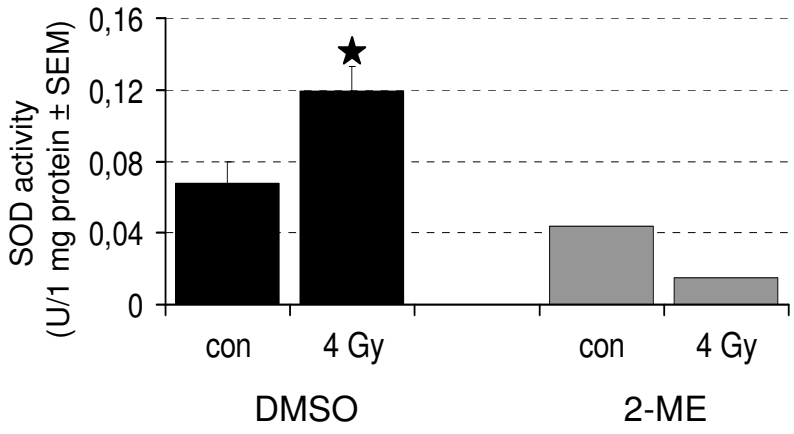


Fig. 3.33: Effect of NF-κB inhibitor on MnSOD translocation to the nucleus. Cells were pretreated with NF-κB inhibitor 1 h before radiation with dose of 25 Gy. At indicated time points after IR cells were lysed and nuclear fraction was isolated. Protein samples were subjected to SDS-PAGE and blots were incubated with antibodies against NF-κB, MnSOD and CuZnSOD.

3.10 2-Methoxyestradiol as SOD inhibitor.

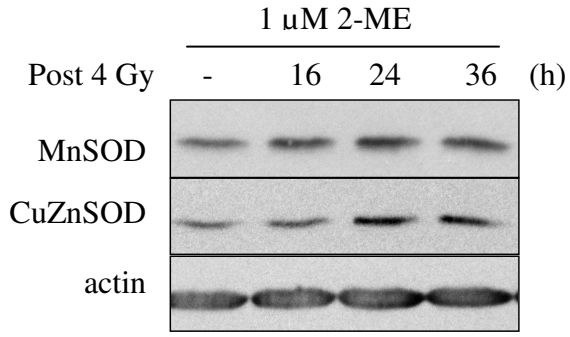
3.10.1 Effect of 2-ME on SOD activity and protein expression.

2-methoxyestradiol has been described as SOD inhibitor, however, the mechanism of the inhibition has not been described so far (Huang *et al.* 2000). The effect of 2-methoxyestradiol on SOD enzyme activity and on its protein expression has been investigated. A549 cells were treated with 2-ME, irradiated with single radiation dose and SOD activity assay was performed. As shown in Fig. 3.34 2-ME reduced basal- and blocked IR-induced SOD activity completely. In contrast, radiation-induced MnSOD and CuZnSOD protein expression after single radiation dose was not affected (Fig. 3.35). However, when cells were irradiated 6 x 3 Gy and treated with 2-ME every 24 h, expression of both SODs were reduced after treatment with 1 µM 2-ME. Treatment with 0.5 µM 2-ME reduced only IR-induced CuZnSOD (Fig. 3.36).

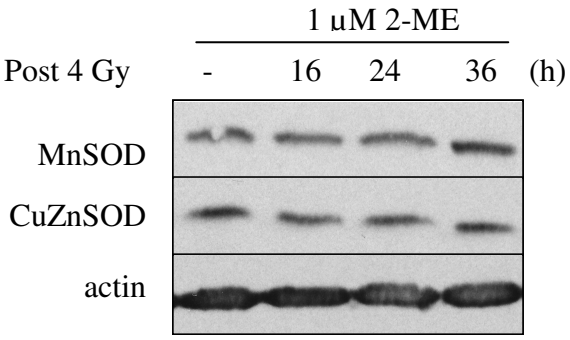


A549

Fig. 3.34: Effect of 2-ME on ionizing radiation induced SOD activity. Cells were treated with 2-ME for 48 h and irradiated with dose of 4 Gy. 16 h after irradiation protein samples were isolated and SOD activity assay was performed as described in Materials and Methods. Asterisk indicates significant enhancement of SOD enzyme activity following irradiation in comparison to unirradiated control (Student's *t*-test/*p<0.05).



A549



H460

Fig. 3.35: Effect of 2-ME on MnSOD and CuZnSOD expression. Cells were treated with 2-ME for 48 h, irradiated with single dose of 4 Gy, subjected to SDS-PAGE and immunoblotted with specific antibodies against MnSOD and CuZnSOD. Actin was detected as loading control.

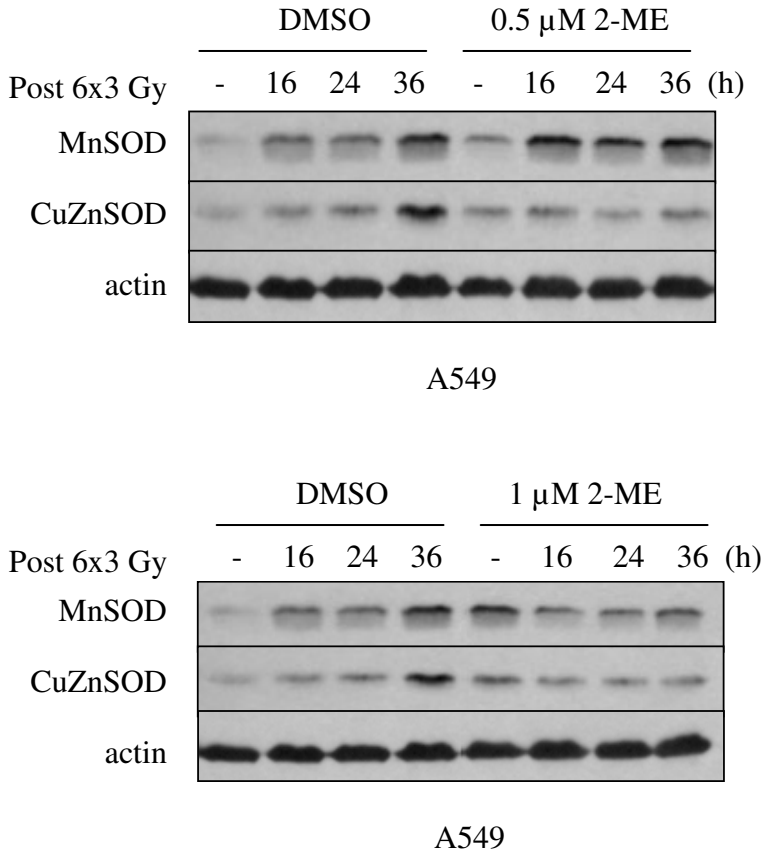
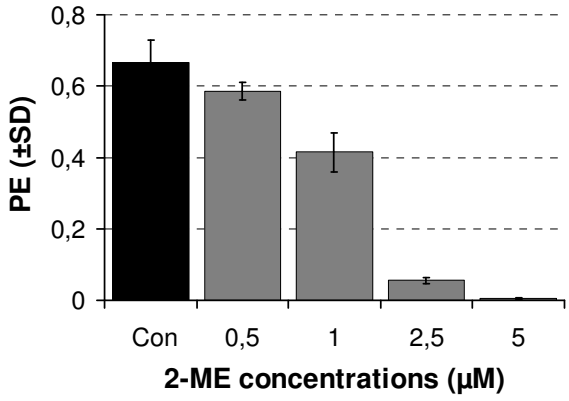


Fig. 3.36: Effect of 2-ME on MnSOD and CuZnSOD expression. Cells were treated with 0.5 (A) or 1 (B) µM 2-ME for 48 h before first radiation fraction, irradiated with 6 x 3 Gy, subjected to SDS-PAGE and immunoblotted with specific antibodies against MnSOD and CuZnSOD. Actin was detected as loading control.

3.10.2 Radiosensitizing effect of 2-ME

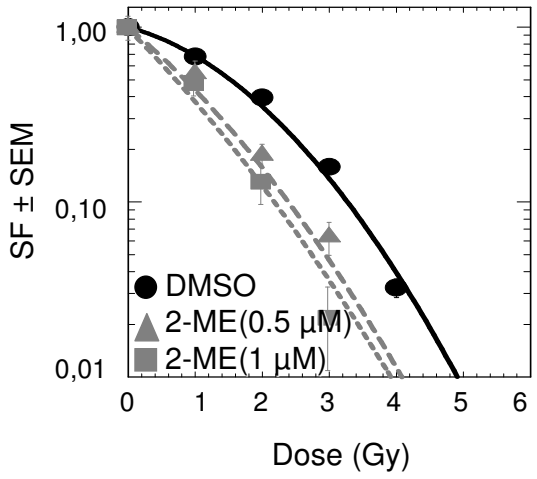
In the line with its enzyme inhibitory activity 2-ME markedly enhanced cellular radiation sensitivity (Huober *et al.* 2000, Zou *et al.* 2006, Zou *et al.* 2007). First, the effect of different concentration of 2-ME on plating efficiency has been analyzed. As shown in Fig. 3.37 0.5 and 1 µM 2-ME decreased plating efficiency by factors of about 10 and 40 %, respectively.



A549

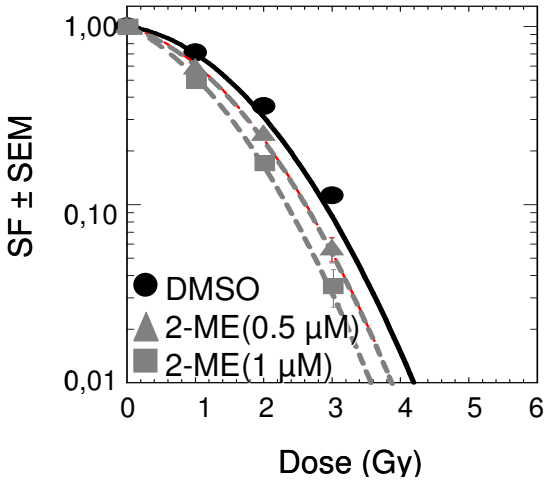
Fig. 3.37: Effect of 2-ME on plating efficiency in A549 cells . Cells were treated with 2-ME, 48 h later cells were plated for colony formation and incubated for 10 days. Data bars shown represent the mean surviving fraction ± SD of 6 parallel experiments.

To study the radiosensitizing effect of 2-ME A549 and H460 cells were treated with 0.5 and 1 µM 2-ME. In A549 cells radiosensitizing effect of 2-ME was induced concentration independent (Fig. 3.38). H460 cells were stronger radiosensitized by higher concentration of 2-ME (Fig. 3.39).



A549

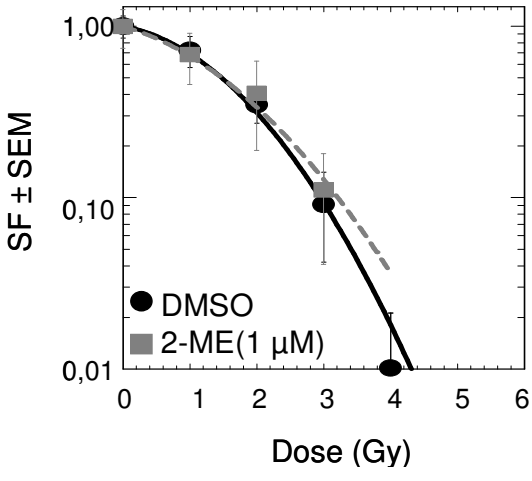
Fig. 3.38: Radiosensitizing effect of 2-ME. A549 cells were treated with 2-ME, 48 h later cells were plated for colony formation, after 24 h irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.



H460

Fig. 3.39: Radiosensitizing effect of 2-ME. H460 cells were treated with 2-ME, 48 h later cells were plated for colony formation, after 24 h irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

In contrast, the radiosensitizing effect of 2-ME was not apperant in H661 cells after treatment with 1 µM 2-ME (Fig. 3.40).



H661

Fig. 3.40: Radiosensitizing effect of 2-ME. H661 cells were treated with 2-ME, 48 h later cells were plated for colony formation, after 24 h irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

3.10.3 Radiosensitizing effect of 2-ME through blockage of IR-induced AKT

Previously, it has been shown that 2-ME inhibits basal phosphorylation of AKT (Gao *et al.* 2005, Zhang *et al.* 2009, Chow *et al.* 2008, Lin *et al*. 2007, Shimada *et al.* 2004, Barchiesi *et al*. 2006). However, the effect of 2-ME on radiation-induced PI3K/ AKT signaling has not been described. A549 cells were treated with 2-ME and irradiated with radiation dose of 4 Gy. The phosphorylation of S472/473 was investigated. Here we show that 1 µM of 2-ME blocked IR induced AKT phosphorylation completely (Fig. 3.41).

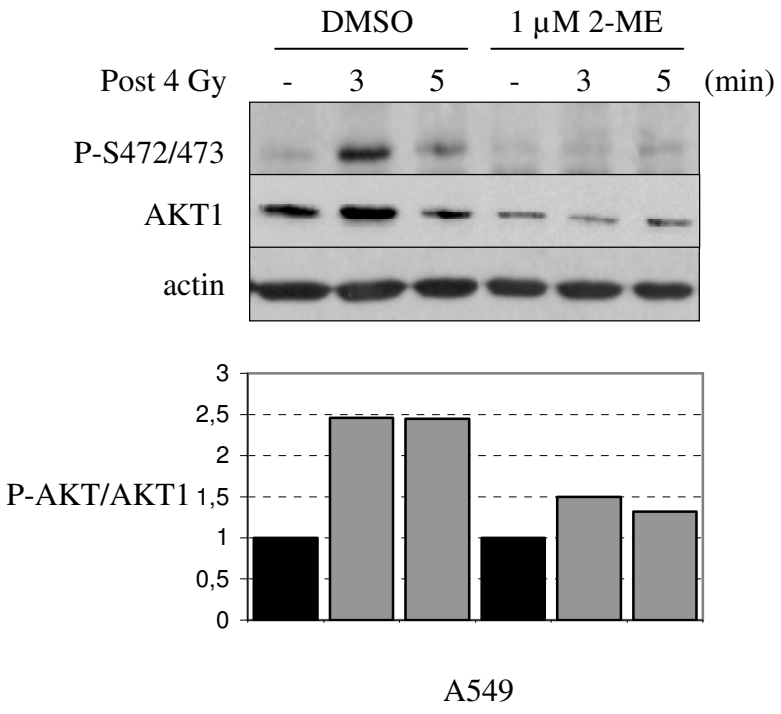
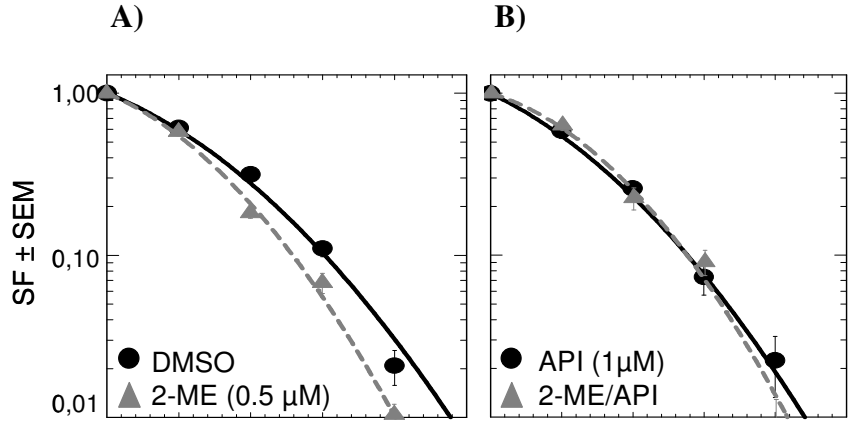


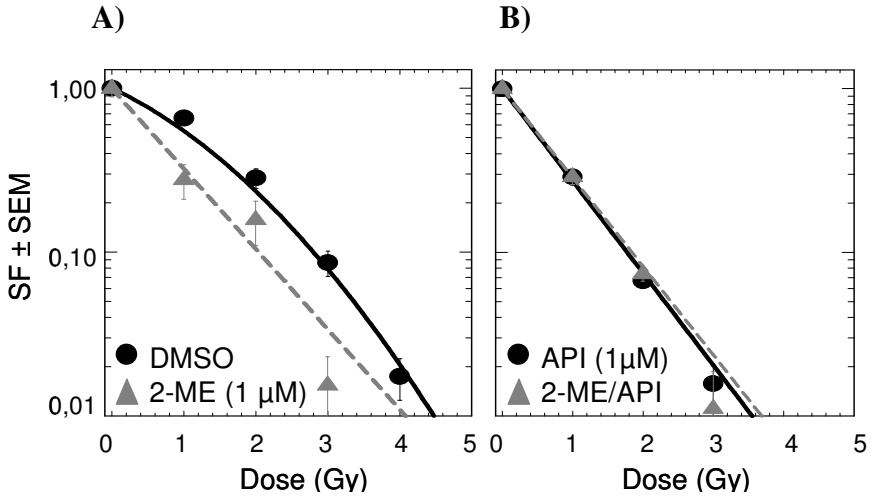
Fig. 3.41: Effect of 2-ME treatment on AKT phosphorylation. Cells were pretreated with 2-ME for 48 h and irradiated with dose of 4 Gy. At indicated time points after IR cells were lysed, subjected to SDS-PAGE and immunoblotted with specific P-AKT antibody. Thereafter, blot was stripped and whole AKT was detected as loading control. As loading control actin was used as well. Based on densitometry the relative level of P-S472/3 was quantified.

To investigate whether radiosensitization by 2-ME is independent or dependent on AKT-signalling, the effect of 2-ME (0.5 µM) on post-irradiation survival was analyzed for A549 and H460 cells pretreated with the AKT inhibitor API-59CJ-OH (1 µM). Confirming the results shown in Fig. 3.38 and 3.39 treatment with 2-ME induced radiosensitization (Fig. 3.42 A and 3.43 A). API treatment alone also induced radiosensitization, but the combination of API pretreatment with subsequent 2-ME administration did not sensitize the cells beyond the API effect (Fig. 3.42 B and 3.43 B).



A549

Fig. 3.42: Radiosensitizing effect of 2-ME in cells pretreated with API. Cells were pretreated with API, 24 h later treated with 2-ME, after 48 h plated for colony formation, irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.



H460

Fig. 3.43: Radiosensitizing effect of 2-ME in cells pretreated with API. Cells were pretreated with API, 24 h later treated with 2-ME, after 48 h plated for colony formation, irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

3.10.4 Radiosensitizing effect of 2-ME through blockage of IR-induced DNA-PKcs and DNA-dsb repair

For further investigations the effect of 2-ME downstream pathway of AKT was analyzed. Specifically, it was investigated whether 2-ME mediated IR-induced AKT phosphorylation blockage results in a reduction of DNA-PKcs phosphorylation at its trans- and autophosphorylation sites T2609 and S2056 (Fig. 3.44). Therefore, A549 and H460 cells were treated with 2-ME and irradiated with dose of 4 Gy and phosphorylation of S2056 and T2609 was determined. As shown in Fig. 3.44 at both phosphorylation sites 1 µM 2-ME markedly blocked IR-induced DNA-PKcs phosphorylation.

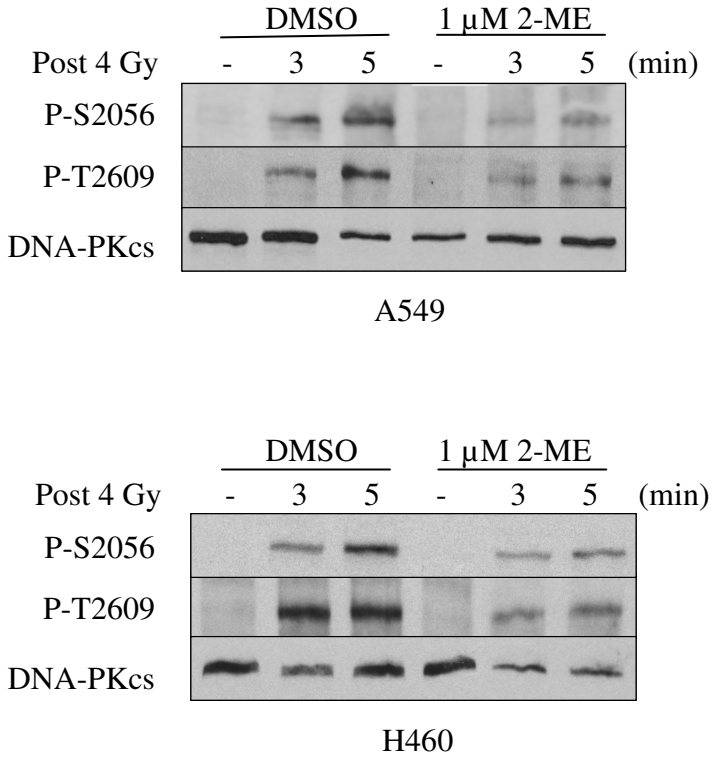


Fig. 3.44: Effect of 2-ME treatment on DNA-PKcs phosphorylation. Cells were pretreated with 2-ME for 48 h and irradiated with dose of 4 Gy. At indicated time points after IR cells were lysed, subjected to SDS-PAGE and immunoblotted with specific P-DNA-PKcs antibodies. Thereafter, blot was stripped and whole DNA-PKcs was detected as loading control.

In addition the effect of 2-ME on the repair of IR-induced DNA-dsb was investigated. In A549 treated with 2-ME and irradiated with dose of 4 Gy. Treatment with 2-ME (0.5 and 1 µM) enhanced the amount of residual DNA-dsb 6 h after radiation exposure (Fig.: 3.45).

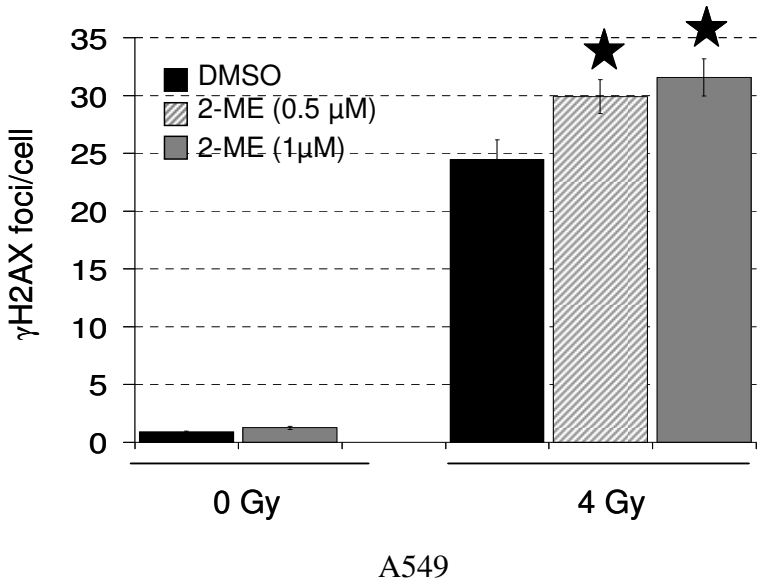


Fig. 3.45: Effect of 2-ME treatment on the repair of IR-induced DNA-dsb. Cells were cultured on glass slides and 24 h later were treated with DMSO or 2-ME. After 2 h cultures were irradiated with single dose of 4 Gy and incubated for 6 h at 37°C. Thereafter, γH2AX focus assay for residual DNA-dsb was performed as described in Material and Methods. Asterisks indicate 2-ME-mediated significant enhancement of residual γH2AX foci (Student's *t*-test/*p<0.05).

3.10.5 Effect of MnSOD/CuZnSOD siRNA transfection on AKT/ DNA-PKcs activity.

To study whether SOD expression interferes with DNA-PKcs activity, A549 cells were transfected with *MnSOD* siRNA and P-S2056 and P-T2609 were investigated. Although, expression of MnSOD was markedly affected by siRNA transfection no effect on DNA-PKcs phosphorylation could be observed (Fig. 3.46).

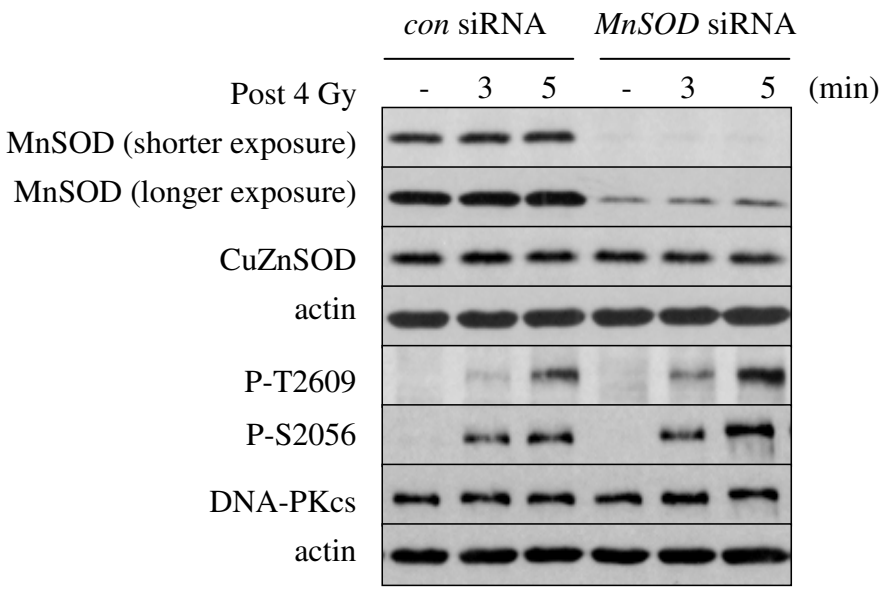


Fig. 3.46: Effect of MnSOD blockage on DNA-PKcs phosphorylation. Cells were transfected with *MnSOD* siRNA. Three days after transfection cells were irradiated with dose of 4 Gy and lysed at indicated time points after radiation, subjected to SDS-PAGE and immunoblotted with specific antibodies for MnSOD and P-DNA-PKcs. As loading controls whole DNA-PKcs and actin were detected.

Thereafter, double transfection with *MnSOD* and *CuZnSOD* siRNA was performed for A549 cells. Expression of MnSOD and CuZnSOD was markedly affected by siRNA transfection. As shown in Fig. 3.47 A no reduction of P-AKT and P-DNA-PKcs after transfection was observed. The same experiment was performed for H460 cells. Likewise, there was no effect of downregulation of MnSOD and CuZnSOD by siRNA on AKT and DNA-PKcs phosphorylation (Fig. 3.47 B).

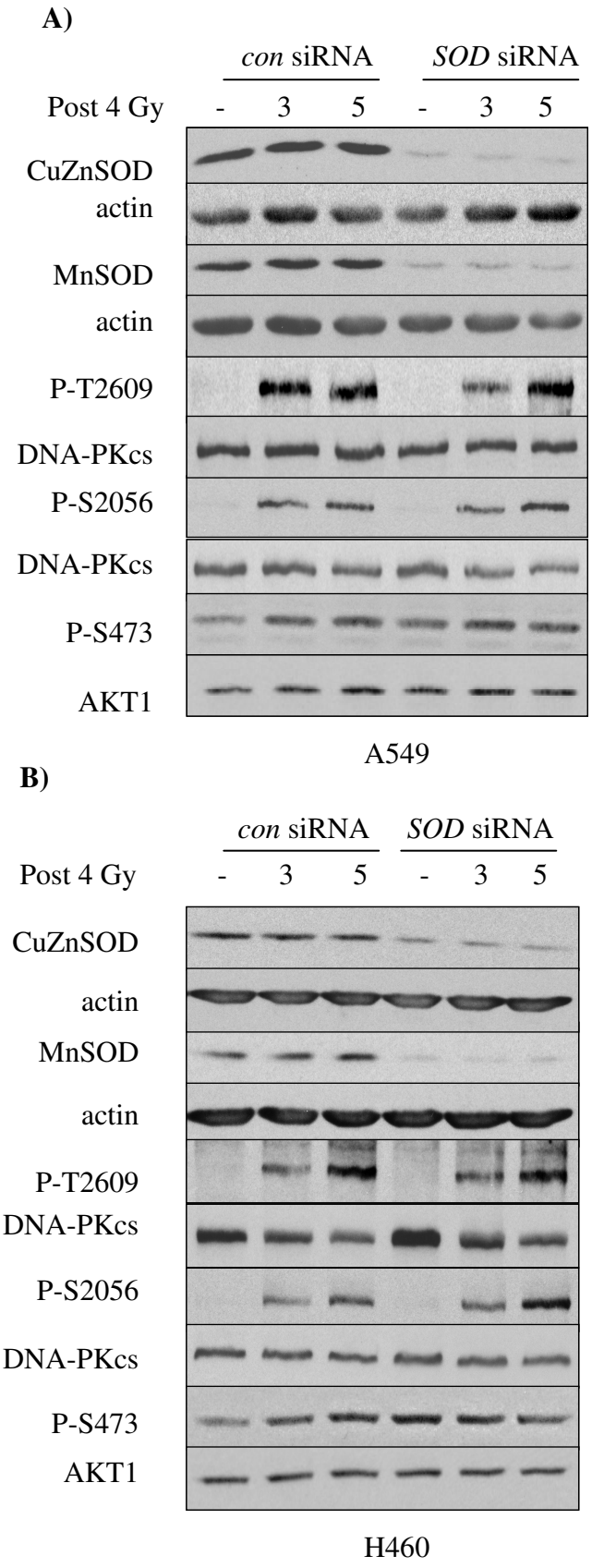


Fig. 3.47: Effect of SOD blockage on AKT and DNA-PKcs phosphorylation. Cells were transfected with *MnSOD* and *CuZnSOD* siRNA. Three days after transfection cells were irradiated with dose of 4 Gy and lysed at indicated time points after radiation, subjected to SDS-PAGE and immunoblotted with specific antibodies for MnSOD, CuZnSOD, P-DNA-PKcs and P-AKT. As loading control whole AKT, whole DNA-PKcs and actin were detected.

3.10.6 Effect of MnSOD/ CuZnSOD siRNA transfection on 2-ME mediated radiosensitization.

It has been reported that SOD activity is involved in protection of cancer cells from radiation (Guo *et al.* 2003, Yamaguchi *et al.* 1994). Therefore, to analyze whether SOD protein expression is essential for cell survival after IR SOD expression in A549 and H460 cells was downregulated by a double siRNA approach. Although, expression of MnSOD and CuZnSOD was markedly affected by siRNA transfection (Fig. 3.48 A and 3.49 A), this effect did not affect clonogenic survival of irradiated cells. Moreover, the no effect of double transfection on 2-ME–mediated radiosensitization was observed. (Fig. 3.48 B and 3.49 B).

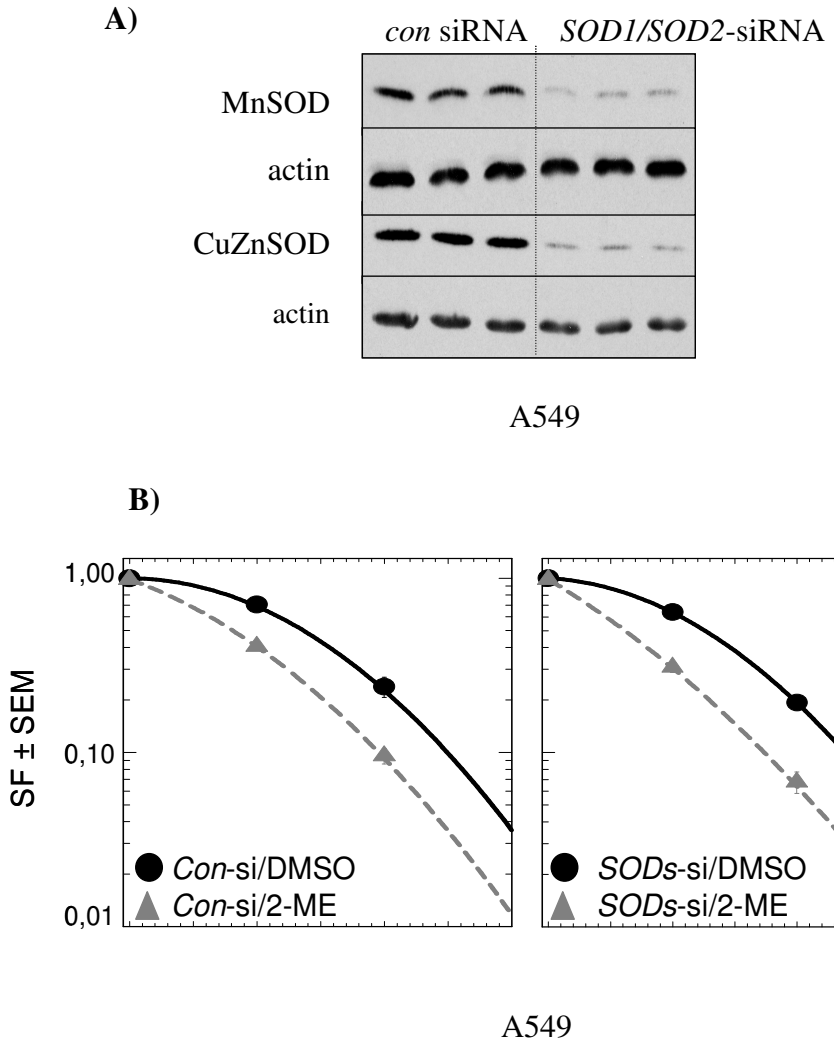


Fig. 3.48: Radiosensitizing effect of 2-ME in A549 cells transfected with siRNA against superoxide dismutases. Cells were transfected with *MnSOD* and *CuZnSOD* siRNA, 24 h later treated with 2-ME, after 48 h plated for colony formation, irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

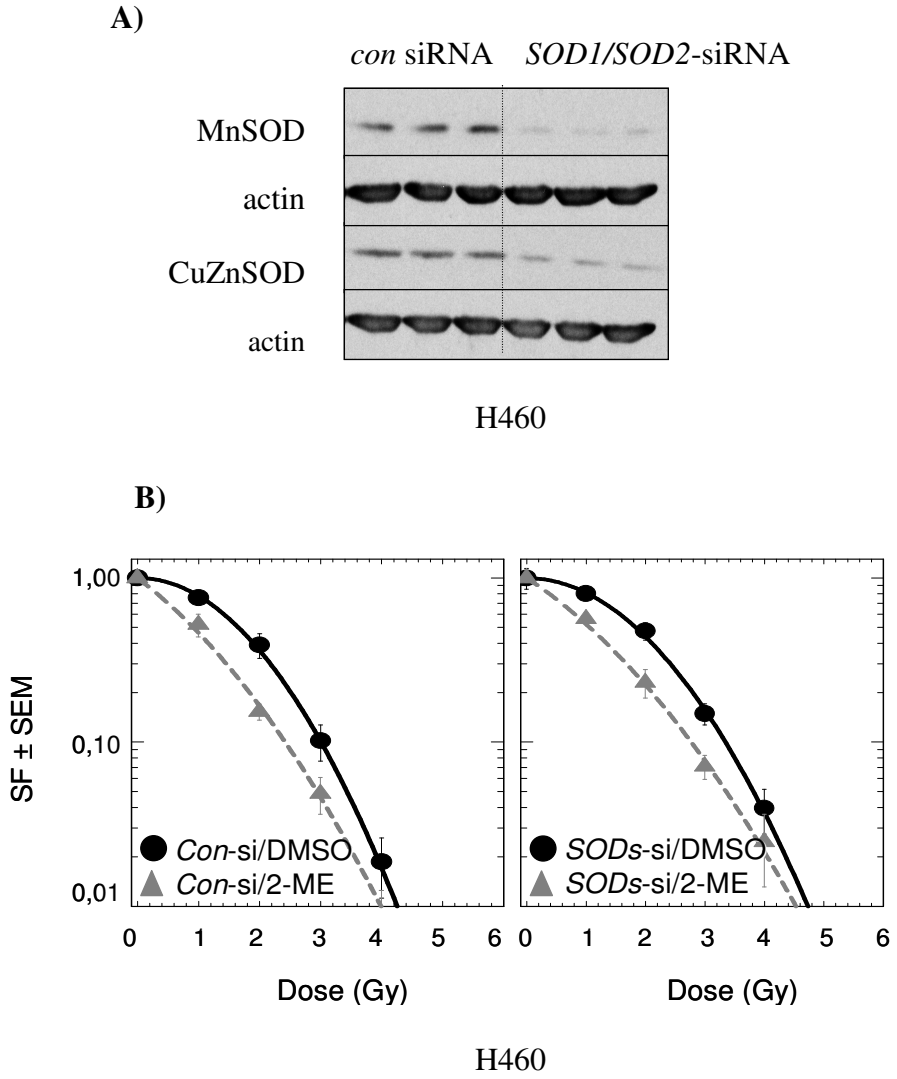
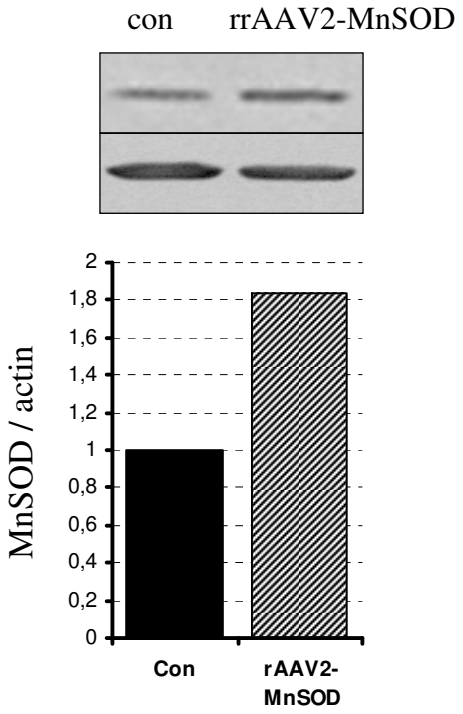


Fig. 3.49: Radiosensitizing effect of 2-ME in H460 cells transfected with siRNA against superoxide dismutases. Cells were transfected with *MnSOD* and *CuZnSOD* siRNA, 24 h later treated with 2-ME, after 48 h plated for colony formation, irradiated with doses of 1 to 5 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

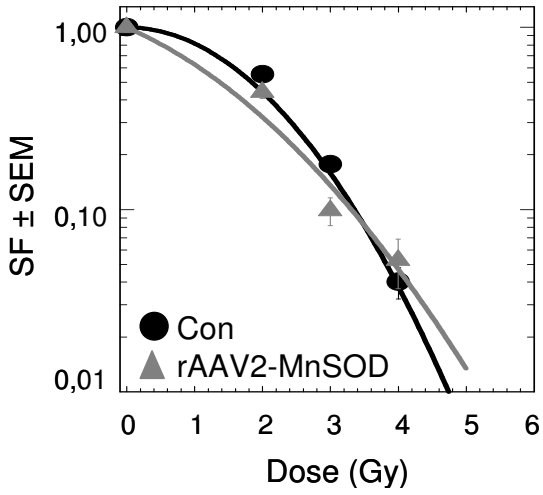
3.10.7 Effect of MnSOD overexpression on radiosensitization

To further study the role of MnSOD in cellular radioprotection H460 cells were transduced using rAAV-2-MnSOD vectors (Veldwijk *et al.* 2004) to overexpress MnSOD. As shown in Fig. 3.50 MnSOD protein level was increased about two times.



H460

Fig. 3.50: Effect of transduction with rAAV2-MnSOD vectors on MnSOD expression. Cells were transduced with rAAV2-MnSOD particles. After three days cells were lysed, subjected to SDS-PAGE and the blot was incubated with antibody against MnSOD. Actin was used as loading control. Based on densitometry ratios of MnSOD/actin normalized to 1 control MnSOD overexpression was apparent in cells transduced with rAAV2-MnSOD vectors.



H460

Fig. 3.51: Effect of transduction with rAAV2-MnSOD vector on radioresistance of H460 cells. H460 cells were transduced with rAAV2-MnSOD vectors, three days later cells were irradiated with doses of 2, 3 and 4 Gy, after 6 h plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

Furthermore, it has been analyzed whether MnSOD overexpresssion can protect cells against ionizing radiation. Although in transduced cells the amount of MnSOD was two times higher than in untransduced control, no radioprotective effect of MnSOD was observed for H460 cells (Fig. 3.51).

3.10.8 Radiosensitizing effect of 2-ME in cells pretreated with ATM and DNA-PKcs inhibitor.

To investigate whether radiosensitization by 2-ME is independent or dependent on DNA-PKcs activity, the effect of 2-ME on post-irradiation survival was analyzed for A549 and H460 cells in presence of ATM and DNA-PKcs inhibitor. NU7026 (NU) and KU55933 (KU) inhibitors were used to block both phosphorylation sites of DNA-PKcs. NU7026 blocked autophosphorylation site of DNA-PKcs S2056 (Fig. 3.52), and KU55933 blocked T2609 which is phosphorylated by ATM (Fig. 3.53).

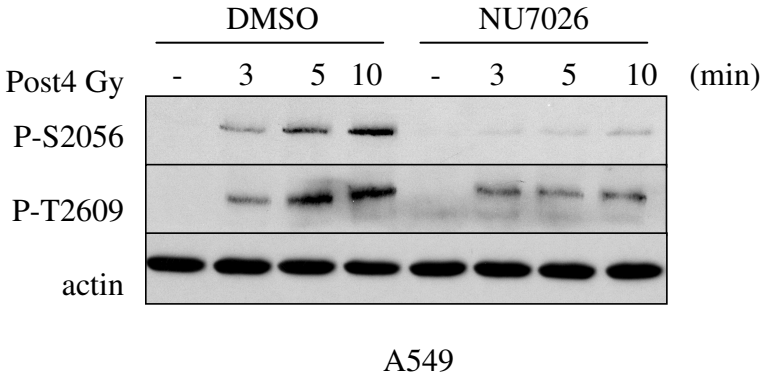
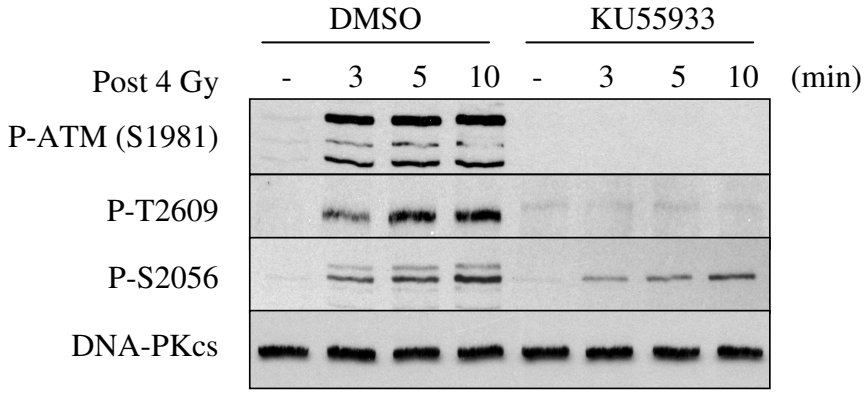


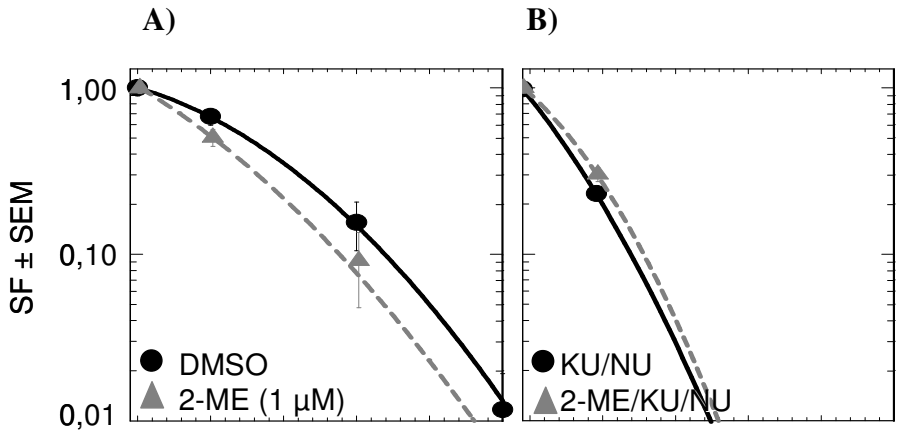
Fig. 3.52: Inhibition of DNA-PKcs autophosphorylation site in A549 cells following NU7026 treatment. Cells were incubated with or without DNA-PKcs inhibitor for 1 h before radiation with dose of 4 Gy. At indicated time point cells were lysed, subjected to SDS-PAGE and immunoblotted using a specific antibodies for P-S2056 and P-T2609. As loading control actin was used.



A549

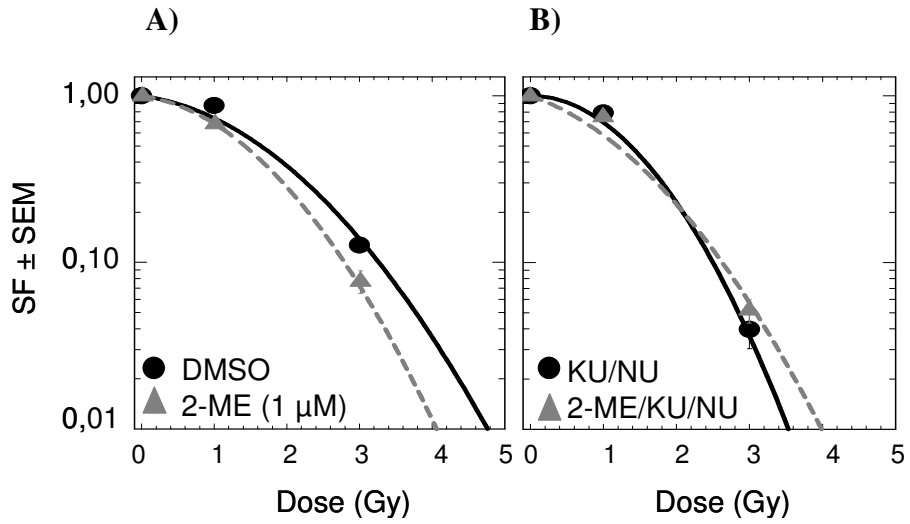
Fig. 3.53: Inhibition of DNA-PKcs transphosphorylation site in A549 cells following KU55933 treatment. Cells were incubated with or without ATM inhibitor for 1 h before radiation with dose of 4 Gy. At indicated time points cells were lysed, subjected to SDS-PAGE and immunoblotted using a specific antibodies for P-ATM, P-S2056 and T2609. As loading control whole DNA-PKcs was used.

Treatment with KU55933 and NU7026 induced a clear radiosensitization, but the combination of KU55933, NU7026 pretreatment with subsequent 2-ME administration did not sensitize the cells further and beyond the KU55933, NU7026 effect (Fig. 3.54 and 3.55).



A549

Fig. 3.54: Radiosensitizing effect of 2-ME in presence of KU55933 and NU7026 inhibitors. H460 cells were pretreated with 2-ME, 48 h later cells were plated for colony formation, treated with ATM and DNA-PKcs inhibitors for 1 h, irradiated with 1 and 3 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.



H460

Fig. 3.55: Radiosensitizing effect of 2-ME in presence of KU55933 and NU7026 inhibitors. H460 cells were pretreated with 2-ME, 48 h later cells were plated for colony formation, treated with ATM and DNA-PKcs inhibitors for 1 h, irradiated with 1 and 3 Gy and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of 6 parallel experiments.

3.11 Effect of radical scavenger on MnSOD and CuZnSOD expression

Radical scavenger from plants removing reactive oxygen species (Duthie *et al.* 2000, Yu *et al.* 2005) act similar to superoxide dismutases. The most potent antioxidant activity among flavonoids showed radical scavenger rutin (Yu *et al.* 2005). The radiation-induced expression of SOD in presence of rutin was analyzed. Therefore, A549 and H460 cells were pretreated with rutin and irradiated with single radiation dose of 25 Gy (Fig. 3.56). Induction of MnSOD and CuZnSOD expression was found 24 and 36 h after radiation treatment. Treatment with rutin, which works similarly to superoxide dismutases in removing free radicals, clearly inhibited the IR-induced SOD expression.

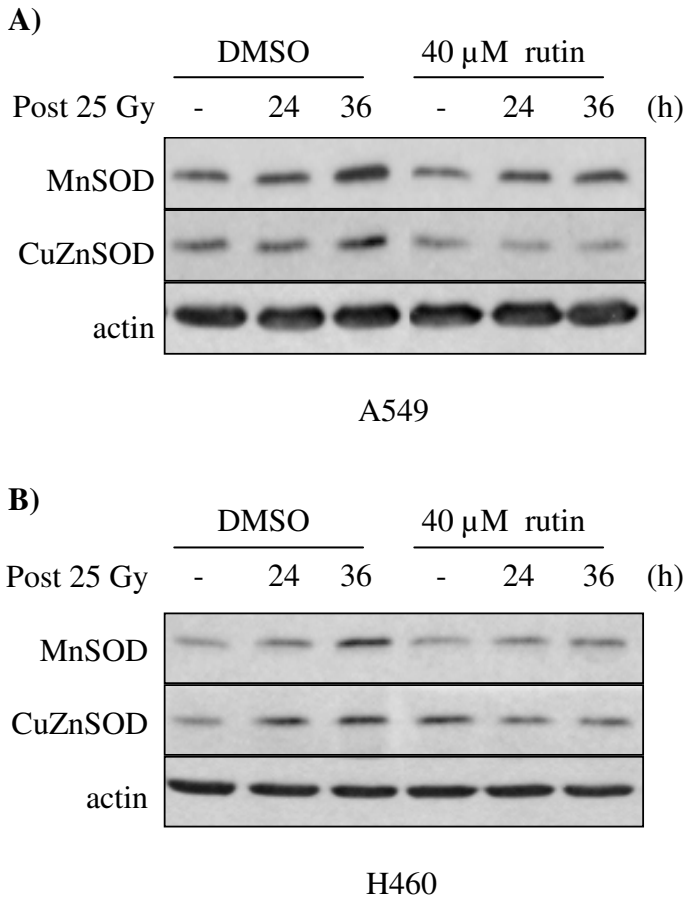
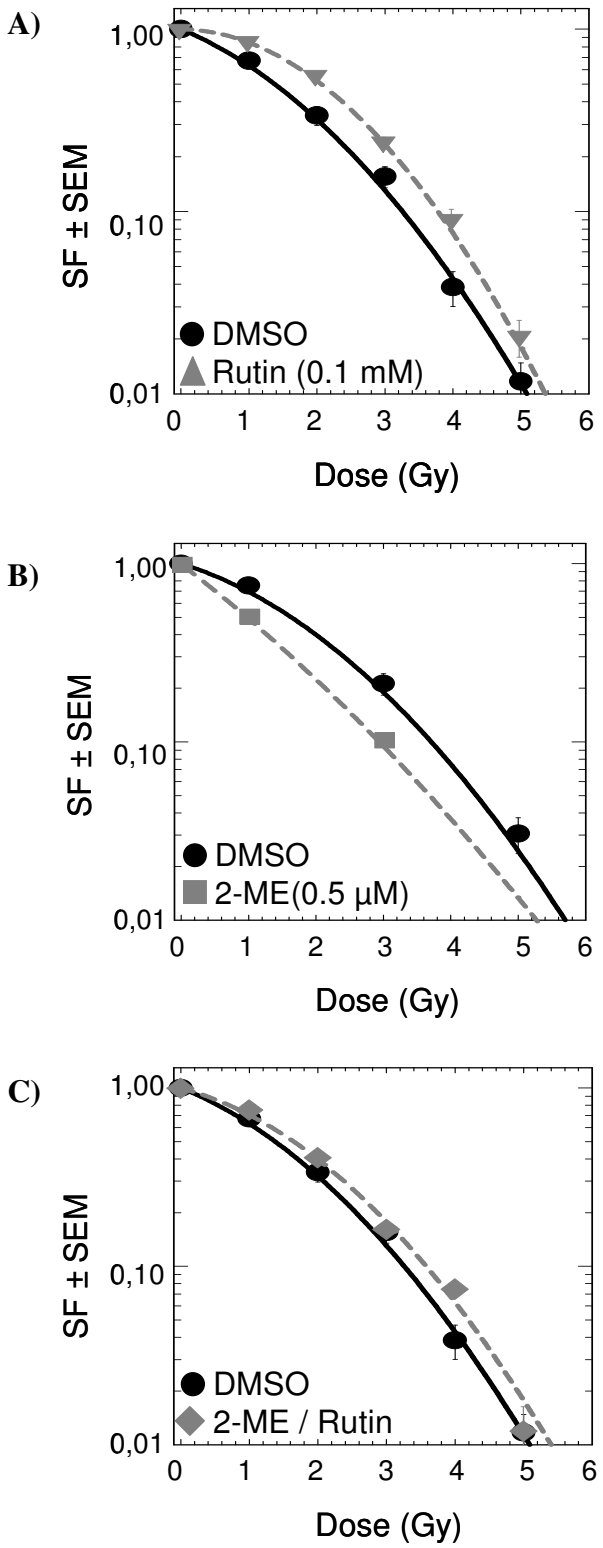


Fig. 3.56: Effect of radical scavenger rutin treatment on MnSOD and CuZnSOD expression. Cells were treated with rutin for 24 h, irradiated with dose of 25 Gy and lysed at indicated time points after IR. Protein samples were subjected to SDS-PAGE and blots were incubated with antibodies against MnSOD and CuZnSOD. Actin was detected as loading control.

The radioprotective effect of rutin was further analyzed by the clonogenic survival assay. A549 cells pretreated with rutin before irradiation with doses of 1 to 5 Gy were clearly radioprotected (Fig. 3.57 A). To further investigate the radioprotective potential of rutin the effect of rutin in presence of 2-ME was observed. Fig. 3.57 B and C shows that rutin completely abolished the radiosensitizing effect of 2-ME.



A549

Fig. 3.57: Effect of rutin on radiosensitivity in presence and absence of 2-ME. A549 cells were plated for colony formation assay, after 24 h were treated with rutin, irradiated with doses of 1 to 5 Gy and incubated for 10 days. (A). Cells were treated with 2-ME, 48 h later cells were plated for colony formation, after 24 h irradiated with doses of 1 to 5 Gy and incubated for 10 days (B). Cells were treated with 2-ME, 48 h later cells were plated for colony formation, after 24 h treated with rutin , irradiated with doses of 1 to 5 Gy and incubated for 10 days. For all experiments based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SEM of six parallel experiments.

4. DISCUSSION

4.1 IR-induced SOD enzyme activity and expression

Exposure to ionizing radiation (IR) induces production of reactive oxygen species (ROS) which results in cells cytotoxicity (Sun *et al*. 2007). As a response to radiation injury cells increase the expression of superoxide dismutases (SOD) (Kuninaka *et al.* 2000). Numerous studies have shown that IR increases the level of SOD expression in different cancer cell lines. Yanagisawa *et al.* (1997) showed that manganese superoxide dismutase (MnSOD) and copper zinc superoxide dismutase (CuZnSOD) are induced after IR with a dose of 4 Gy in nasopharyngeal epidermal carcinoma cell line (KB). Likewise, antioxidant enzymes in a radioresistant variant clone from U251 human glioblastoma cell line which is one of the most radioresistant tumors were induced up to 5-fold when compared to the parent cells after radiation (Lee *et al.* 2004). Together, it seems that SOD expression after irradiation protects tumor cells against cytotoxicity.

In the present study both superoxide dismutases were induced after a single low dose of ionizing radiation. Fractionated radiation exposure has been reported to induce radioresistance in esophageal cancer EC109 cells (Xie *et al.* 2009). Furthermore, in radioresistant variants isolated from MCF-7 human carcinoma cells fractionated ionizing radiation treatment increased MnSOD expression level which was much higher than in the control MCF-7 cells (Guo *et al.* 2003). In the present research, radiation with a single high dose did not result in efficient induction of MnSOD and CuZnSOD expression in comparison to fractionated radiation. This result indicates, in the context of radiation induced SOD expression that the number of radiation fractions appears to be more important than the total dose.

IR induces free radicals which increase not only the expression of SOD but also its enzyme activity (Durovic *et al.* 2008). It was found in the present study, that MnSOD activity increased after exposure to radiation but not CuZnSOD. The pattern of induction was different in all three lung carcinoma cell lines. Although there was no induction of MnSOD and CuZnSOD expression after radiation in H661 cells, the enzyme activity increased. The induction 1 min after radiation exposure in A549 was much lower than in H460 cells. The level of CuZnSOD in both cell lines is similar but MnSOD level is much higher in H460 cells. Thus, MnSOD activity can increase more in H460 cells immediately after radiation treatment. In A549 cells the maximum level of SOD activity was observed 16 h after IR, when already new MnSOD protein could be produced and increase in MnSOD protein expression could be observed. Such late time points of enhanced MnSOD activity has been observed in fibroblasts

exposed to 10, 20, 40 and 80 Gy (Akashi *et al.* 1995). There are several reports which confirm these results. Sun *et al.* (2007) showed that 24 and 48 h after radiation dose of 6 Gy MnSOD activity was increased but the level of CuZnSOD activity remained unchanged. Likewise, Xu *et al.* (2007) showed the induction of MnSOD activity but not CuZnSOD after ionizing radiation exposure. MnSOD seems to be a more effective enzyme than CuZnSOD in protecting cells against IR. Overexpression of CuZnSOD showed only slight radioprotective effect in Chinese hamster ovary (CHO) cells, while the MnSOD dependent radioprotective effect was clearly shown (Sun *et al.* 1998). Lack of radioprotective effect of CuZnSOD was also reported by Greenberger *et al.* (2003). These results can be explained by an insufficient amount of manganese in cytosol which resulted in only poor activation of MnSOD. On the other hand, the mitochondrial localization of SOD seems to play an important role as well. CuZnSOD located in mitochondria showed to have also a protective role against radiation exposure (Greenberger *et al.* 2003). In the present study, results of transfection with *MnSOD* and *CuZnSOD* siRNA suggest that not only CuZnSOD but also MnSOD are not major components of cellular radioprotection mechanism in tumor cells under *in vitro* conditions. Another possibility is that the small amount of SOD which still will be present in cells after transfection is enough to protect cells against IR. It has been published that both MnSOD and CuZnSOD are involved in radioprotection of normal tissue (Veldwijk *et al.* 2009, Yan *et al.* 2008). However, there are also published data which confirm that SOD does not play a role in radioprotection of cancer cells. Human cervix carcinoma HeLa-RC cells transfected with recombinant adeno-associated virus overexpressing MnSOD or CuZnSOD were not protected against radiation (Veldwijk *et al.* 2004). In the present study this result could be confirmed. Overexpression of MnSOD in H460 cells did not protect cells against IR. These data suggest that MnSOD and CuZnSOD do not contribute to radioprotection of cancer cells *in vitro*.

Radical scavengers have been shown to have strong antioxidant activity (Duthie *et al.* 2000, Yu *et al.* 2005). Among flavonoids rutin seems to show the most potent antioxidant activity (Yu *et al.* 2005). In the present report, rutin reduced IR-induced SOD activity by removing ROS and showed strong radioprotective effect by excluding completely radiosensitization of 2-methoxyestradiol (2-ME).These data suggest that rutin has stronger antioxidant activity than SOD against IR-induced free radicals in cancer cells *in vitro*.

4.2 Regulation of SOD expression

Numerous data demonstrated SOD induction by IR exposure (Yanagisawa *et al*. 1997, Kuninaka *et al*. 2000). However, the exact mechanism of SOD activation has remained

unexplored so far. The membrane receptor which could be responsible for regulation of SOD expression is also unknown. However, it has been already reported that *MnSOD* is one of the NF-κB downstream target genes (Saccani *et al.* 2001, Kiningham *et al.* 2001). ROS induced by radiation activate NF-κB which results in radioresistance of cancer cells (Criswell *et al.* 2003, Magne *et al.* 2006). In response to radiation, NF-κB members activate the expression of MnSOD leading to tumor cell protection (Guo *et al.* 2003, Murley *et al.* 2004). In the present study, blockage of NF-κB resulted in downregulating MnSOD and CuZnSOD. Regulation of CuZnSOD by NF-κB transcription factor was already shown by Rojo *et al.* (2004). It was demonstrated that NF-κB can be activated by phosphorylated AKT (Wang *et al.* 2004, Ozes *et al.* 1999, Romashkova and Makarov 1999) which is regulated in PI3K dependent manner in response to oxidative stress (Konishi *et al.* 1997). Furthermore, it was reported that expression of CuZnSOD is regulated via the PI3K/AKT pathway (Rojo *et al.* 2004). Results presented in this work clearly demonstrated that not only CuZnSOD but also MnSOD is regulated by the PI3K/AKT pathway.

Furthermore, the PI3K/AKT pathway can be induced in epidermal growth factor receptor (EGFR) dependent manner (Toulany *et al.* 2005). EGFR is a tyrosine kinase receptor which is activated by radiation exposure (Harari and Huang 2002). Overexpression of EGFR found in numerous cancers results in resistance to radiation treatment (Harari and Huang 2002). Overexpression of SOD results also in radioprotective effect on tumor cells (Sun J *et al.* 1997, Greenberger *et al.* 2003). Nishiguchi *et al*. (1994) reported that treatment of cells with one of the EGFR ligand EGF can induce expression of SOD. This suggests that EGFR activity could also regulate SOD expression in a ligands independent manner following radiation exposure. However, the present report did not confirm these results (Nishiguchi *et al*. 1994). Data obtained in this study clearly suggest that EGFR activity does not contribute to regulation of SOD expression. Similar results have been obtained for another tyrosine kinase receptor, IGF-1R induced by radiation treatment as well (Cosaceanu *et al.* 2006) and involved in tumor survival and growth (West 2009, Karamouzis *et al.* 2006). IGF-1R activity is also involved in regulation of the PI3K/AKT pathway (Vasilcanu *et al.* 2004) which has been shown to regulate SOD expression. Thus, in the present work it has been hypothesized that IGF-1R can be a membrane receptor regulating SOD expression. Results shown in the present study suggest that IGF-1R activity is not involved in MnSOD regulation but is involved in CuZnSOD regulation. Another tyrosine kinase receptor ErbB2 induced by radiation treatment (Bowers *et al.* 2001) and involved in radioresistance of tumor cells (Stackhouse *et al.* 1998) is also required in regulation of the PI3K/AKT pathway (Qi *et al.* 2009, Ou *et al.* 2008).

Therefore, in the present report it has been suggested that ErbB2 can be a membrane receptor regulating SOD. Blockage of ErbB2 activity by inhibitor or siRNA transfection did not affect MnSOD but strongly blocked CuZnSOD expression.

These results indicate that EGFR does not contribute to regulation of SOD expression. ErbB2 and IGF-1R involved in activation of the PI3K/AKT pathway regulate also CuZnSOD expression. However, the role of ErbB2 and IGF-1R in CuZnSOD regulation via the PI3K/AKT pathway following IR exposure has remained unknown (Fig. 5.1).

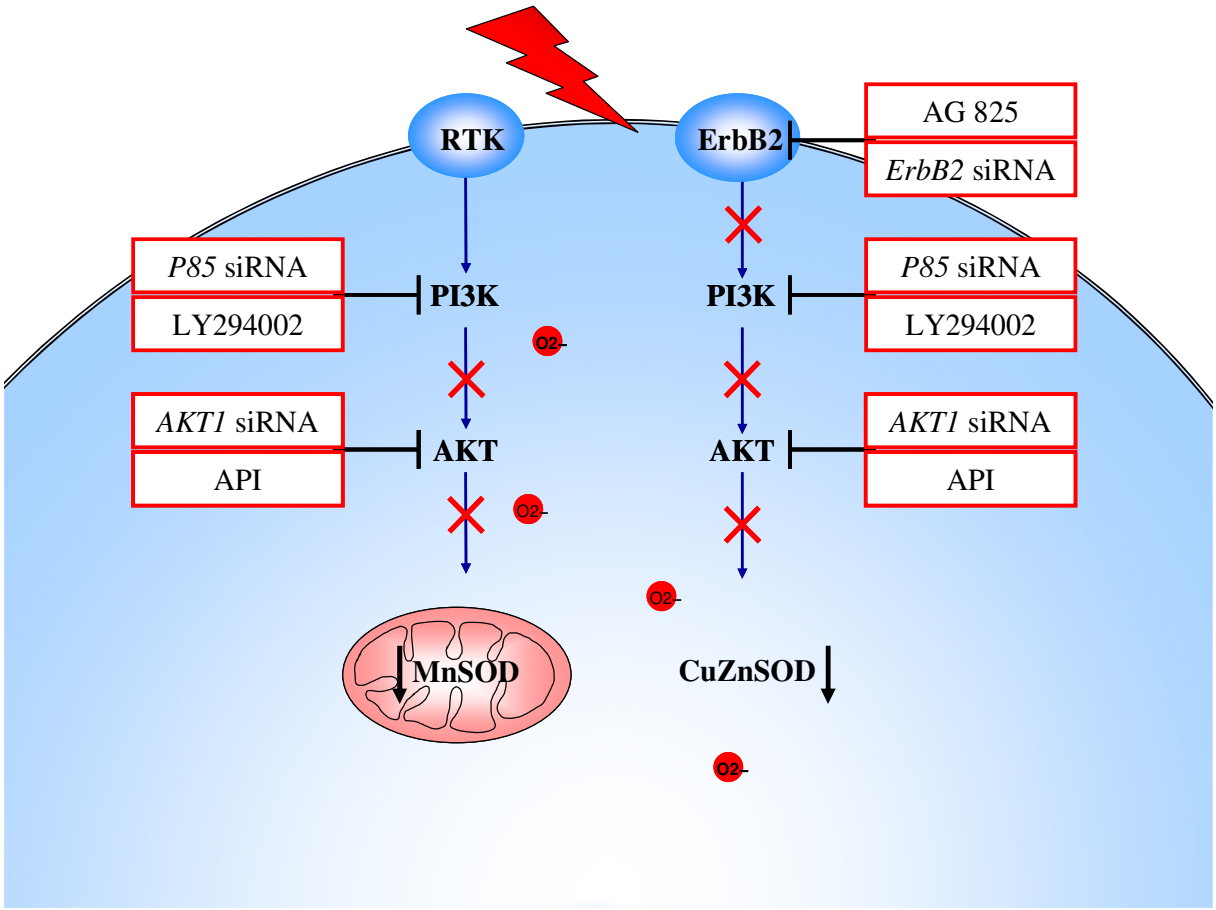


Fig. 5.1: Schematic illustration of the pathway involved in regulation of SOD expression

4.3 MnSOD translocation to the nucleus following irradiation

It is known that the activity of transcription factors is regulated by redox status (Pinkus *et al.* 1996). By activating ROS radiation induces numerous transcription factors, including the nuclear factor-κB (NF-κB) (Criswell *et al.* 2003). It has been reported that one of the NF-κB downstream target genes is *MnSOD* (Saccani *et al.* 2001, Wang *et al.* 2002, Kiningham *et al.* 2001). However, in this report blockage of NF-κB resulted not only in reduced MnSOD

expression but CuZnSOD expression. Further investigations of the link between SOD and NF-κB showed that after binding to NF-κB MnSOD is translocated to the nucleus following ionizing radiation exposure (Fig. 5.2). MnSOD was found in the nucleus under control condition or following irradiation. So far, it has been described that MnSOD is located in mitochondria (Hirose *et al.* 1995). The copper zinc containing SOD is cytosolic protein, which is also partially localized in nuclei (Chang *et al.* 1988). However, in the present study almost no CuZnSOD was found in the nucleus. Li *et al.* (2009) observed that MnSOD was colocalized to the cytoplasm in response to oxidative stress by bile salts stimulation. Parallel the activity of MnSOD was decreased although MnSOD expression was increased. One of the reasons that MnSOD in cytosol was enzymatically inactivated could be a small amount of manganese in cytosol. Manganese is required for MnSOD activation. However, cytosolic CuZnSOD activity also did not increase in cytoplasm in response to oxidative stress following irradiation. Yet, when CuZnSOD was localized in mitochondria it showed radioprotective effect (Greenberger *et al.* 2003). MnSOD translocated with NF-κB to the nucleus can be a new protein synthesized in cytoplasm or protein which has just been translocated to the cytosol as a response to oxidative stress and mitochondria dysfunction which can be induced by radiation treatment. In the nucleus extracellular superoxide dismutase (ECSOD) was also found. Usually, ECSOD is secreted to the medium (Ookawara *et al.* 2002). It has been reported that ECSOD is also translocated to the nucleus. Yet, the mechanism of translocation seems to be different. The heparin-binding domain of ECSOD serves as nuclear localization signal (NLS) (Ookawara *et al.* 2002). However, the roles of both superoxide dismutases in the nucleus can be similar. Ookawara *et al.* (2003) reported that nuclear translocation of ECSOD was clearly enhanced in response to oxidative stress. It is known that DNA is cleaved by ROS (Kaneto *et al.* 1994). It has been hypothesized that ECSOD can be involved in protecting genomic DNA from ROS in the nucleus. The other possibility of ECSOD's role in the nucleus is modulation of signal transduction as a scavenger of superoxide anions or supplier of hydrogen peroxide (Ookawara *et al.* 2002). A similar role in the nucleus can also play MnSOD. The basal level of MnSOD is constantly present in the nucleus like ECSOD and the level of both SOD increase in the nucleus in response to oxidative stress. However, since it is known that MnSOD can be activated only in mitochondria and not in cytosol, it is unclear whether the activation of MnSOD in the nucleus is possible.

The role of NF-κB in MnSOD nuclear translocation remains still unclear. Both MnSOD and NF-κB are activated in response to oxidative stress and both can be involved in cellular response to the stress by activating prosurvival pathways, NF-κB by regulation of

transcription of genes involved in survival, MnSOD regulating signal pathways by changing the redox status in cells. It is also not known whether NF-κB is the only protein involved in MnSOD translocation.

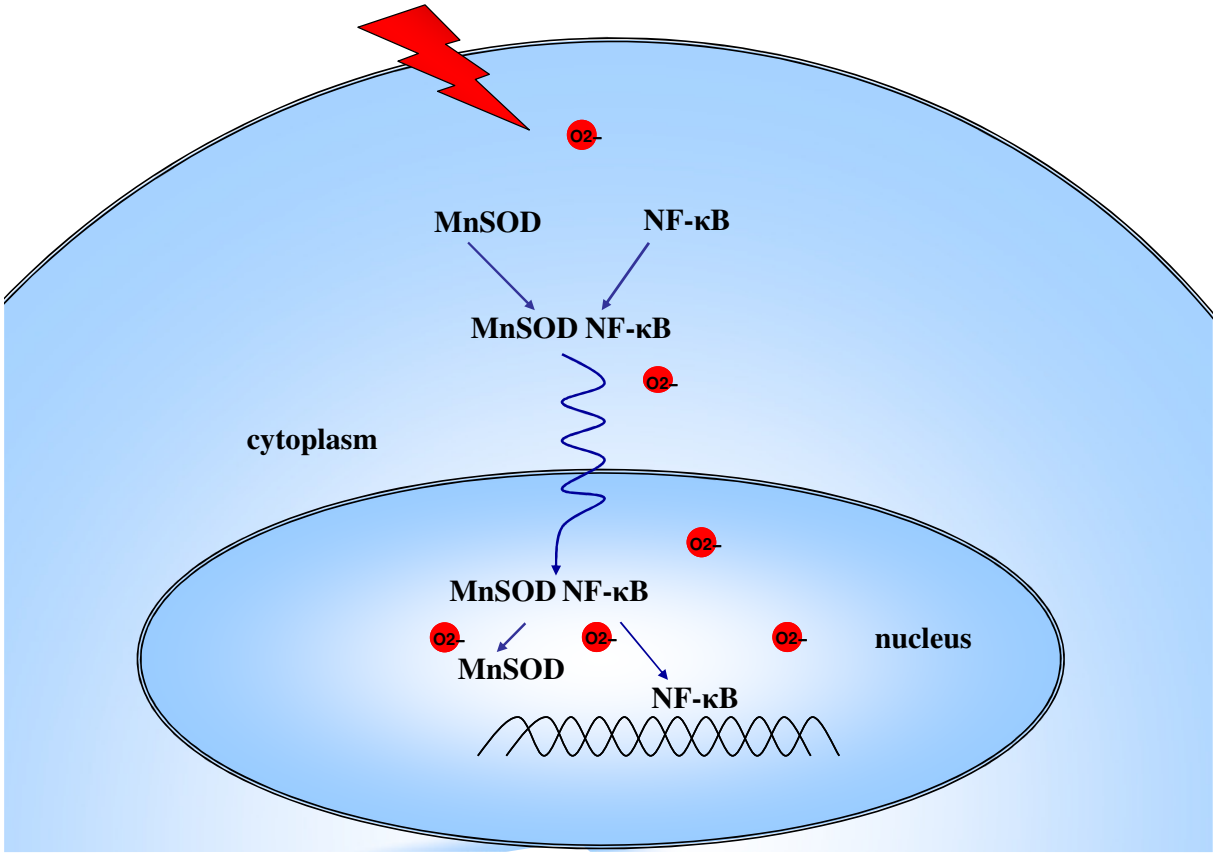


Fig. 5.2: Schematic illustration of mechanism of IR-induced MnSOD nuclear translocation.

4.4 Mechanism of radiosensitization by 2-ME

SOD has been shown to have a protective role against IR in cancer cells (Sun *et al.* 1997). Detoxification of free radicals by SOD is involved in cellular radiation response. Therefore, SOD seems to be an important target as anticancer therapy. It has been reported that endogenous estrogen metabolite 2-methoxyestradiol (2-ME) inhibits MnSOD and CuZnSOD (Huang *et al.* 2000). Furthermore, 2-ME preferentially kills leukaemia cells and does not show cytotoxic activity to normal cells (Huang *et al.* 2000). Conflicting data to this report were published by Kachadourian *et al.* (2001). They indicate that 2-ME does not inhibit SOD. In the present work it was observed that the SOD activity induced by clinically relevant doses of ionizing radiation can be blocked by 2-ME. This inhibition of the SOD activity is in line with findings reported by Huang *et al.* (2000) who described 2-ME as SOD activity inhibitor.

However, the effect of 2-ME on IR-induced SOD expression has not been investigated so far. In the present study it was demonstrated that 2-ME does not affect expression of SOD induced by a single radiation dose. However, a strong effect of 2-ME on MnSOD and CuZnSOD expression could be observed after fractionated radiation treatment. Fractionated radiation as well as fractionated treatment with inhibitor required longer incubation with 2-ME and led to a higher end concentration of the inhibitor which could result in stronger effect of 2-ME on SOD expression.

Since it has been reported that IR induced SOD (Kuninaka *et al*. 2000) and overexpression of SOD plays a role in radioresistance of tumor cells (Sun *et al.* 1997) it was suggested that 2-ME can sensitizes cancer cells to radiation treatment by blockage of SOD. The data presented herein supported the radiosensitization effect of 2-ME which was already demonstrated by others. Huober *et al* (2000) observed that administration of a nontoxic estrogen metabolite 2-ME, significantly enhanced the radiation effect in non-small-cell lung cancer without any toxicity. Furthermore, 2-ME radiosensitized colon carcinoma cells (Zou *et al.* 2006) and significantly enhanced radiation-induced cell death in glioma cells (Zou *et al.* 2007). Thus, results presented in this study on radiosensitization are in good agreement with the published data. However, non of these studies (Huober *et al.* 2000, Zou *et al.* 2006, Zou *et al.* 2007) addressed the question of whether radiosensitization of cancer cells by 2-ME is a consequence of SOD inhibition.

It has been shown previously that 2-ME inhibits basal phosphorylation of AKT (Gao *et al.* 2005, Zhang *et al.* 2009, Chow *et al.* 2008). However, the effect of 2-ME on the radiation-induced PI3K/ AKT signaling has not been described so far. In the present research, 2-ME clearly abolished radiation-induced AKT phosphorylation. This result indicates that SOD activity may not be the only target of 2-ME which results in radiosensitization. The PI3K/AKT pathway is a prosurvival pathway which can activate numerous genes encoding protein whose blockage can induce radiosensitization of cancer cells after treatment with 2-ME. Furthermore, the present report showed that in cells radiosensitized by targeting of AKT no further radiosensitization could be induced by subsequent administration of 2-ME indicating that 2-ME radiosensitizes cells through PI3K/AKT targeting.

PI3K/AKT is involved in the activation of number of proteins. One of these proteins is DNA-dependent protein kinase catylitic subunit (DNA-PKcs). In few studies it has been shown that AKT-signalling is an important mediator of radiation-induced stimulation of DNA repair through NHEJ repair pathway (Kao *et al.* 2007, Golding *et al.* 2009). In this context a clear function of AKT in activation of DNA-PKcs has been demonstrated by Toulany *et al*. (2008).

In the present report, 2-ME also inhibited trans- and autophosphorylation of DNA-PKcs. Thus, as a consequence of impaired DNA-PKcs activity DNA-dsb repair through NHEJ is affected resulting in increased residual γH2AX-foci in irradiated cells pretreated with 2-ME. This conclusion is supported by data published by Zou *et al.* 2006 which demonstrated that γH2AX-foci were strongly increased by 2-ME pretreatment. Furthermore, similar effect of 2-ME could be observed in xenografts after IR (Zou *et al.* 2006). 2-ME blocks SOD activity. However, no effect of SOD blockage on phosphorylation of AKT and DNA-PKcs was noted in the present report indicating that 2-ME affects DNA-PKcs activity via blockage of the PI3K/AKT pathway. Blockage of SOD does not further induce 2-ME mediated radiosensitization. This indicates that SOD is not the target of 2-ME leading to radiosensitization of cancer cells. The present work has shown that in cells radiosensized by targeting of DNA-PKcs no further radiosensitization can be induced by subsequent administration of 2-ME. This indicates that blockage of DNA-PKcs is involved in 2-ME mediated radiosensitization (Fig. 5.3).

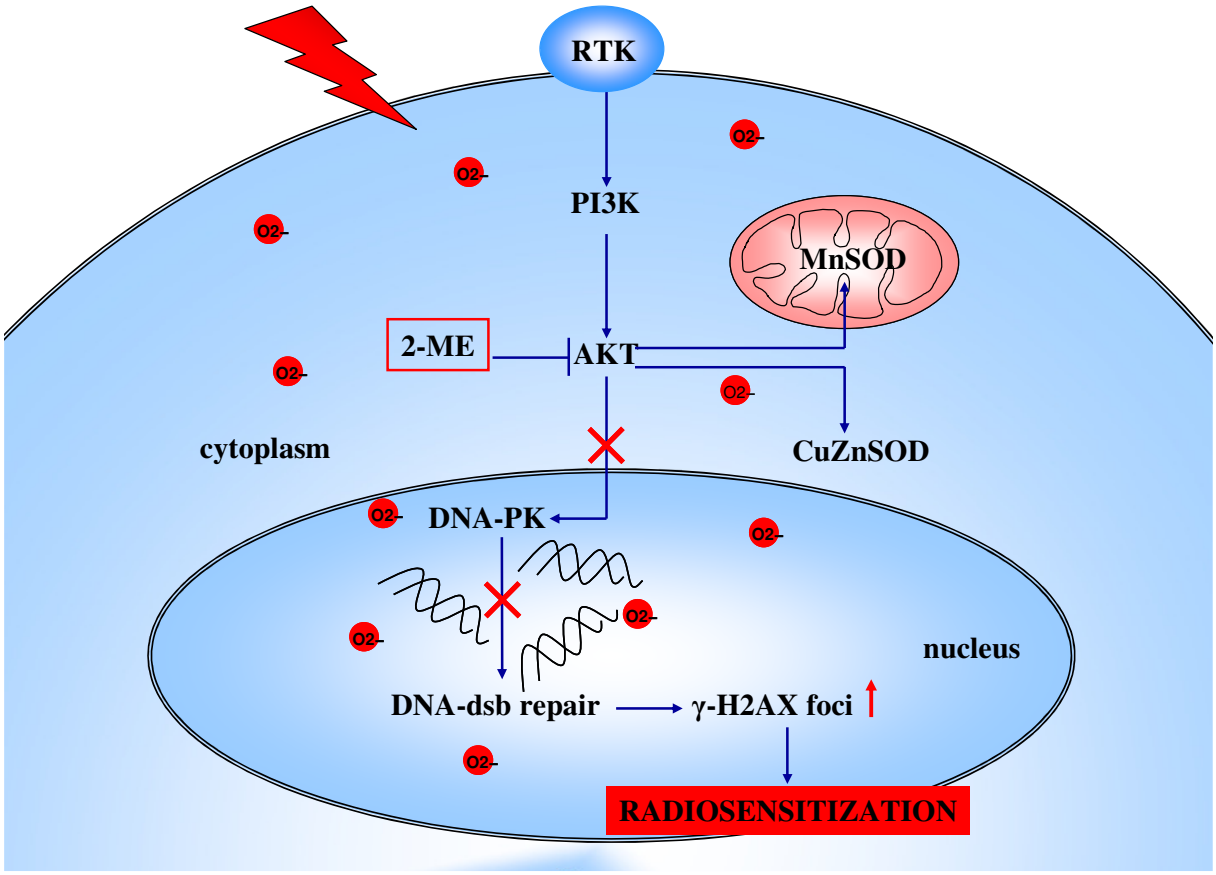


Fig. 5.3: Schematic illustration of the pathway involved in 2-ME mediated radiosensitization

4.5 Concluding discussion

Taken together the data presented in this work and in the literature, radiation-induced SOD seems to have no marked protective role against ionizing radiation in tumor cells in vitro. 2-ME-mediated radiosensitization could be demonstrated to be independent of its effect on SOD but dependent on its inhibitory effect on AKT-signalling and AKT's function in stimulating DNA-PKcs and DNA-dsb repair (Fig. 5.4).

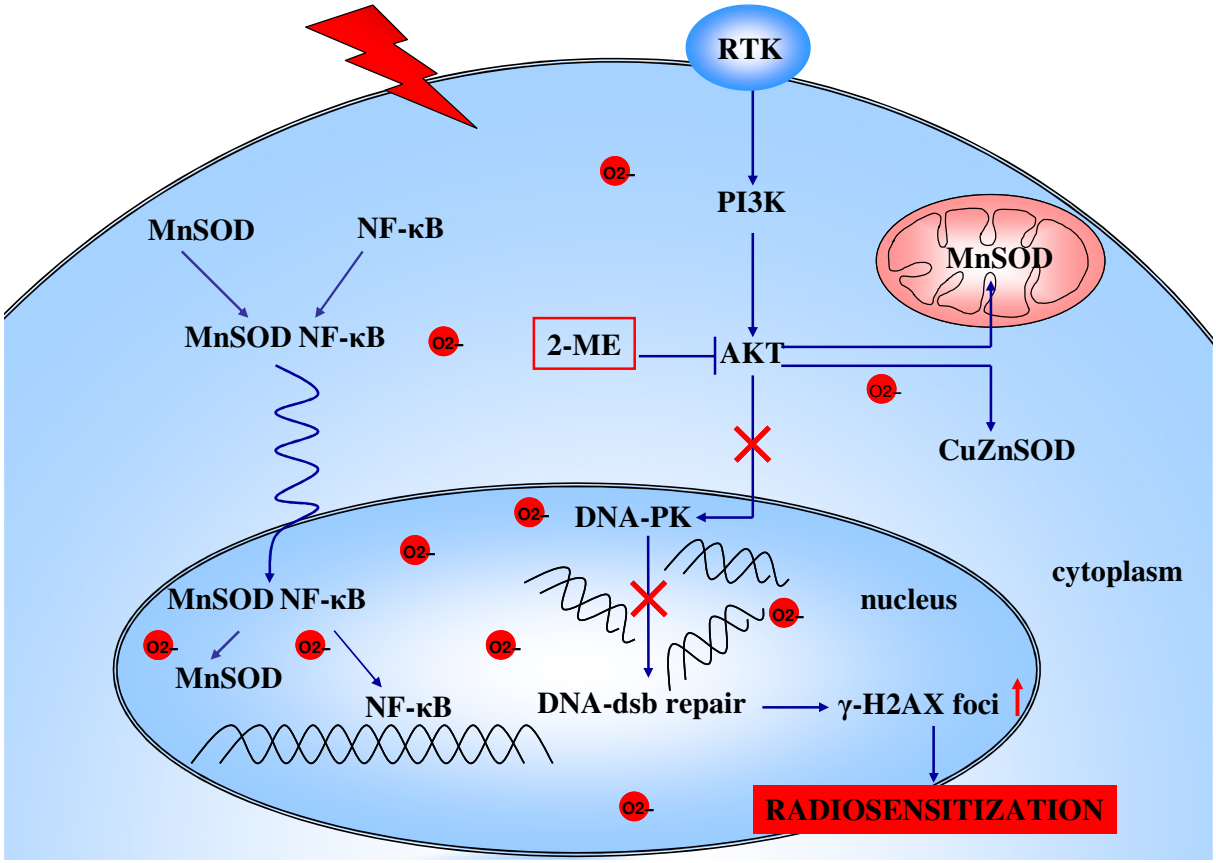


Fig. 5.4: Schematic illustration of the pathway involved in 2-ME mediated radiosensitization, mechanism of IR-induced MnSOD induction and nuclear translocation.

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CURRICULUM VITAE

Personal details

Name: Urszula Florczak
Date of birth: August 2, 1980
Nationality: Polish

Education

10.1999 – 07.2004 Study of Biotechnology, Agricultural University of Poznan, Poland

MSc Thesis
Department of Biochemistry and Biotechnology

Searching for genetic diversity among individuals of Metasequoia glyptostroboides species.

08.2004 – 03.2005 Leonardo da Vinci scholarship
Institute of Human Genetics, Eberhard Karls University, Tübingen, Germany

Department of Otolaryngology, Eberhard Karls University, Tübingen, Germany

04.2005 – 09.2005 Wissenschaftliche Hilfskraft

Department of Otolaryngology, Eberhard Karls University, Tübingen, Germany

06. 2006 – 11.2009 **PhD Thesis**

Division of Radiobiology and Molecular Environmental Research, Department of Radiooncology, Eberhard Karls University, Tübingen, Germany

Role of superoxide dismutase system in the radiation response of tumor cells.

Poster presentations

10th. International Wolfsberg Meeting on Radiation Biology/Oncology; Ermatingen, Switzerland, 2007.
Florczak U, Toulany M, Baumann M, Rodemann HP
Ionizing radiation induced PI3K/AKT activity regulates superoxide dismutase expression.

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PI3K/AKT dependent superoxide dismutase expression protects tumor cells against ionising radiation.

11. Jahrestagung der Gesellschaft für Biologische Strahlenforschung; Tübingen, Germany 2008
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2-methoxyestradiol radiosensitizes lung carcinoma cell lines through suppression of IR-induced AKT/DNA-PKcs activity.

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