Mechanism and Role of EGFR Tyrosine Kinase Inhibition in Radiation Response of Human Tumor and Normal Cells

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for my Wife and Son

SUMMARY

Cancer is a public health problem worldwide and the main cause of mortality. Surgery, radiotherapy and chemotherapy are the three major cancer treatment modalities. Applying advanced technical developments in radiation oncology has improved the quality of cancer treatment by radiotherapy alone as well as in combination with chemotherapy. Nevertheless, further progress in clinical efficiency of radiotherapy can only be expected when in addition to technological advances biological parameters of the radiation response profile of tumors are taken into account for the development of treatment strategies. Therefore clarifying the underlying molecular mechanisms of radiation responses and identifying molecular targets for intervention will create the potential to develop specific therapeutic strategies in radiation oncology based on individual biological parameters of the tumors to be treated.

Accelerated repopulation of tumors during fractionated radiotherapy is a phenomenon that limits the success and effectiveness of radiation treatment. One proposed mechanism for tumor repopulation is the potential of ionizing radiation to activate the epidermal growth factor receptor (EGFR) which is linked to several components of mitogenic and survival signaling pathways mediating resistance to ionizing radiation and failure in tumor treatment.

Based on the prominent role of EGFR in accelerated repopulation as well as cellular radioresistance, molecular-targeting approaches of this receptor were proposed to enhance efficacy of radiotherapy. For this purpose, differential pharmacological and biological approaches have been developed favoring two strategies: Monoclonal antibodies against ligand binding domain of EGFR and low molecular weight receptor tyrosine kinase (RTK) inhibitors.

The aim of the present study was to investigate the molecular principles of radioresistance, radiation-induced EGFR-autophosphorylation and activation of downstream signal transduction pathways in EGFR-overexpressing human tumor cells. Since it is known that in addition to EGFR-overexpression, mutations in the *RAS* gene are not only very frequent in human tumors but also influence cellular radiation sensitivity, the analyses were performed with human tumor cells with either K-*RAS*-wildtype (K-RAS_{wt}) or K-*RAS*-mutated (K-RAS_{mt}) status. Special emphasis was given to the radiosensitizing potential of the selective EGFR TK inhibitor BIBX1382BS and its molecular mode of action.

The following major results were obtained:

1. As shown for a panel of human tumor cell lines and fibroblasts exposure to ionizing radiation mediated stimulation of EGFR autophosphorylation in a ligand independent manner.
2. Blockage of EGFR-tyrosine kinase activity by BIBX1382BS led to a differential antiproliferative effect for all cells tested.
3. Inhibition of EGFR-TK-activity by BIBX1382BS resulted in enhanced radiation toxicity only in tumor cells presenting a point mutation in the K-*RAS* gene.
4. Analyses of the three major EGFR downstream pathways (PI3K-AKT, MAPK, and JAK-STATs) revealed that blockage of EGFR-TK by BIBX1382BS primarily results in an inhibition of the phosphatidylinositol 3- kinase (PI3K)-AKT pathway leading to enhanced radiation sensitivity.
5. The radioresistance of K-RAS_{mt} tumor cells was found to be dependent on autocrine/paracrine secretion of EGFR ligand, *i.e.* amphiregulin (AREG). Due to constitutively active K-*RAS* these cells produce significantly elevated levels of secreted AREG which in turn leads to an autocrine activation of the EGFR-PI3K-AKT survival pathway.
6. Expression of K-*RAS*-siRNA in K-RAS_{mt} cells blocked autocrine activation of EGFR-PI3K-AKT pathway and enhanced radiation sensitivity.
7. Blockage of EGFR-tyrosine kinase activity by BIBX1382BS affected DNA repair mainly by significantly reducing nuclear activation of DNA-PKcs (an important enzyme in NEHJ-repair) resulting in increased micronuclei formation.

The data presented provided for the first time direct evidence that radiosensitization of human tumor cells by EGFR-targeting approaches applying the EGFR-specific TK-inhibitor BIBX1382BS requires the presence of a K-*RAS* mutation. These findings specifically point to a mechanism that promotes radioresistance in K-RAS_{mt} human tumor cells via EGFR dependent but Ras-GTP independent autocrine activation of PI3K/AKT pathway through modulation of DNA repair processes, *e.g.* NHEJ.

In conclusion the present study provides molecular and biochemical evidence which may help to explain at least in part the heterogeneity of EGFR-targeting approaches for induction of enhanced radiation sensitivity of EGFR overexpressing tumor cells and underlines the

importance of additional mutations in related pathways which may promote or abolish the expected effect.

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LIST OF ABREVIATIONS

APS	Amuniumpersulfate
AREG	Amphiregulin
ATM	Ataxia telangiectasia mutataed
ATP	Adenosintriphosphate
BSA	Bovine serum albumin
cDNA	Complementary DNA
CM	Conditioned media
COX2	Cyclo-oxygenase 2
CPM	Counts per minute
CREB	Cyclic APM response element binding protein
DMEM	Dublecco´s modified eagle medium
DMF	Dose modifying factor
DMSO	Dimethylsolfoxide
DNA	Deoxyribonuccleic acid
DNA-PK	DNA-dependent protein kinase
DSBs	Double strand breaks
dsDNA	Double strand DNA
DTT	Dithiothreitol
ECL	Enhanced chemilominescence
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGFRvIII	EGFR variant III
ERK	Extra-cellular signal regulated kinase
FCS	Fetal calf serum
FTI	Farnesyl transferase inhibitor
GDP	Guanosinediphosphate
Grb2	Growth factor receptor binding 2
GST	Glutation-S-transferase
GTP	Guanosinetriphosphate
Gy	Gray
$H20_{dd}$	Twice-destilled, deionised water
HEPES	N-(2-Hydroxyethyl)-piperaszin-N‘-2-Ethansulfonic acid
HER	Human EGF-related
H-Ras	Harvey Ras
HSF	Human skin fibroblast
IB	Immunoblotting
IgG	Immunoglobulin
IMRT	Intensity modulated radiation therapy
IP	Immunoprecipitation
IR	Ionizing radiation
JAK	Janus kinase
kDa	Kilodalton
K-Ras	Kirsten Ras
Linac	Linear accelerator
MAbs	Monoclonal antibodies
MAP	Mitogen-activated protein
MAPK	MAP kinase
MEK	MAP kinase/ ERK kinase
MNi	Micronuclei

MRN	Mre11- Rad50-Nbs1
NFkB	Nuclear factor kB
NHEJ	Non-homologous end joining
N-Ras	Neuroblastoma Ras
NSCLC	Non-small cell lung cancer
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol-3,4-diphosphate
PIP_3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase-B
PVDF	Polyvinylidene fluride
PY	Phospho-tyrosine
Raf	Homologue to v-raf (murine sarcoma viral oncogene)
Ras	Homologue to v-ras (rat sarcoma viral oncogene)
Ras_{mt}	Ras mutated
Ras_{wt}	Ras wildtype
RBD	Raf binding domain
RNA	Ribonuccleic acid
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription PCR
SCC	Squamous cell carcinoma
SCCHN	Squamous cell carcinoma of head and neck
SD	Standard deviation
SDS	Nantriumdodecylsulfate
SDS-PAGE	SDS polyacrilamide gel electrophoresis
SE	Standard error
SF	Surviving fraction
SHC	Src homology and collagen
siRNA	Small interference RNA
Src	Homologue to v-src (sarcoma viral oncogene)
STAT	Signal transducer and activator of transcription
3D-CRT	Three-dimensional conformal radiotherapy
TCA	Trichloroacetic acid
TEMED	N,N,N´,N´- Tetarmethylethylenediamine
TGFα	Transforming growth factor alpha
TKI	Tyrosine kinase inhibitor
VEGFR	Vascular endothelial growth factor receptor
WB	Western blotting

1 INTRODUCTION

1.1 Cancer and treatment modalities

Cancer is a major public health problem worldwide. The world health organization (WHO) estimates that more than 11 million people are diagnosed with cancer every year. It is proposed that there will be 16 million new cases every year by 2020. Currently cancer causes 7 million deaths every year or 12.5% of deaths worldwide.

Surgery, radiation therapy (RT), and chemotherapy are the three major cancer treatment modalities. About 50% of all individuals developing cancer will receive some form of radiation therapy for treatment. Ionizing radiation (IR) can be applied as single therapy, as concomitant therapy with chemotherapy, or as an adjuvant therapy after surgical removal of a tumor to prevent the growth of new tumor tissue from residual site and lastly as palliative therapy. Nowadays, standard radiotherapy (RT) is performed applying high energy X-ray photons produced by linear accelerators (Linac) (Van Dyk, 1999).

Early experiments showed that irradiation leads to a loss of viscosity in DNA solutions. Subsequently this has been shown to result from DNA strand breaks. There are two categories of DNA strand breaks induced by IR, single-strand breaks (SSB) and double strand breaks (DSB). Base damage and different crosslinks between DNA strands and nuclear proteins are the other types of DNA lesions induced by irradiation. In a typical mammalian cell the number of DNA lesions that are detected immediately after irradiation with a dose of 1 Gray (Gy) has been estimated to be approximately 40 DSBs, 1000 SSBs and 2700 base damages. Each strand break is a consequence of approx. 2.7 base damages (Ward, 1995). Thus, IR at clinically used doses (2-3 Gy/fraction/day) induces a vast amount of DNA damage. Among different lesions induced by irradiation, DSBs are the critical lesions for IR induced cell killing.

However, in addition to the intended effect of tumor cell killing the early and late or somatic and genetic effects in normal tissue are included by IR which are the close limiting factors of radiation therapy (Williams *et al.,* 2003). Radiation-induced second malignancies could be part of late genetic effect which increases with radiation dose and affect the function and quality of life of cancer survivors (Mohanti & Bansal, 2005). In addition, several mechanisms can make tumor more radioresistant than normal tissue. Tumor repopulation during radiation fractionation, genetic instability, hypoxia, DNA repair, binding of free radicals by glutathione and other sulfhydril molecules, increased glutathione-s transferase and other enzymes that eliminate free radicals as well as changes in expression of anti-apoptotic proteins *e.g.* Bcl_2 are

part of the mechanisms involved in radiation resistance of tumors (Tannock, 1996; Cho *et al.,* 2005).

In a first attempt to overcome this problem, chemotherapy alone or in combination with radiotherapy has been introduced and become common in the radical treatment of cancers at many sites. Many drugs used for chemotherapy function by damaging DNA as well. However, clinical gains for chemotherapy alone or concomitant therapy with radiotherapy have been small. In addition to sever normal tissue toxicity, tumor resistance to this approach either at the beginning of treatment or during treatment has been reported (Di Nicolantonio *et al.,* 2005).

Therefore, in order to further improve in cancer treatment by conventional methods, *i.e.* chemotherapy and radiotherapy, mechanistically based approaches as new targeted therapy are required.

1.2 Molecular targeted therapy

Clarifying the molecular mechanism of malignant transformation and exploring the differences between malignant and noncancerous cells create the potential to specifically interfere with these events. The ultimate goal of tumor therapy is to interrupt proliferation and metastasis of cancer cells while the function of normal cells largely undisturbed. Traditional cytotoxic chemotherapy however does not discriminate between normal and tumor cells. In contrast RT is a local cytotoxic treatment without systemic effects and thus provides many advantages for effective tumor therapy. Radiation therapy in addition to DNA damage induces a complex of signaling processes like activation or transcription of different proteins, DNA repair process and apoptosis. Therefore the biological differences between normal and malignant cells which are mediating cellular radiation responses offer the identification of specific targets for developing optimal RT protocols. In theory this could be a promising therapeutic approach for several reasons. First, since the cellular targets of these agents and mechanism(s) of action are different for those of cytotoxic drugs, it is possible to combine chemotherapy without crossresistance as shown for epidermal growth factor receptor targeting (EGFR) inhibitors and different chemotherapeutic agents (Shin *et al.,* 2001; Xiong *et al.,* 2004; Chan *et al.,* 2005). Second, alteration in the expression and/or the activity of genes that regulate survival and mitogenic signals not only can directly cause perturbation but also may affect the sensitivity of cancer cells to conventional chemotherapy and radiotherapy (Ryan and chabner, 2000; Melisi *et al.,* 2004). Therefore molecular targeting is a promising option to increase the radiation response of tumors and decrease normal tissue reactions, *i.e.*

to achieve a therapeutic gain. Various compounds for different molecular targets, *e.g.* EGFR, COX2, VEGFR, Ras…have been developed and are in different stages of preclinical and clinical trails (Cohen-Jonathan, 2000; Abdollahi *et al.,* 2003; Nakata *et al.,* 2004; Baumann *et al.,* 2004). So far among different molecular targeting strategies EGFR seems to be the most promising target.

1.3 Epidermal growth factor receptor targeting

The EGFR was the first receptor identified of a family of membrane receptors known as type I receptor tyrosine kinases. This receptor family is comprised of four members: EGFR itself or ErbB1 (HER1), ErbB2 (HER2/*neu*), ErbB3 (HER3), and ErbB4 (HER4) (Casalini, 2004; Olayioye, 2000). Although specific soluble ligands that bind to extracellular domain of EGFR, erbB3 and erbB4 have been identified, no ligand has been identified for erbB2. In addition to the extracellular ligand-binding domain and transmembrane segment, erbB receptors (except ErbB3) contain an intracellular protein tyrosine kinase domain with a regulatory carboxyl terminal segment (Yardan, 2001). Under physiologic conditions, a variety of EGFR family ligands drive the formation of homo- or heterodimeric complexes among the four ErbB receptors, which provides a mechanism of signal amplification and diversification.

EGFR/ErbB1 is a 170 kDa transmembrane glycoprotein. In tumor cells this receptor can be activated by additional mechanisms than ligand binding. First, receptor overexpression in tumor may lead to ligand-independent receptor dimerization (Hirsch *et al.,* 2003). In tumors like glioblastomas, the mutant form of EGFR (EGFRvIII) arising from gene rearrangements results in ligand-independent constitutive receptor activation and impaired receptor down-regulation (Klingler-Hoffmann *et al.,* 2003; Li *et al.,* 2004; Luwor *et al.,* 2004). Heterologous ligand-dependent mechanisms are also at play as demonstrated by the finding that stimulation of G-protein–coupled receptors results in EGFR activation via metalloproteinase-mediated cleavage of precursor membrane- bound EGFR ligands (Gschwind *et al.,* 2001). A ligand-independent EGFR activation via the urokinase plasminogen receptor has also been identified (Liu *et al.,* 2002). These findings indicate that tumor cells in contrast to normal cells may have additional EGFR activation mechanisms beyond receptor overexpression, mutations and autocrine ligand production.

At the signal-processing level, activation of the intrinsic receptor tyrosine kinase is induced and tyrosine autophosphorylation occurs. These events result in the recruitment and phosphorylation of several intracellular substrates as well as the binding of docking and adaptor molecules to specific phosphotyrosine sites on receptor molecules. A major

downstream signaling of the ErbB family is the Ras-Raf-MAPK pathway (Lewis *et al.,* 1998) Activation of Ras initiates a multistep phosphorylation cascade that leads to the activation/phosphorylation of ERK1 and ERK2. P-ERK1/2 regulate transcription of proteins that are linked to the cell proliferation, survival and transformation. Second important target in EGFR signaling is phosphatidylinositol 3-kinase (PI3K) and the downstream protein-serine/threonine kinase Akt (Chan *et al.,* 1999). Another route for EGFR signaling is via the stress-activated protein kinase pathway, involving protein kinase C and janus tyrosine kinase (JAK)/signal transducer and activator of transcription (STAT) pathways (Boudny and Kovarik, 2002). As shown in Fig. 1-1 the activation of these pathways translates in the nucleus into distinct transcriptional programs that mediate a variety of cellular responses, including cell division, survival (or death), motility, invasion, adhesion and cellular repair.

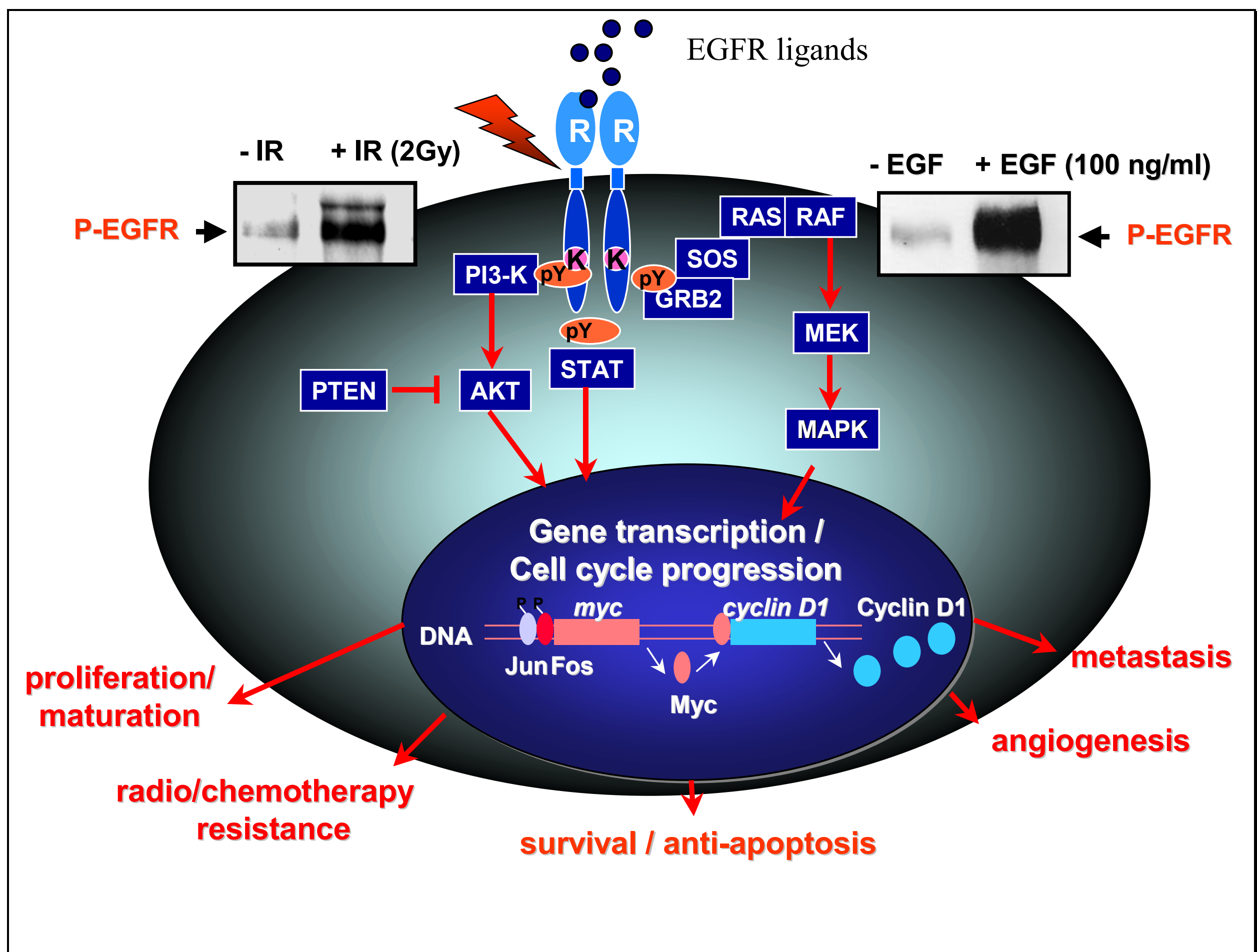


Fig. 1-1: Ligand-dependent and ligand-independent but radiation-induced signaling through EGFR and its functional consequences.

Intriguingly exposure of tumor cells to IR in the therapeutic dose range activates EGFR via autophosphorylation (Contessa *et al.,* 1999; Lammering *et al.,* 2001; Lammering *et al.,* 2003)

which, leads to the stimulation of different downstream signal transduction pathways as explained above. Among different pathways, activated PI3K-AKT pathway with many downsream targets, *i.e.* regulators of apoptosis (BAD, caspase-9), glucose metabolism (glycogen kinase), various transcription factors (CREB and the forkhead family of transcription factors), and finally the I-kappaB kinases (IKKs) is the most important downstream survival pathway (Fahy *et al.,* 2003).

There is now growing evidence that members of the epidermal growth factor receptor family (erbB1-4) play a significant role in modulating the cellular response to ionizing radiation and a positive correlation has been found between *EGFR* expression and increased tumor chemo and radioresistance. (Akimoto *et al.,* 1999; Barker *et al.,* 2001; Ang *et al.,* 2002). Therefore several biological and pharmacological approaches for targeting of the receptor have been introduced. For this purpose the following four strategies are at different stages of development (El-Rayes and LoRusso, 2004):

1. Monoclonal antibodies (MAbs) against the EGFR
2. Inhibition of receptor tyrosine kinase (RTK) activity
3. Inhibition of receptor trafficking to the cell membrane
4. Inhibition of receptor synthesis through anti-sense oligonucleotides

Of these approaches, MAbs and RTK inhibitors are in the most advanced stages of clinical development. Nevertheless, the underlying mechanisms of these antagonists are so far not understood.

Furthermore differential classes of erbB1/EGFR inhibitors may have differential potential to improve outcome of radiotherapy in the same tumor model (Krause *et al.,* 2005). The implication of this finding is that different classes of agents may have to be tested separately in individual tumor types. This differential activity profiles could also be an indication that these agents do not have completely overlapping mechanism of action. Some of the known differences between EGFR low molecular weight TK inhibitors and MAbs that could account for the different responses are listed in table 1. (Mendelsohn and Baselga, 2003)

Table 1.1: Advantages and disadvantages of TKIs compared with MAbs

KIs are oral; MAbs require intravenous administration.
KIs are low MW and distribute more rapidly than MAbs.
KIs crossreact with other kinases; MAbs are truly specific.
KIs lack immune function that can be mediated by MAbs.
KIs exhibit dose-limiting systemic toxicity not seen with MAbs.
KIs do not downregulate EGFR, but MAbs do.

Abbreviations: KIs, kinase inhibitors; MAbs, monoclonal antibodies; MW, molecular weight; EGFR, epidermal growth factor receptor

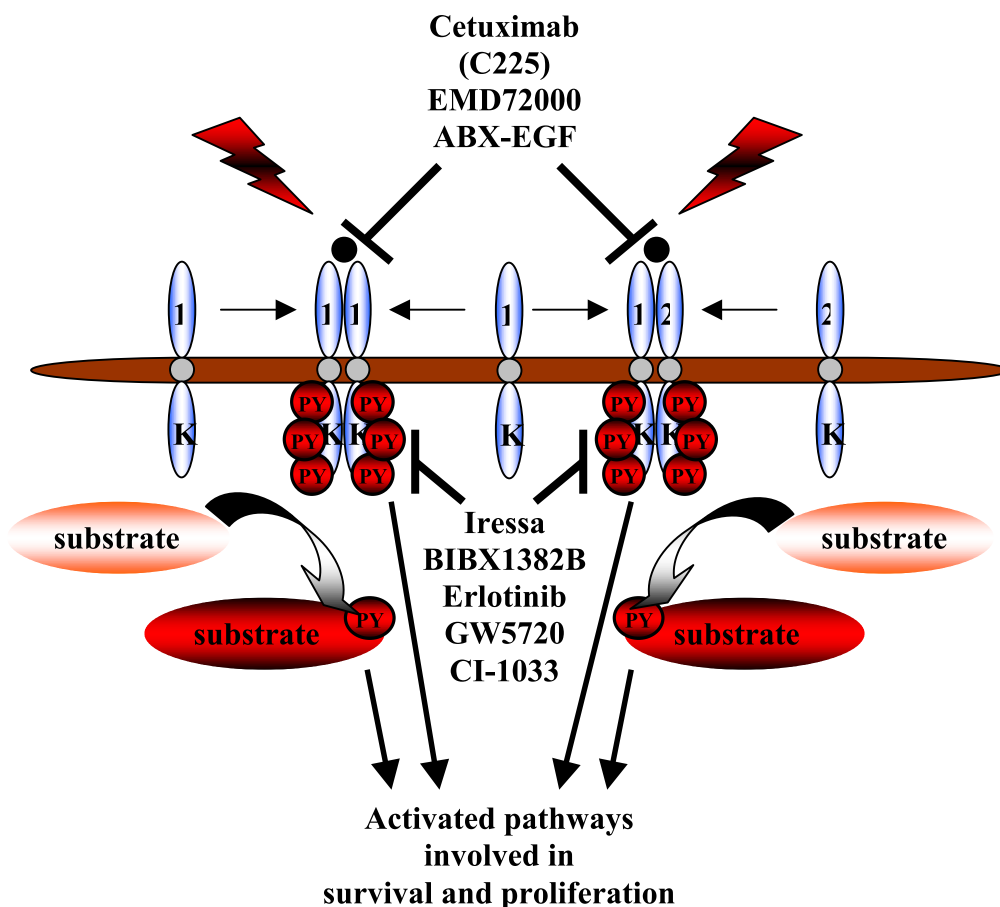


Fig. 1-2: Schematic illustration of EGFR mediated signal transduction and inhibitory action of antagonists. Activation of EGFR by its ligands or IR results in a homodimerization of two EGF recepors or heterodimerization with another member of the erbB family, followed by autophosphorylation in different tyrosin residues. This induces amplification and diversification of signal and activation of pathways involved in proliferation, growth and survival. Different classes of EGFR inhibitors block the activation of the receptor.

Targeting of erbB2 is an example indicating the importance of EGFR dimerization in cellular signaling. As shown in Fig.1-2, EGFR stimulation with either ligands or IR induces formation of receptor homodimerization and erbB2 containing heterodimerization (Olayioye *et al.,* 2000). In spite of overexpression of erbB2 in a significant proportion of tumors, *i.e.* breast cancers (Pauleti *et al.,* 2000), no soluble ligand has been identified for this receptor. Nevertheless erbB2 neutralizing antibody, trastuzumab, has been introduced as potential radiosensitizer and tumor suppressor in mammary carcinomas (Liang *et al.,* 2003; Matsuni *et al.,* 2005). Heterodimerization of EGFR with erbB2 may explain the antitumor effects of trastuzumab. In tumors overexpressing erbB2, heterodimerization of EGFR transactivates erbB2 and induces downstream signal transduction pathways. Consequently, antibodies against erbB2, like trastuzumab, block heterodimerization and affect tumor proliferation and survival. The studies indicating simultaneous phosphorylation of erbB2 and EGFR in metastatic breast cancer (Gschwantler-Kaulich *et al.,* 2005) as well as co-overexoression of EGFR with erbB2 (DiGiovanna *et al.*, 1998) confirm this idea.

1.3.1 EGFR neutralizing antibodies

MAbs recognize the extracellular domain of EGFR and compete with ligands in binding to the receptor. Binding of antibody to the receptor with more affinity in comparison to receptor ligands induces dimerization and internalization of the receptor but inhibits ligands stimulated TK activity and down stream signaling pathways. Among the various monoclonal antibodies against EGFR, C225 (Cetuximab) and EMD72000 are best investigated and in clinical trails. Inhibition of cell cycle progression, angiogenesis, metastasis, DNA repair and enhancement of apoptosis as the mechanisms of action of C225 on proliferation and survival have been proposed (Perrotte *et al.,* 1999; Bonner *et al.,* 2000; Mendelsohn 2001). Recently published data from our laboratory showed that C225 blocks IR induced EGFR import to the nucleus and subsequently inhibits DNA-PKcs activity which is accompanying with inhibition of DNA repair and radiosensitization (Dittmann *et al.,* 2005a; Dittmann *et al.,* 2005b).

Results from peclinical *in vitro* and *in vivo* studies with C225 as single therapy or in combination with radiotherapy are promising (Huang *et al.,* 1999; Saleh *et al.,* 1999; Milas *et al.,* 2000; Bianco *et al.,* 2000; Huang and Harari, 2000; Bonner *et al.,* 2000; Milas *et al.,* 2004). Likewise clinical trials of this antibody in combination with radio/chemotherapy in different tumors, *i.e.* NSCLC and SCC of head and neck have been performed (Robert *et al.,* 2001; Gatzemeier *et al.,* 2003; Bonner *et al.,* 2004). Although some of these trials have shown encouraging results (cited above), conflicting data has been reported as well (Raben *et al.,*

2005). In addition to the conflict data, mechanism of antitumor activity of this antibody is tumor cells like gliomas (Eller *et al.,* 2005) harboring EGFR mutation and lacking ligand binding domain is not clear. Therefore more investigations for understanding the mechanism of action of these antibodies are necessary.

1.3.2 EGFR tyrosine kinase inhibitors

Another approach to inhibit EGFR makes use of small molecules designed to inhibit EGFR TK activity and/or critical downstream signaling components. Hypothetically, this approach could inhibit signaling mediated by ligands as well as signaling that is ligand independent. These inhibitors appear to block TK activity by occupying the ATP binding sites on the intra-cellular domain of receptor. Four different classes of RTK inhibitors have been identified and these include: (1) reversible EGFR inhibitors (*e.g.* Gefitinib and erlotinib), (2) irreversible EGFR inhibitors (*e.g.* EKB-569), (3) reversible dual-erbB inhibitors (*e.g.* GW572016), (4) irreversible pan-erbB inhibitors (*e.g.* CI-1033) (El-Rayes and LoRusso, 2004). The 80% homology between EGFR and erbB2 has allowed the generation of receptor TK inhibitors GW572016 and CI-1033 which inhibit enzymatic activity of EGFR and erbB2 receptors (Rowinsky, 2004). The rational for introducing dual-erbB inhibitors is the hetrodimerization of EGFR and erbB2. Since both receptors are simultaneously overexpressed, therefore targeting both at the same time by one inhibitor or combination of specific inhibitors for each receptor might increase the treatment efficiency. Chemically different TK inhibitors are derivatives of anilinoquinazoline. Gefitinib (Iressa) and erlotinib (OSI-74) are already in different stages of clinical trials. The association between Gefitinib and IR has resulted in cytotoxic enhancement shown both *in vitro* and *in vivo* in different tumors, *e.g.* head and neck (Huang *et al.,* 2002; Magne *et al.,* 2002) colon, ovary, NSCLC, and breast origins (Bianco *et al.,* 2002; Solomon *et al.,* 2003)

However, as discussed for anti-EGFR monoclonal antibodies, for RTK inhibitors in spite of promising preclinical studies as monotherapic agents or in combination with radio/chemotherapy (Sirotnak *et al.,* 2000) , several conflicting data has also been reported (Baumann *et al.,* 2003; Giocanti *et al.,* 2004). As an example; in contrast to the report by Solomon *et al.* indicating enhanced antitumor effect of single and multiple fractions of radiation in combination with Gefitinib in A431 cells, the investigation by Giocanti *et al.* showed that this inhibitor in the same cell line as well as in A549 and Hela cells does not show any radiosensitizing effect (Giocanti *et al.,* 2004). In addition, using a tumor xenograft model in nude mice, *i.e.* the human head and neck tumor cell line FaDu, conflicting results of

the effect of combined radiotherapy and EGFR antagonism have been reported by Baumann *et al.* (Baumann *et al.,* 2003). In these animal experiments no beneficial effect of blocking EGFR-tyrosine kinase activity by the small molecule BIBX1382BS in combination with fractionated radiotherapy using local tumor control as experimental endpoint was observed (Baumann *et al.,* 2003; Krause *et al.,* 2004), although the inhibitor significantly affected cell proliferation.

Therefore in spite of many clinical trials as cited above with different EGFR tyrosine kinase inhibitors to improve local tumor control and the heterogeneous data observed so far, it is necessary to investigate the molecular prerequisites for effective treatment outcomes. Thus, the specific pathways attacked by the antagonists used and the search for accompanying mutations in related pathways which can modify the efficacy of the targeting approach need to be investigated in more detail.

1.3.2.1 BIBX1382BS as an EGFR tyrosine kinase inhibitor

BIBX1382BS *(*M_W 397.87 pyrimido[5,4-D]- pyrimidine-2,8-diamine,*N*8-(3-chloro-4-fluorophenyl)-*N*2- (1-methyl-4-piperidinyl)-2 as a specific EGFR TK inhibitor and new anticancer agent with following chemical structure (Fig. 1-3) was synthesized by Boehringer Ingelhein Austeria (Vienna, Austria) (Nuijen *et al.,* 2000).

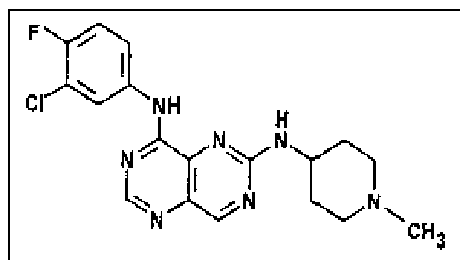


Fig. 1-3: Chemical Structure of EGFR TK inhibitor BIBX1382BS

BIBX1382BS exerts its activity by a potent and selective inhibition of human EGFR tyrosine kinase activity. As the antitumor activity of this drug most likely depends on a continuous inhibition of EGFR function, oral administration is aimed as the primary route of administration (Nuijen *et al.,* 2000). The antitumor activity of BIBX1382BS has been evaluated in murine tumor models and in human cell lines both *in vitro* and *in vivo*. Various degrees of antitumor effect of this inhibitor ranging from a reduced growth rate to complete growth suppression has been detected at the human EGFR-expressing head and neck SCC cell lines, HN5 FaDu, HN15 and HNX-OE in subcutaneous mouse tumor models. In EGFR over expressing established subcutaneous human SCC A431, BIBX1382BS caused full growth

suppression during a 3 weeks treatment period with dosage decreasing to 10 mg/kg/day. However in EGFR expressing KB cell line no anti-tumor effect of the inhibitor has been reported (Dittrich *et al.,* 2002).

Objectives of the study:

Based on different investigations into the antitumor potential of BIBX1382BS alone or in combination with irradiation (Nuijen *et al.,* 2000; Dittrich *et al.,* 2002; Baumann *et al.,* 2003; Krause *et al.,* 2004; Krause *et al.,* 2004) the present investigation was focused on the following objectives:

1. Does inhibition of EGFR TK activity by BIBX1382BS modulate proliferation and radiosensitivity of human tumor cells?
2. What is the EGFR dependent mechanism of radioresistance in K-*RAS* mutated human tumor cells?
3. Which EGFR downstream pathway is modulated and mediates the potential radiosensitizing effect of BIBX1382BS?

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Laboratory chemical and biochemicals

Acetic acid	Merk
Acetone	Merk
Acrylamide	Roth
Agarose	Sigma
APS	Aldrich
β-Glycerol phosphate	Sigma
β-Mercaptoethanol	Sigma
β-Propanol	Merk
Bromphenol blue	Pharmacia Biotech
BSA	Roth
Coomassie® Brilliant Blue G 250	Serva
Coomassie® Brilliant Blue R 250	Serva
Cytochalasin-B	Sigma
DAPI	Serva
DMSO	Sigma
DTT	Sigma
EDTA	Sigma
Ethanol	Merk
Ethidium bromide	Roth
Formaldhyde	Merk
Giemssa	Merk
HCL	Roth
HEPES	Sigma
Kaleidscope prestained standards	Bio-Rad
KCl	Merk
Lipofectamine™ 2000	Invitrogen
Mowiol	Sigma
NP-40	Sigma
Penicilin-Streptomycin	Gibco
Ponceau S	Sigma
Scintillation cocktail	Roth
SDS	Serva
Sodium chloride	Merk
Sodium fluride	Sigma
Sodium orthovanadate	Sigma
TCA	Fluka
TEMED	Sigma
TRIZMA-Base	Sigma
TRIZMA-HCL	Sigma
Trypsin	Serva
Tween 20	Roth

2.1.2 Radiochemicals

[γ –^{32}P]ATP	6000 Ci/mmol	Amersham Biosciences
methyl-^{3}H- Thymidine	49 Ci/mmol	Amersham Biosciences

2.1.3 Kits and other materials

Cell culture materials	Falcon, UK/ Greiner, Solingen, Germany
ECL detection kit	Amersham Pharmacia Biotech, UK
First strand cDNA Synthesis kit	Roche, Mannheim, Germany
Gel-blotting paper (Watmann)	Schleicher & Schüll, Dassel, Germany
LightCycler SYBR Green I kit	Roche, Mannheim, Germany
NucleoSpin RNA isolation kit	Macherey-Nagel, Dueren, Germany
Phosphatase inhibitor cocktail 1	Sigma, Taufkirchen, Germany
Protease inhibitor	Roche, Manheim, Germany
Protein A sepharose	Amersham Biosciences, Freiburg, Germany
Protein assay kit	Bio-Rad, Munich, Germany
PVDF 0.20 μm membrane	Schleicher & Schüll, Dassel, Germany
Ras-activation-assay-Kit	Upstate Biotechnology, USA
Sterile filter 0.20 μM and 0.45 μM	Sartorious, Hannover, Germany
X-ray film	Agfa-Gevaert, Belgium

2.1.4 Instruments

β-counter	Wallac, Freiburg, Germany
Cell Analyser System (CASY 1)	Schärfe Systems, Reutlingen, Germany
Centrifuges	Eppendorf, Germany
Electrophoresis Units	Hoefer, USA
ELIZA reader	Anthos Labtec, Salzberg, Austria
LightCycler	Roche, Mahnheim, Germany
Microscopes	Zeiss, Germany
PCR	Applied Biosystems, USA
X-ray machine (RS-225)	Gulmay, England

2.1.5 Stock solutions for buffers

Blotting buffer (anode)	3.1 g	Boric acid
	4 ml	SDS 10 %
	200 ml	Methanol
	ad l Lit	H_2O_{dd}
		pH 9.0 (NaOH)
Blotting buffer (cathode)	3.1 g	Boric acid
	4 ml	SDS 10 %
	50 ml	Methanol
	ad l Lit	H_2O_{dd}
		pH 9.0 (NaOH)

Cell lysis buffer	50	mM	Tris-HCL pH 7.5
	50	mM	Glycerophosphate
	150	mM	NaCl
	1	mM	NaF
	1	mM	DTT
	1	mM	$NaVO_4$
	10	%	Glycerol
	1	%	Tween 20
	1	%	Phosphatase inhibitor
	1	Tab/10ml	Protease inhibitor
Coomassie[R] blue destaining solution	10	%	Methanol
	5	%	Acetic acid
Coomassie[R] blue staining solution	50	%	Methanol
	10	%	Acetic acid
	0.25	%	Coomassie[R] Blue
DNA loading buffer	0.25	%	Bromphenol blue
	0.25	%	Xylencyanol
	30	%	Glycerol
	100	mM	EDTA
			pH 8.0
MLB	25	mM	HEPES pH 7.5
	150	mM	NaCl
	1	mM	$NaVO_4$
	25	mM	NaF
	10	mM	$MgCl_2$
	1	mM	EDTA
	0.25	%	Sodium deoxycholate
	10	%	Glycerol
	1	%	NP-40
	1	%	Phosphatase inhibitor
	1	Tab/10ml	Protease inhibitor
PBS	13.7	mM	NaCl
	2.7	mM	KCl
	80.9	mM	Na_2HPO_4
	1.5	mM	KH_2PO_4
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_2O_{dd}
Running buffer (5x)	72.05	g	Glycin
	15.15	g	Tris-Base

	5	g	SDS
	ad 1	lit.	H_2O_{dd}
			pH ≈ 8,6
Separating gel puffer (4x)	18,17	g	Tris-Base
	4	ml	SDS (10 %)
	ad 100	ml	H_2O_{dd}
			pH 8.8 (12N HCL)
Stacking gel puffer (4x)	18.17	g	Tris-Base
	4	ml	SDS (10 %)
	ad 100	ml	H_2O_{dd}
			pH 6.8 (12N HCL)
Strip buffer	62.5	mM	Tris-HCL pH 6.8
	2.0	%	SDS
	100	mM	β-Mercaptoethanol
TBST	10	mM	Tris-HCL pH 7.5 (NaOH)
	100	mM	NaCl
	0.1	%	Tween 20

2.1.6 Growth factors and inhibitors

To access the specific activities of EGFR dependent signal transduction pathways the following EGFR specific ligands at concentrations indicated were used:

AREG	10-100 ng/ml	Sigma, Taufkirchen, Germany
EGF	100 ng/ml	Sigma, Taufkirchen, Germany
TGFα	10-100 ng/ml	Sigma, Taufkirchen, Germany

In addition, to investigate the effect of IR or ligands induced EGFR autophosphorylation on activation of downstream signal transduction pathways the following inhibitors at the concentrations indicated were used:

BIBX1382BS	5 µM	EGFR TK inhibitor	Boehringer Ingelhein, Austria
LY294002	10 µM	PI3K inhibitor	Calbiochem, Schwalbach, Germany
Maumycin A	5 µM	Farnesyl Transferase Inhibitor (FTI)	Calbiochem, Schwalbach, Germany
PD153035	500 pM	EGFR TK inhibitor	Calbiochem, Schwalbach, Germany
PD98059	20 µM	MEK inhibitor	Calbiochem, Schwalbach, Germany
PP2	10-40 µM	Src inhibitor	Calbiochem, Schwalbach, Germany

With respect to EGFR -TK inhibitor BIBX1382BS specifically under study herein, it has been shown that 3 µM BIBX1382BS efficiently in EGFR positive breast cancer cell line T47D inhibits transforming growth factor alpha-stimulated phosphorylation of ErbB1. 10 µM of the inhibitor was necessary to inhibit ErbB2 activity stimulated by agonist antibodies (Egeblad *et al.,* 2001). Based on these results and pilot experiments testing the effect of BIBX1382BS in different concentrations on EGF induced EGF receptor autophosphorylation, BIBX1382BS in the present study at concentration of 5 µM was used.

2.1.7 Small interfering RNA (siRNA)

The smart pool siRNAs against K-*RAS* and H-*RAS* as well as control siRNA were prepared by Dharmacon (Chicago-USA).

2.1.8 Antibodies

The following antibodies were used in Western/immunoblot and immuoprecipitation experiments.

Primary antibody	Description	Origin
Actin	Rabbit, monoclonal	Sigma
AKT	Mouse, monoclonal	BD, Transduction Laboratories
DNA-PKcs	Mouse monoclonal	BD, Pharmingen
EGFR	Mouse, monoclonal	BD, Transduction Laboratories
ERK1/2	Rabbit, polyylonal	Promega
Grb2	Mouse, monoclonal	BD, Transduction Laboratories
H-Ras	Mouse, monoclonal	Santa Cruz
K-Ras	Mouse, monoclonal	Sigma
P-AKT/PKB	Mouse, monoclonal (Ser-472/3)	BD Pharmingen
Pan-Ras	Mouse, monoclonal H, K and N-Ras	Santa Cruz
P-DNA-PKcs	Rabbit, monoclonal (Thr-2609)	Rockland
P-ERK1/2	Rabbit, polyclonal (Thr-202/Tyr-204) MAPK	Promega
P-H2AX	Mouse, monoclonal phospho-H2AX (Ser139)	Upstate
P-Tyr (PY20)	Mouse, monoclonal	BD, Transduction Laboratories
SHC	Mouse, polyclonal	BD, Transduction Laboratories
STAT1	Rabbit, polyclonal	BD, Transduction Laboratories
STAT3	Mouse, monoclonal	BD, Transduction Laboratories

Secondary antibody	concentration	Origin
Donkey anti-Rabbit	1:2000-1:5000	Amersham Pharmacia Biotech
Sheep anti-mouse	1:2000-1:10000	Amersham Pharmacia Biotech

2.1.9 Neutralizing antibodies

The following neutralizing antibodies directed against EGFR ligands were used at the concentration of 10 µg/ml 2 h before stimulation.

AREG neutralizing antibody	R&D, Nordenstadt, Germany
TGFα neutralizing antibody (Ab-3)	CalBiochem, Schwalbach, Germany

2.1.10 Cell culture media

Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 mg/ml glucose from Gibco supplemented with 44.04 mM $NaHCO_3$.

Minimum Essential Medium (MEM) containing L-glutamine from Biochrom, supplemented with 26.2 mM $NaHCO_3$.

RPMI-1640 containing L-glutamine from Gibco, supplemented with 26.8 mM $NaHCO_3$.

For cryoprotection of cells DMEM, MEM or RPMI-1640 supplemented with 20% FCS containing 5% DMSO as cryoconservative was used.

2.1.11 Cell lines

The following established human cell lines of different tumor entities and normal as well as transformed human skin fibroblast were used.

Cell line	Description	Origin/ Reference
A549	Human non-small cell carcinoma of lung	ATCC, CCL-185
MDA-MB-231	Human squamous cell carcinoma of breast	ATCC, HTB-26
PC-3	Human squamous cell carcinoma of prostate	ATCC, CRL-1435
FaDu	Human squamous cell carcinoma of pharynx	ATCC, HTB-43
SiHa	Human squamous cell carcinoma of cervix	ATCC, HTB-35
HH4-ded	Transformed human skin fibroblast	Dittmann *et al.,* 1998
HSF-7	Normal human skin fibroblast	Gueven *et al.,* 2001

2.2 Methods

2.2.1 General cell culture techniques

All cell lines were grown in a humidified 93% air, 7% CO_2 incubators (Heraeus, Binder) and routinely assayed for mycoplasma contamination using DAPI staining. A549, MDA-MB-231, SiHa, HSF-7 and HH4-ded were cultured in DMEM. FaDu and PC-3 were cultured in MEM and RPMI-1640 respectively. Media were supplemented with 10% FCS.

2.2.2 Inhibitors treatment

BIBX1382BS, PD153035, PD98059, PP2, LY294002 and Manumycin were dissolved in DMSO, stock concentrations of 10 mM and stored at -70°C. For treatment, stock solution of inhibitors were first 100 times in serum-free medium diluted and administrated to cell cultures. Control cultures received appropriate medium containing DMSO at the concentration of 0.1 %. For combined treatment with IR and growth factors cells were pre-incubated for 30 min prior to corresponding stimulation.

2.2.3 Irradiation

Cell cultures were irradiated at room temperature using a Gulmay RS-225 X-ray generator operating at 200 kVp and 15 mA at a dose rate of 3 Gy/min.

2.2.4 Proliferation assay

Cell proliferation was examined over a growth period of 5 days. 24 hours after seeding, cell cultures were treated or not with BIBX1382BS and incubated for appropriate time intervals. Thereafter cells were trypsinized and counted with Cell Analyser System (CASY 1).

2.2.5 Clonogenic assay

To analyze clonogenic cell survival, cells were plated at a density of 500 cells per 100 mm culture dish with or without further treatments. Ten days later culture dishes were stained (Coomassie). Colonies with more than 50 cells were counted and survival fractions were calculated on the basis of colony counts and calculation of plating efficiency.

2.2.6 Flow cytometry

Cells were seeded, grown for 48 h and serum starved. 24 h after starvation cells were pretreated with BIBX1382BS for 30 min and irradiated. Immediately after IR medium was changed with fresh medium containing 10% FCS and 5 μM BIBX1382BS. After 24 h cells were trypsinised, counted, washed with PBS, resuspended in 70% ethanol and stored in –20°C until cell cycle analysis. Cell cycle analysis and determination of the proportion of cells in subG1 (apoptotic cells) was performed according to standard protocol (Ohneseit *et al.,* 2005).

2.2.7 Protein analysis methods

2.2.7.1 Lysis of cells

Prior to lysis, cell grown to 80-90% confluence were pretreated with antagonists and/or agonists as indicated in figure legends. At indicated time points after stimulation cells were washed two times with ice-cold PBS and lysed with lysis buffer. Lysates were precleared by centrifugation at 14000 rpm for 15 min at 4°C.

2.2.7.2 Protein quantification

For protein quantification the Bio-Rad protein assay was used according to manufactures recommendations. BSA was used as standard.

2.2.7.3 Immunoprecipitation and Western blotting

For immunoprecipitations, 2 mg whole lysate was incubated with antibody against protein of interest and 50 µl of 50% protein A-sepharose overnight at 4 °C. Precipitates were washed five times with 200 µl lysis buffer, suspended in SDS sample buffer, boiled for 5 min and subjected to SDS-PAGE.

In Western blotting analysis without immunoprecipitation, 100-200 µg of whole lysates were directly subjected to SDS-PAGE

2.2.7.4 SDS-polyacrylamide-gelelectrophoresis (SDS-PAGE)

SDS-PAGE was conducted as described previously (Sambrook, 1990)

2.2.7.5 Transfer of proteins on nitocellulose membrane

For immunoblot analysis proteins were transferred to nitrocellulose membrane for 2 -3 hours at 0.8 mA/ cm^2 of gel using a semidry blotting device. Following blotting proteins were stained with Ponceau S (0.1% in 5% acetic acid) in order to visualize and mark standard protein bands. The membrane was destained in water.

2.2.7.6 Immunoblott detection

After electroblotting the transferred proteins are bound to the surface of the nitrocellulose membrane, providing access for reaction with immunodetection reagents. Remaining binding sites were blocked by 3% BSA or 5% nonfat dry milk for 1 h at RT. Thereafter, membranes were probed with primary antibody overnight and followed by 1 h (6x10 min) washing with TBS-T. The membranes were incubated with secondary antibody and washed again as before. Antibody-antigen complexes were identified using Horseradish Peroxidase (HRP) coupled to secondary anti-IgG antibody. Luminescent substrates were used to visualize peroxidase activity. For detection of signals, X-ray films were used. To confirm loading control after detecting of activated protein, membranes were stripped for 30 min at 50°C, blocked and re-probed with different antibodies against whole protein or actin.

2.2.7.7 Ras Activation Assay

Ras-Activation-Assay-Kit was purchased from Upstate Biotechnology. Ras activity was determined according to the supplier's instruction. Cells were lysed with MLB. 1 mg of cell lysate was precleared with glutathione agarose. 10 µl of a 50% slurry of Raf-1 RBD (Ras Binding Domain)-agarose was incubated with the lysate at 4 °C for 30 min. The agarose was collected by micro-centrifugation and washed with MLB three times and boiled in 20 µl of SDS sample loading buffer. GTP-bound Ras protein was subjected to SDS-PAGE, blotted with 5% nonfat dried milk and detected with pan-Ras antibody.

2.2.8 Radioactive assays

2.2.8.1 EGFR kinase assay

Precleared lysates were immunoprecipitated by means of anti-EGFR antibody. As described (Gueven *et al.*, 1998) immunoprecipitations were washed two times with lysis buffer, one time with washing buffer (100 mM Tris PH 7.5, 0.5 M LiCl) and two times with kinase buffer (10 Mm Tris PH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5 Mm DTT). The Kinase reaction was started by by addition of 20 µl kinase buffer containing 1 µM ^{32}P-ATP to the precipitate and

incubated for 30 min at 30 °C. The kinase reaction was stopped by adding 20 µl of SDS sample buffer. Electrophoresis of the samples was performed by 6% SDS-PAGE. EGFR kinase activity was visualized by autoradiography.

2.2.8.2 DNA-PK activity

The lysates were immunoprecipitated by means of anti-DNA-PK antibody (Dittman *et al.,* 2003). Immunoprecipitations were washed as explained above for EGFR kinase assay. The Kinase reaction was started by addition of 30 µl kinase buffer containing 1 µg GST-$TP53_{1-50}$ peptide as substrate plus 1 µCi γ -^{32}P-ATP to the precipitate and incubated for 30 min at 30 °C. Subsequently reaction was stopped by adding 20 µl of SDS sample buffer. Electrophoresis of the samples was performed by 10% SDS-PAGE. DNA-PK activity was visualized by autoradiography.

2.2.8.3 Incorporation of ^{3}H- Thymidine into DNA

5 x 10^4 cells were seeded per well in 6 well plates. After 24 h cells were washed with PBS and incubated with serum free medium. 24 hours later serum free medium was replaced with CM containing 1 µCi/ml ^{3}H-thymidine as indicated in figure legends for 10 hours. For extraction of ^{3}H-thymidine labeled DNA, medium was aspirated and cells were washed with 1 ml ice cold PBS and incubated with 1 ml of ice cold 5% TCA for 30 min at 4 °C followed by solubilization and neutralization with 0.5 ml of 0.5 N NaOH in 0.5% SDS. 4 ml of scintillation cocktail was added to 400 µl of the solublized cell solution and tritium incorporation was quantified with a β-counter.

2.2.9 RNA extraction and first strand cDNA synthesis

Total RNA was extracted according to the protocol of the NucleoSpin kit and quantified using a UV spectrophotometer ($A_{260\ nm}$ = 1 corresponds to 40 µg/ml RNA). RNA was reversely transcribed using the First Strand cDNA Synthesis kit according to the manufacturers' protocol. In brief, 2 µl (0.8 µg/µl) oligo-p$(dT)_{15}$ primer and 1 µg RNA were mixed and appropriate volume of sterile water was added to a final volume of 10.2 µl. After RNA denaturation (70°C, 10 min) 9.8 µl of mixed buffer [2 µl of 10x reaction buffer (100 mM Tris, 500 mM KCL; pH 8.3), 2 µl of 25 mM $MgCl_2$, 2 µl of 10 mM deoxyribonucleoside triphosphate, 1 µl of 50 units/µl RNase inhibitor and 0.8 µl AMV reverse transcriptase] were added. Samples were incubated in a Perkin Elmer 2400 thermocycler for 10 min at 25°C followed by 60 min at 42°C and finally at 99°C for 5 min. By adding 80 µl of sterile water the volume of cDNA was increased to 100 µl and stored in –20°C.

2.2.10 Real-time RT-PCR

Quantitative real-time RT-PCR with 5 µl of the cDNA was performed on a LightCycler instrument with the LightCycler FastStart DNA Master SYBR Green I quantification kit in a total volume of 20 µl. The following primers were used for *AREG* and *TGFA* as published (Adam *et al.,* 1999):

Primer	**Sequence (5´-3´)**	**Product (bp)**
AREG-forward	AGAGTTGAACAGGTAGTTAAGCCCC	421
AREG-reverse	GTCGAAGTTTCTTTCGTTCCTCAG	
TGFA-forward	TCAGTTCTGCTTCCATGGAACC	317
TGFA-reverse	TTTCTGAGTGGCAGCAAGCG	

Real-time PCR was conducted with the following profile: initial heating to 95°C for 10 min followed by 40 cycles of amplification with denaturation at 95°C, annealing at 63°C (*AREG*)

or 61°C (*TGFA*) for 3 s, extension for 30 s at 72°C. Fluorescence data were collected and mRNA was quantified by using the second derivative maximum method of quantification (lightCycler software version 3.5). A standard melting curve analysis was performed to check the quality of amplification. A standard curve and negative controls were included in each run. The standard curve was composed of serial dilutions of a pool of culture cell lines RNA. To generate the standard curve, calibrators with the following concentration of RNA were used: 250, 50, 25, 5, 2.5 and 0.25 ng/ml. As negative control a sample without RNA was added. For normalization of the data we performed quantitative RT-PCR for *GAPDH* with the following primers: Specific annealing temperature for *GAPDH*-primers was 68°C.

Primer	**Sequence (5´-3´)**	**Product (bp)**
GAPDH-forward	CCAGTGAGCTTCCCGTTCAGC	471
GAPDH-reverse	CCCATCACCATCTTCCAGGAGC	

2.2.11 *RAS* siRNA transfections

Transfection of A549 and MDA-MB-231 cells with K-*RAS* and H-*RAS*-siRNAs was performed using Lipofectamine™ 2000 according to the manufacturer´s protocol. Based on time course pilot studies, suppression of K-Ras and H-Ras proteins was analysed at day 4 after transfection with 50 nM *RAS* siRNA or control siRNA by SDS-PAGE and immunoblotting.

2.2.12 Cytokinesis blocked micronucleus assay

The cells were seeded at a density of $0.2x10^6$ cells in 25 cm^2 tissue culture flasks. After seeding, the medium was changed with fresh medium. 24 hours later cells were pre-treated 30 min before IR with inhibitor or vehicle, irradiated and incubated with cytochalasin-B (2 µg/ml). Thereafter at indicated time point, cells were collected by centrifugation and incubated with a hypotonic solution containing 0.075 M KCl for 4-6 min. After centrifugation at 1000 rpm, cells were fixed in a mixture of methanol and glacial acetic acid (3:1). Cell suspensions were carefully dropped onto cooled slides. Slides were air dried and stained in 4% Giemsa in distilled water and MNi were scored in binucleated cytokinesis-blocked cells.

2.2.13 Statistics and Densitometry

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

Densitometric quantification analyses of immuno-blots were performed with Scion Image computer software.

3 RESULTS

3.1 MODULATION OF PROLIFERATION AND RADIATION SENSITIVITY VIA TARGETING OF EGFR AUTOPHOSPHORYLATION APPLYING SPECIFIC TYROSINE KINASE INHIBITOR BIBX1382BS

3.1.1 Concentration dependent Blockage of EGFR autophosphorylation by BIB1382BS

To evaluate the potential of BIBX1382BS on inhibition of EGFR tyrosine phosphorylation serum-depleted culture of A549 cells were pre-treated with different concentrations (0.5, 1, 2.5 and 5 µM) of the inhibitor 30 minutes before stimulation with 100 ng/ml of EGF as natural ligand of the receptor. Treatment of cells with EGF resulted in an autophosphorylation of EGFR, which was detectable 5 minutes after treatment. BIBX1382BS inhibited autophosphorylation of the receptor in a dose dependent manner. 5 µM of BIBX1382BS reduced P-EGFR below the level of unstimulated control (Fig. 3-1).

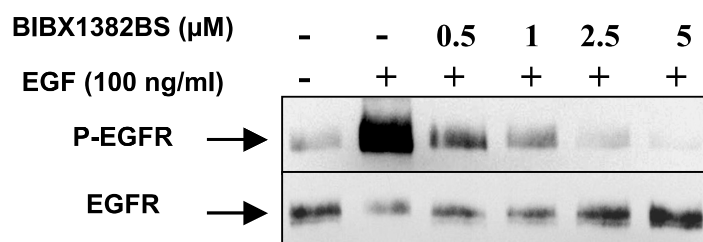


Fig. 3-1: Blockage of EGF-induced autophosphorylation of EGFR. 24 h serum-depleted A549 cells were pre-treated or not with different concentrations of BIBX1382BS as described in *Methods*. Following EGF stimulation, EGFR was immunoprecipitated and autophosphorylation of receptor was analyzed using phosphotyrosin antibody. After detection of P-EGFR, the blot was stripped and re-probed with antibody directed against total EGFR.

3.1.2 Antiproliferative effect of BIBX1382BS

The proliferation activity was evaluated for the cell lines A549 and FaDu following addition of BIBX1382BS (5 µM) to the culture medium of exponentially growing cells. As shown in Fig. 3-2, BIBX1382BS (5 µM) led to a strong antiproliferative effect in FaDu cells, but only to a moderate inhibitory effect in A549 cells.

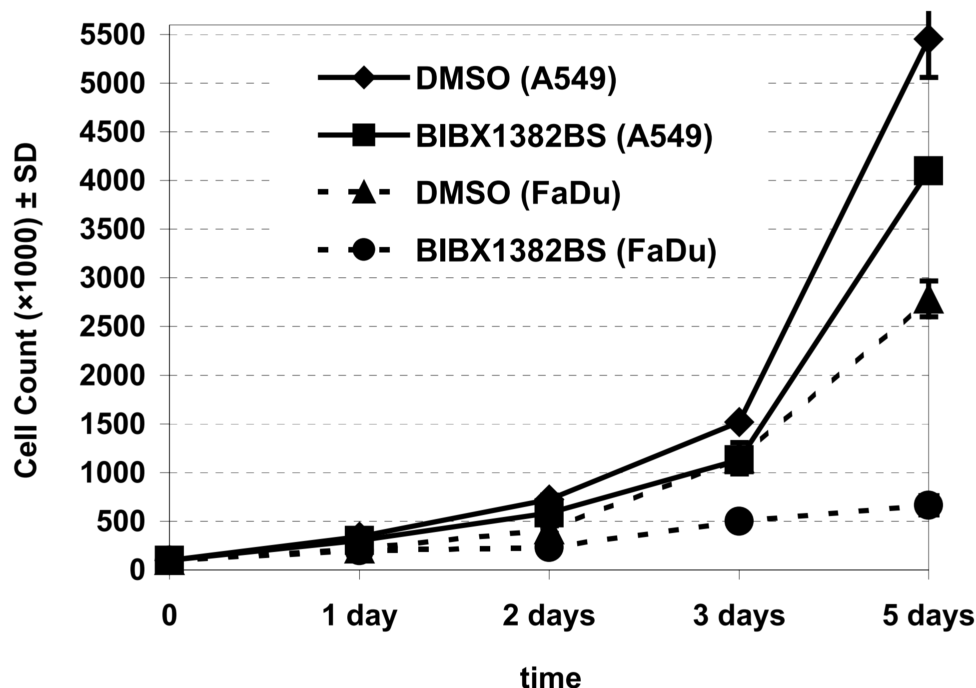


Fig. 3-2: Antiproliferative effect of BIBX1382BS on cell proliferation in FaDu and A549 cells *in vitro*. A549 and FaDu cells were plated in 60 mm culture dishes in DMEM containing 10% FCS. After 24 hours medium was changed and fresh medium supplemented with 5 µM BIBX1382BS or vehicle was added to the culture. After the time periods indicated cell counts were performed applying the CASY 1 system. Data points shown represent the mean ± standard deviation (SD) of four experiments.

Likewise, the antiproliferative effect of BIBX1382BS was evaluated for the human tumor cell lines A549, FaDu, MDA-MB-231 and HTB-35 as well as normal and transformed human skin fibroblast HSF-7 and HH4-ded cells. Cells seeded for colony formation were treated with different concentrations of BIBX1382BS (1, 3 and 5 µM). As shown in Fig. 3-3, 5 µM of EGFR tyrosine kinase inhibitor BIBX1382BS led to differential antiproliferative effect in different cell lines tested. In HSF-7 and HH4-ded BIBX1382BS did not induce any antiproliferative effect while in tumor cells a dose dependent blockage of proliferation by the inhibitor was observed. The strongest inhibitory effect of BIBX1382BS on proliferation was observed in FaDu cells. 1 µM of the inhibitor resulted in an app. 90 % inhibition of clonogenic activity.

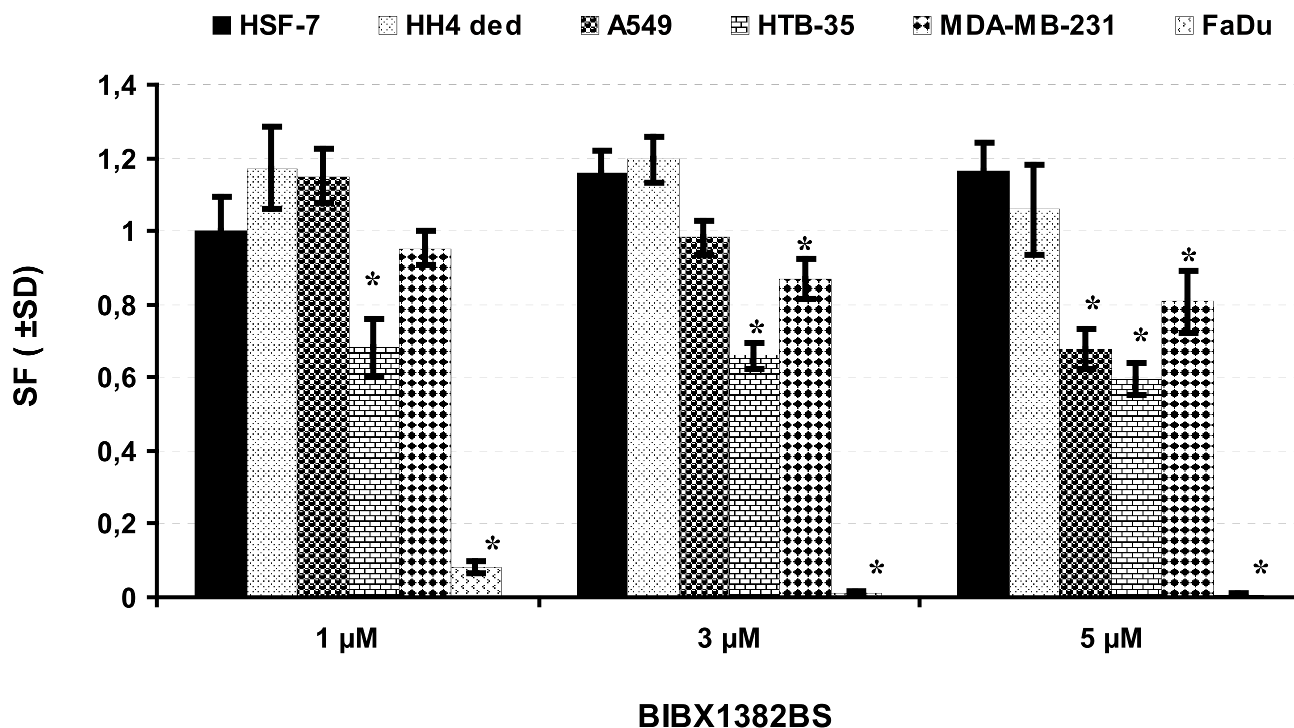


Fig. 3-3: Clonogenic survival of indicated cell lines after treatment with different concentrations of BIBX1382BS. Log phase cells were plated for colony formation. 24 hours after plating cells were treated with different concentrations of BIBX1382BS. Colonies formed within 10 days were stained and counted. Based on the colony counts and plating efficiency the surviving fraction was calculated. Asterisks indicate significant difference (Student's t-test/ $p<0.05$) in proliferation of cells treated the inhibitor in comparison to control cells treated with vehicle.

3.1.3 Modulation of radiation-induced EGF receptor autophosphorylation by BIB1382BS

The ability of ionizing radiation (IR) to activate EGFR via autophosphorylation was investigated in different tumor cells as well as normal and transformed human skin fibroblast 24 hours after serum starvation. A single dose of 2 Gy caused an immediate activation of EGFR in all cell lines tested within 5 min post irradiation. This radiation-dependent activation of the EGFR could be blocked by treatment with BIBX1382BS (5 µM) 30 min prior to radiation exposure (Fig. 3-4).

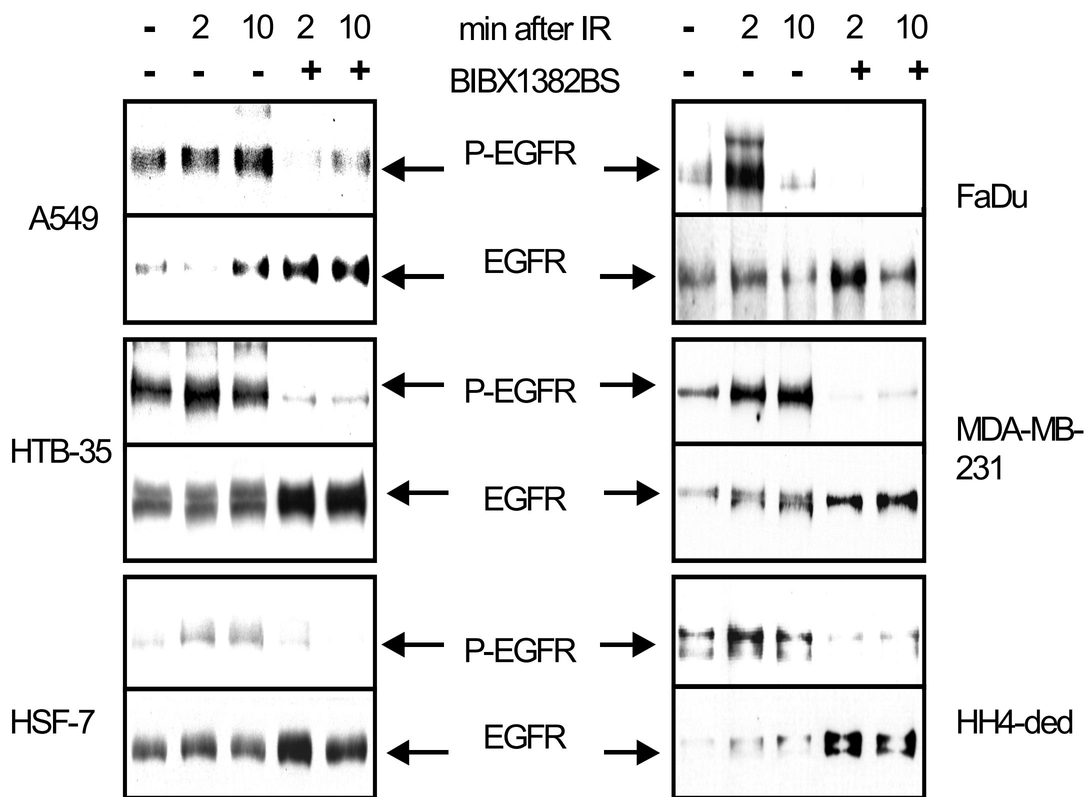


Fig. 3-4: Blockage of radiation-induced EGFR autophosphorylation by BIBX1382BS. 24 h serum-depleted cells were pre-treated or not with BIBX1382BS (5 µM) for 30 min as described in *Methods*. Autophosphorylation of EGFR was analyzed upon stimulation by a single dose of 2 Gy ionizing irradiation at the post-irradiation time points indicated.

3.1.4 Radiosensitizing Effect of BIBX1382BS

Applying panel of different tumor cell lines tested BIBX1382BS modulates toxicity of single and fractionated dose irradiation differentially. Radiation sensitivity was significantly affected only in A549 and MDA-MB-231 cells while the FaDu, HTB-35, HH4-ded. and HSF-7 cells were not sensitized to radiation by this compound. As demonstrated in Fig. 3-5, combined BIBX1382BS treatment and fractionated-dose irradiation (4 x 2 Gy) of A549 cells resulted in a significant radiosensitization with a dose modifying factor (DMF) of 1.72. A less pronounced, but still significant radiosensitizing effect (DMF 1.26) could also be observed for MDA-MB-231 cells (Fig. 3-5).

As shown in Fig. 3-4 the clinically relevant dose of irradiation (2 Gy) generally induces autophosphorylation of EGFR, which is abrogated by pre-treatment with BIBX1382BS. In contrast enhancement of radiation toxicity by this inhibitor is not observed in all cell lines tested. These results clearly indicate that the potential of BIBX1382BS in blockage of radiation induced EGFR phosphorylation is not predicting parameter for the radiosensitization of the inhibitor. Thus modulation of radiation toxicity by BIBX1382BS may most likely depend on specific genotype of cell lines tested.

One of the most important difference between the components of EGFR signal transduction pathways of radiosensitized cell lines (A549, MDA-MB-231) and non-radiosensitized cell lines (FaDu, HTB-35, HH4ded, HSF-7) is K-*RAS* mutation. Oncogenic mutation in codon 12, 13 and 61 of *RAS* gene prevents GTP hydrolysis, resulting in constitutively active Ras protein. Such mutations changing the protooncogene *RAS* to an active oncogene are found in approximately 30% of human tumor cells (Lowy and Willumsen, 1993; Downward, 1998) and may function as therapeutic targets in tumors that harbour these mutations (Adjei, 2001; Eskandarpour *et al.,* 2005). To include more evidences that cell lines presenting K-*RAS* mutation can be radiosensitized by BIBX1382BS, post-irradiation survival of human prostate cancer cell line, PC-3 containing a point mutation of K-*RAS* in codon 12 (Rajesh *et al.,*1999) was analyzed. Data shown in Fig. 3-6 indicates that BIBX1382BS significantly enhances radiation effect in this cell line.

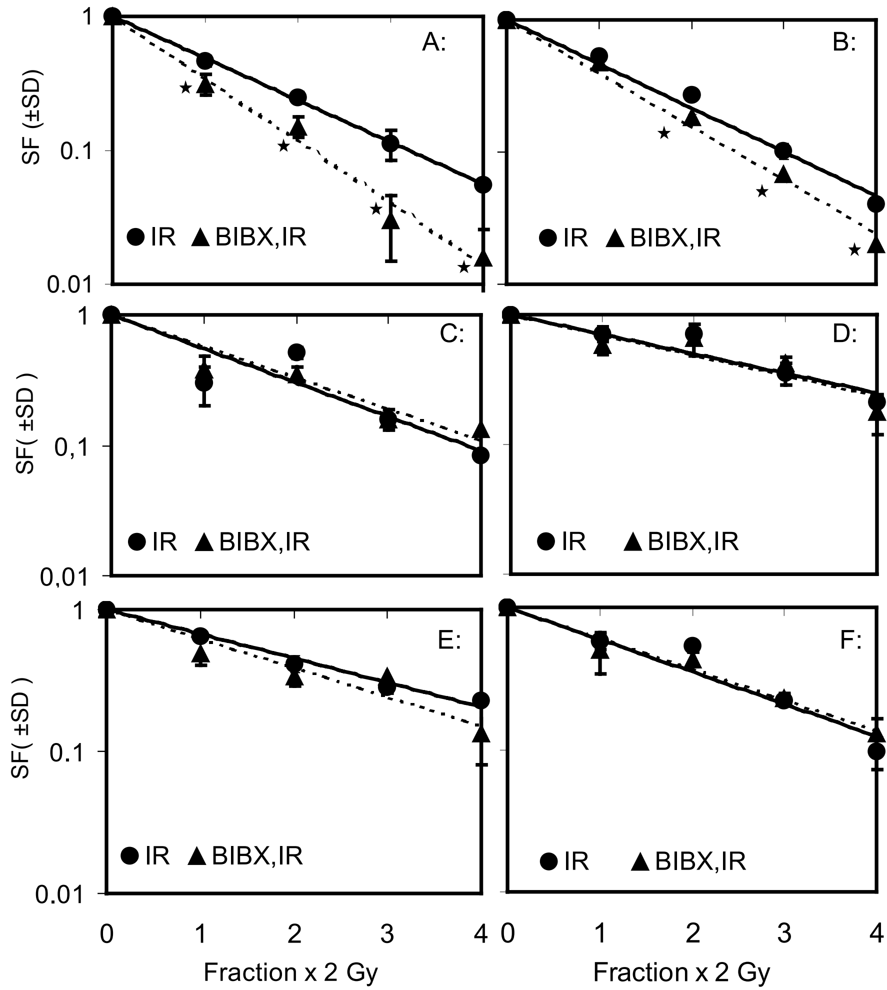


Fig. 3-5: Effect of BIBX1382BS on fractionated radiation response. A: A549 cells; B: MDA-MB-231 cells; C: FaDu cells; D: HTB-35 cells; E: HSF-7 cells; F: HH4ded cells. Semiconfluent cells were exposed to BIBX1382BS 30 min before each fraction of irradiation (2 Gy fractions per day, 4 fractions in total) and plated for colony formation 6 hours after each fractionated irradiation. (•) Surviving fraction of cells exposed to irradiation alone; (▲) surviving fractions of cells exposed to irradiation and BIBX1382BS treatment. Asterisks indicate significant differences (Student's t-test/ $p<0.05$) between irradiated controls and irradiated cells treated with BIBX1382BS

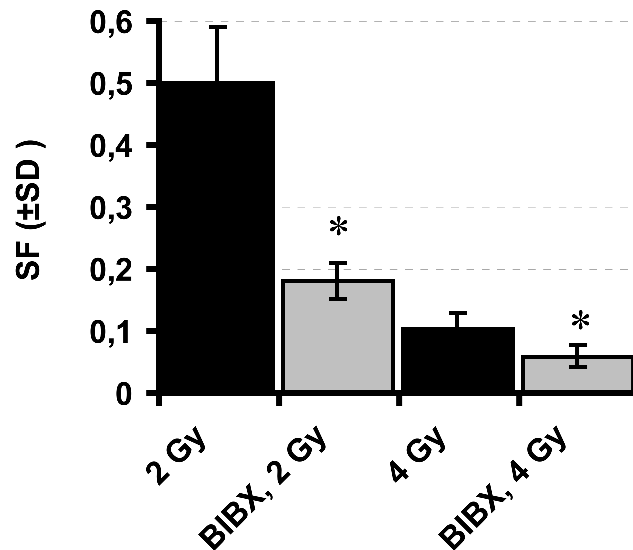
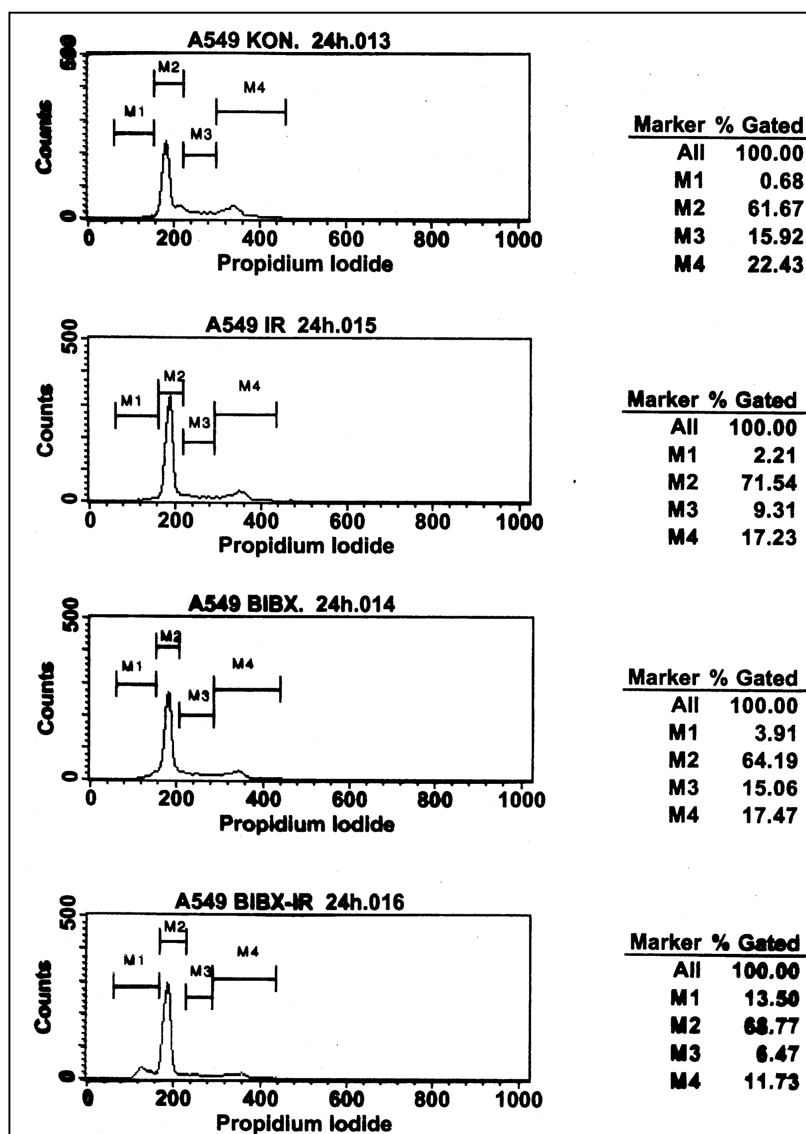


Fig. 3-6: Survival fractions of irradiated PC-3 cells as a function of BIBX1382BS treatment. Log-phase cells PC-3 were irradiated with a single dose of 2 and 4 Gy alone or in combination with BIB1382BS (5 µM). 6 h after irradiation cells were plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SD of 6 parallel experiments. Asterisks indicate significant difference (Student's t-test/ $p<0.05$) between irradiated controls and irradiated cells treated with BIBX1382BS.

This data suggest that blockage of radiation induced EGFR phosphorylation alone by BIBX1382BS does not enhance radiation toxicity and the observed radiosensitizing effect of this inhibitor may depend on presence of additional mutation in K-*RAS* gene.

3.1.5 Effect of BIBX1382BS on cell cycle progression

To investigate the effect of BIBX1382BS on cell cycle progression, flow cytometric analysis following IR and in combination with BIBX1382BS was performed. Therefore, exponentially growing A549 cells were treated with 5 µM BIBX1382BS, 2 Gy IR or combination of BIBX1382BS and IR. As shown in Fig. 3-7 in comparison to non-irradiated control, radiation dose of 2 Gy enhanced the proportion of cells population in G1-phase from 61 to 71 % (G1-arrest). Pre-treatment with BIBX1382BS did not mediate marked changes in cell cycle distribution. As determined by the proportion of cells representing the sub-G1-peak (apoptotic cells) it can be concluded that BIBX1382BS treatment alone induces apoptosis in about 2 % of all cells. Radiation-induced apoptosis is observable in about 4 % of total cells only. Combined treatment with BIBX1382BS and IR resulted in about 13 % of apoptotic cells indicating that apoptosis is not the major cause of radiosensitization by BIBX1382BS.



M1: subG1 M2: G1 M3: S M4: G2-M

Percentage of apoptotic fraction (subG1-cells):

Control	0.68
BIBX	2.21
IR	3.91
BIBX, IR	13.50

Fig. 3-7: Cell cycle distribution of A549 cells after treatment with BIBX1382BS, IR or combined treatment. Exponentially growing cells were treated with BIBX1382BS (5 µM), IR (2 Gy) or in combination and flow cytometric analysis was performed 24 hours after IR.

3.1.6 Pattern of Ras activation following EGF treatment and irradiation

The results demonstrated so far for the tumor cell lines A549, MDA-MB-231 as well as prostate cancer cell line PC-3 revealed that tumor cells presenting a K-*RAS* mutation could be sensitized to radiation by BIBX1382BS whereas tumor cell lines presenting normal *RAS*, like

FaDu, HTB-35, and HH4ded or normal fibroblasts (HSF-7) were not sensitized. Therefore, as an important EGFR-dependent regulator of proliferation, differentiation and cell survival the activity profile of Ras-protein under basal and stimulated (ionizing radiation or EGF) conditions in the presence or absence of BIBX1382BS (5 µM) was tested. As indicated in Fig. 3-8, A549 and MDA-MB-231 cells presenting a K-*RAS*-mutation (Gilhooly and Rose, 1999; Kimura *et al.,* 2004) showed already under basal conditions high level of activated Ras (Ras-GTP). In contrast to these two cell lines, under non-stimulated, basal condition FaDu and HTB-35 cells presented no or only slightly detectable activated Ras. EGF (100 ng/ml) differentially induced Ras-GTP. Activated Ras was moderately induced in irradiated A549 and MDA-MB-231 cells while in FaDu and HTB-35 cells EGF led to a pronounced appearance of Ras-GTP within 5 min (Fig. 3-8).

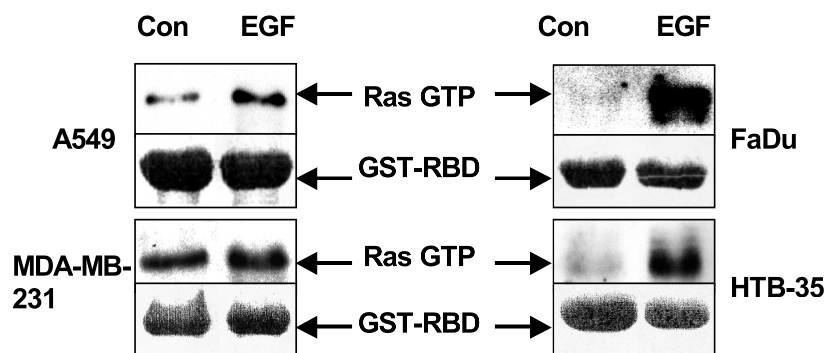


Fig. 3-8: Pattern of Ras activity following EGF treatment in K-*RAS* mutated (A549, MDA-MB-231) and wild type *RAS* (FaDu, HTB-35) cells. 24 h serum-starved cells were treated with EGF (100 ng/ml, 5 min). GTP-bound active $p21^{ras}$ was isolated from lysates by affinity precipitation with a GST-RBD fusion protein followed by immunoblot analysis with anti-$p21^{ras}$ antibody. The fusion protein (42 kDa) was detected by Commassie staining of the polyacrylamide gel.

The different response profile of Ras-GTP in *RAS*-mutated and *RAS* wild type cells is also documented after radiation exposure. Activated Ras was moderately induced in irradiated A549 and MDA-MB-231 cells by a factor of 2.5 and 1.3, respectively. In FaDu-cells not showing any basal Ras-GTP, radiation exposure led to a pronounced appearance of activated Ras within 5 min. Radiation-stimulated Ras-GTP in FaDu cells was completely blocked by pretreatment with BIBX1382BS whereas in A549 and MDA-MB-231 cells it was only reduced slightly below the level of unirradiated controls (Fig. 3-9A). As tested for non-irradiated A549 cells high basal level of Ras-GTP was not modulated by BIB1382BS treatment (Fig. 3-9B). Thus, this data indicate that reduction of radiation-induced Ras-GTP is

rather due to blockage of the EGFR-dependent pathway than to modulation of basal activated Ras.

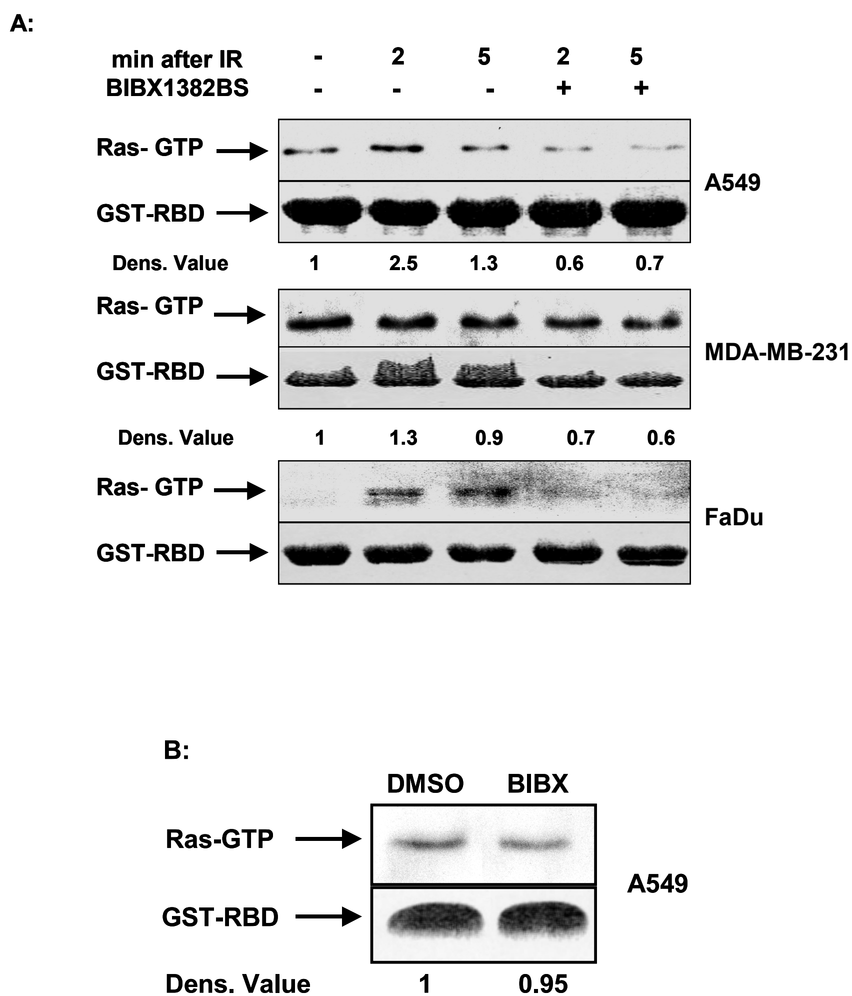


Fig. 3-9: Effect of BIBX1382BS on Ras-GTP in A549, MDA-MB-231 and FaDu cells. A) 24 h serum-starved cells were exposed to ionizing radiation (2 Gy) with and without BIBX1382BS (5 µM). GTP-bound active $p21^{ras}$ and fusion protein (42 kDa) were detected as described in Fig. 3-8. The activity profile for the various treatment conditions were quantitatively analysed by densitometry (NIH image program). On the basis of basal Ras-GTP (set 1.0) densitometry values of Ras-GTP intensities under the different conditions were calculated. Due to absence of Ras-GTP under basal non-irradiated condition, the induction of Ras-GTP in FaDu cells by irradiation could not be quantified by densitometery method applied. B) Same procedure of Ras-GTP detection was done as explained for part A to show whether reduction of Ras-GTP shown in A549 is via reduction of basal level of Ras-GTP or due to blockage of the EGFR dependent pathway.

3.2 MODULATION OF RADIATION INDUCED ACTIVATION OF EGFR DEPENDENT DOWNSTREAM SIGNAL TRANSDUCTION PATHWAYS BY BIBX1382BS

To investigate specifically the molecular differences, which may underlie BIBX1382BS-mediated different radiation responses of *RAS*-mutated and non-*RAS*-mutated tumor cells, this study especially focused on the analysis of signaling cascades down-stream of EGFR mainly in *RAS*-mutated (RAS_{mt}) A549 cells and *RAS* wild type (RAS_{wt}) FaDu cells. As reported, ligand dependent or independent EGFR activation stimulates mainly three downstream pathways such as the Ras-Raf/MAPK pathway, the JAK-STAT pathway and the PI3K-AKT pathway (Olayioye *et al.,* 1999; Contessa *et al.,* 1999; Reardon *et al.,* 1999; Bonner *et al.,* 2000; Hynes *et al.,* 2001; Arteaga, 2002; Dent *et al.,* 2003). Stimulation of these pathways activates specific genes mediating enhanced cell cycle traversal, inhibition of cell death, increased cell adhesion and mobility potential. AKT (Brognard *et al.,* 2001; Tenzer *et al.;* 2001; Fahy *et al.;* 2003) and MAPK/ERKs (Seong *et al.,* 2002) are the best characterized kinases downstream of EGFR known to promote survival signals following radiation exposure. Through stimulation and proliferation via MAPK or inhibition of apoptosis via AKT the importance of these pathways in the regulation of cell survival following radiation exposure has been reported (Liang *et al.,* 2003).

For cell lines tested the addition of EGF (100 ng/ml) to serum-depleted cultures resulted in an autophosphorylation of EGFR and subsequent activation of ERK1/2 (Thr 202/Tyr 204) as well as AKT (Ser-472/3) (Fig. 3-10), which was detectable 5 minutes after treatment. To evaluate the inhibition of EGFR tyrosine phosphorylation by BIBX1382BS, cells were pre-treated with 5µM of this antagonist 30 minutes before stimulation with EGF. As a result in both cell lines autophosphorylation of EGFR as well as phosphorylation of the down-stream components AKT and ERK1/2 was markedly inhibited by BIBX1382BS (Fig .3-10).

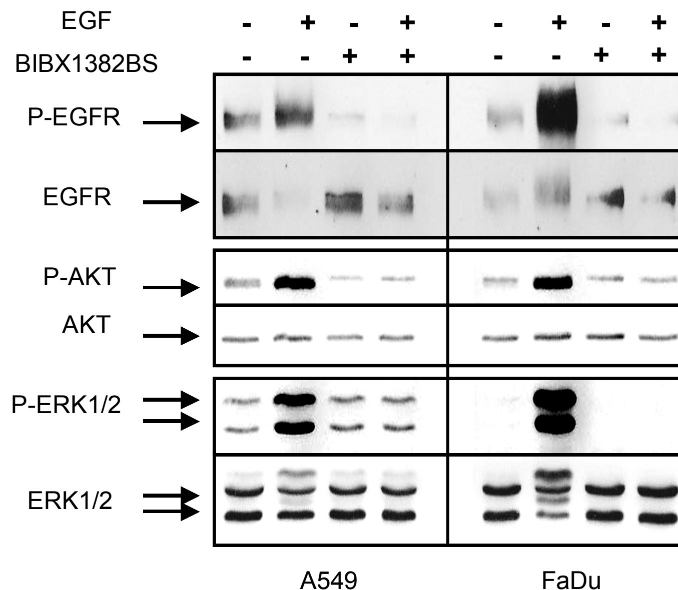


Fig. 3-10: Blockage of EGF-induced autophosphorylation of EGFR and downstream pathways. 24 h serum-depleted cells were pre-treated or not with BIBX1382BS (5 µM) as described in *Methods*. Following EGF stimulation, autophosphorylation of EGFR and the level of ERK1/2 and AKT phosphorylation was analyzed after immunoprecipitation by specific antibodies directed against the phosphorylated proteins. Following detection of P-EGFR, P-ERK1/2 and P-AKT, the blots were stripped and re-probed with antibodies directed against total EGFR, ERK1/2 and AKT proteins to control protein loading.

3.2.1 EGFR/Ras/Raf/MAPK pathway

Ligand dependent or independent activation of EGFR leads to activation of multiple signal transduction pathways including protein kinase cascades. One of this kinase cascades is Ras/Raf/MAPK pathway which plays a role in the regulation of proliferation, differentiation and cell matrix interactions. ERK1 and ERK2 are dually phosphorylated on threonine and tyrosine residues by the upstream MAP kinase, MEK. ERK1/2 then phosphorylate and activate a variety of substrates including transcription factors, protein kinases and phosphotyrosine protein phosphatases leading to positive and negative regulation of signaling cascades (Xing and Imagawa, 1999).

Under normal culture conditions a strong basal level of P-ERK1/2 was apparent in both cell lines (RAS_{mt} A549 and RAS_{wt} FaDu). Upon BIBX1382BS treatment level of P-ERK1/2 was differentially affected in A549 and FaDu cells. After a treatment time of 2.5 h P-ERK1/2 was reduced in A549 cells by about 60-70 % (Fig. 3-11). In contrast, for FaDu cells a complete inhibition of P-ERK1/2 could be observed already after 30 min. Thus, A549 and FaDu cells presented a pronounced difference in the sensitivity of pro-proliferative MAPK pathway to BIBX1382BS treatment.

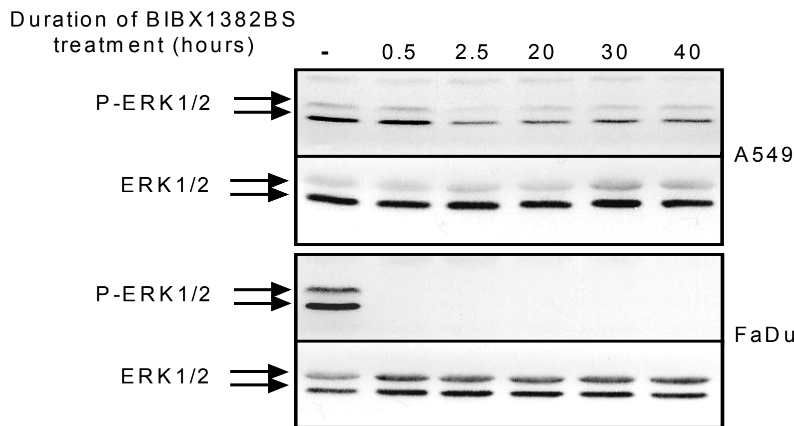


Fig. 3-11: Inhibition of basal ERK1/2 phosphorylation in A549 and FaDu cells as a function of tyrosine kinase inhibitor treatment. Log-phase cells (A549, FaDu) under normal culture conditions were incubated with or without BIBX1382BS (5 µM) for the time intervals indicated. At each time point cells were lysed and subjected to SDS-PAGE and immuno-blotting using a specific antibody for P-ERK1/2 as described in *Methods*. Loading control was performed by means of an antibody directed against total ERK1/2 protein.

Radiation exposure resulted in a phosphorylation of ERK1/2 in both cell lines although at slightly different time kinetics (Fig. 3-12). In FaDu cells an immediate response with a maximum 5 min post-irradiation was apparent and induced P-ERK1/2 activity returned to control levels within 25 min post-irradiation. In A549 cells ERK1/2 phosphorylation reached peak levels at about 15 min and maintained at least until 25 min post irradiation. Pre-treatment with BIBX1382BS for 30 min led to a complete inhibition of ERK1/2 activity in FaDu cells but could not reduce the level of phosphorylated ERK1/2 in A549 cells at time points 15 and 25 min after radiation exposure.

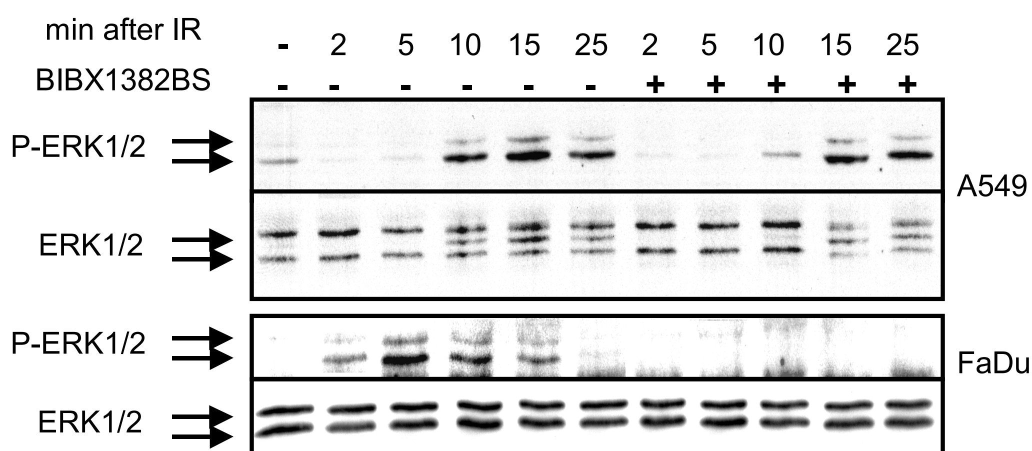


Fig. 3-12: Differential function of tyrosine kinase inhibitor on radiation-induced ERK1/2 phosphorylation in A549 and FaDu cells. 24 h serum-starved cells (A549, FaDu) were incubated with or without BIBX1382BS for 30 min and irradiated with a single dose of 2 Gy. Cells were lysed at the times indicated and subjected to SDS-PAGE and incubated with antibodies against phospho-MAPK. Based on densitometry ratios of P-ERK1/2:ERK1/2 (normalized to 1.0 control) maximum P-ERK1/2 level in A549 cells was apparent at 15 min (3-fold) after radiation exposure.

Inhibition of MAPK/ERKs pathway in modulation of radiation response of tumor cells has been reported in several studies. Some investigations indicate enhancement of radiation response via blockage of ERK1/2 phosphorylation (Carter *et al.,* 1998; Hagan *et al.,* 2000; Seong *et al.,* 2002) while many reports indicate a failure of blockage of this pathway with respect to enhancement of cell killing by ionizing radiation (McKenna *et al.,* 1990; Bernhard *et al.,* 2000; Gupta *et al.,* 2000; Gupta *et al.,* 2001). To determine whether ERK1/2 activation might play a role in radioresistance of A549 cells, a specific ERK1/2 kinase (MEK) inhibitor, PD98059 was used. As compared to the EGFR-antagonist BIBX1382BS the specific MEK-kinase inhibitor PD98059 completely blocked radiation-induced ERK1/2 phosphorylation and activation in A549 cells (Fig. 3-13).

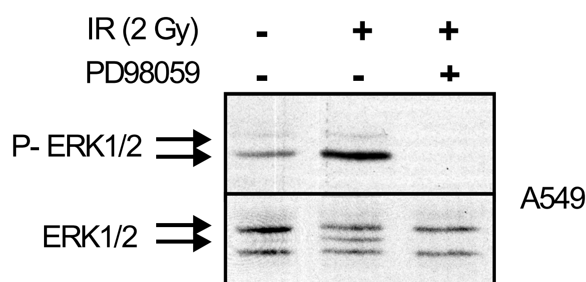


Fig. 3-13: Inhibition of radiation induced ERK1/2 phosphorylation in A549 cells as a function of MEK inhibitor PD98059. 24 h serum-starved A549 cells were incubated with or without PD98059 (20 µM) for 30 min and irradiated with a single dose of 2 Gy. 15 min post-irradiation cells were lysed and subjected to SDS-PAGE and immunoblotting was performed as described above.

However, no effect of PD98059 on radiation induced clonogenic cell death in A549 cells was observed after single dose irradiation (Fig. 3-14). These findings exclude ERK1/2 activation as an important factor involved in radioresistance of A549 cells and subsequent radiosensitization of BIBX1382BS.

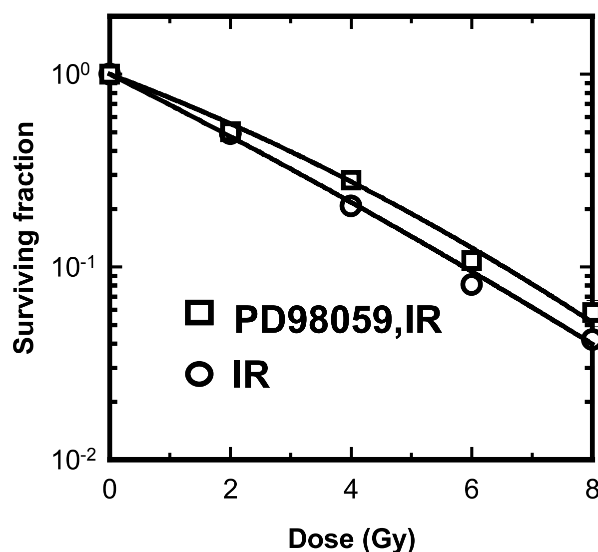


Fig. 3-14: Dose response curve of A549 cells irradiated with single doses IR alone and in combination with MAPK kinase inhibitor PD98059. 24 h serum-starved A549 cells were incubated with PD98059 (20 µM) or equal volume of DMSO (vehicle) for 30 min and irradiated with a single dose of 2 to 8 Gy. 6 h after irradiation cells were plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated.

3.2.2 EGFR dependent and independent activation of ERK1/2 following irradiation

There is accumulating evidence suggesting that time course and intensity of ERK1/2 activation can be profoundly dependent on stimulating upstream agonists (Lee *et al.,* 2000). Recently Audent *et al.* reported that differences in time course of ERK1/2 activation by GPCR ligands depends on stability of receptors in an ERK stimulating conformation (Audent *et al.,* 2005). Activation of ERK1/2 following single dose irradiation with 2 Gy in different cell lines in the presence and absence of BIBX1382BS was determined. As shown in Fig. 3-15, radiation (2 Gy) induces ERK1/2 phosphorylation in all cell lines except MDA-MB-231. Permanent activation of ERK1/2 in MDA-MB-231 cells is presumably because of described K- *RAS* mutation in this cell line (Kimura *et al,* 2004). Pretreatment with BIBX1382BS could partially (in A549 cells) and completely (in FaDu cells) block this activation. Based on data shown in Fig. 3-12 and Fig. 3-15 it can be proposed that radiation may induce two types of *immediate (transient)* and *delayed (sustained)* phosphorylation of the ERK1/2 proteins. Based on IR induced immediate phosphorylation of EGFR, which is blocked by BIBX1382BS, the immediate activation of ERK1/2 which is also responsive to BIBX1382BS treatment (i.e. in FaDu cells and 10 min time point in A549 cells) may depend on EGFR while delayed but

sustained activation of ERK1/2 seems to be independent of radiation-induced EGFR activation

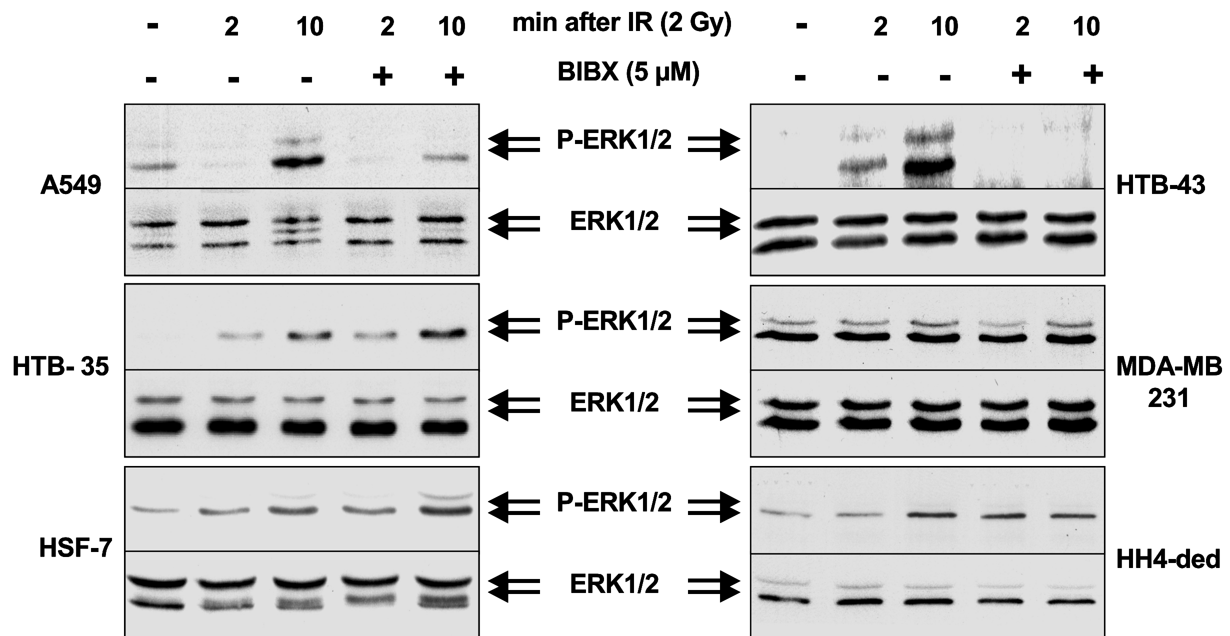


Fig. 3-15: Differential effect of BIBX1382BS on IR induced phosphorylation of ERK1/2. 24 h serum-depleted cells were pre-treated or not with BIBX1382BS (5 µM). Phosphorylation of ERK1/2 was analyzed upon stimulation by a single dose of 2 Gy at the post-irradiation time points indicated. For loading control total ERK1/2 protein was detected by specific antibody.

3.2.3 Radiation induced GRB2 co-immunoprecipitation to SHC in A549 and FaDu cells

A key downstream event in transmission of mitogenic signal by the activated EGFR is the association and subsequent tyrosine phosphorylation of adaptor proteins i.e. SHC and Grb2 (Lowenstein *et al.,* 1992; Sorkin, 2001). SHC binds to specific phosphotyrosine on the EGF receptor and via binding to Grb2 transmits the signal from the receptor and activates downstream proteins leading to phosporylation of the cytoplasmic proteins ERK1/2. To test whether inability of BIBX1382BS to block radiation induced ERK1/2 phosphorylation in A549 cells is due to inability of the inhibitor to abrogate IR induced phosphorylation of specific EGFR tyrosine residues, serum starved A549 and FaDu cells were stimulated via EGF (100 ng/ml) or IR (2 Gy). As shown in Fig. 3-16 both EGF and IR lead to phosphorylation of SHC. In both cell lines BIBX1382BS treatment has similar inhibitory effect on SHC phosphorylation induced either by EGF or IR. To analyse the pattern of co-immunoprecipitation of Grb2 to SHC, blots were incubated with antibody against GRB2. Stimulation of both A549 and FaDu cells via IR or EGF treatment enhances co-

immunoprecipitation of Grb2 to SHC and pretreatment of cells with EGFR inhibitor blocks this complex formation (Fig. 3-16).

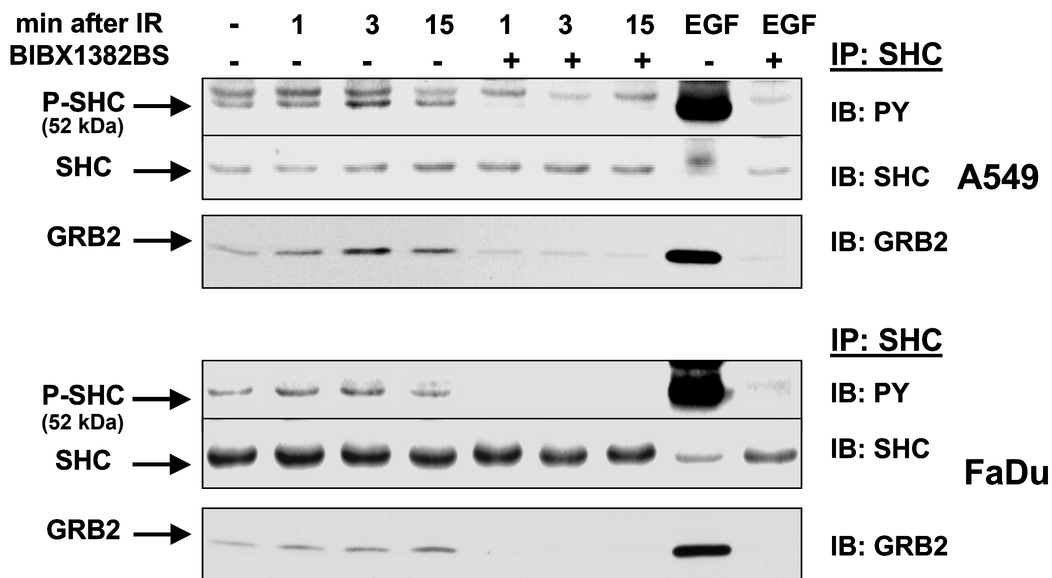


Fig. 3-16: Co-immunoprecipitation of GRB2 to SHC induced by IR and EGF is blocked by BIBX1382BS in A549 and FaDu cells. 24 h serum-depleted A549 and FaDu cells were pre-treated or not with BIBX1382BS (5 µM). Total lysate (2 mg protein) was immunoprecipitated with SHC antibody and immunoblotted initially with antiphosphotyrosine antibody subsequently stripped and re-blotted with SHC antibody to have loading control. To show complex formation of GRB2 with SHC required for ERK1/2 phosphorylation, the lower part of the blot was incubated with anti-GRB2 antibody. IP: immunoprecipitaion IB: immunoblotting

3.2.4 Cytoplasmic SRC tyrosine kinase is upstream of MAPK kinase and is involved in radiation induced ERK1/2 activation

Radiation induced ERK1/2 activation in A549 cells is prolonged and seems to be independent of EGFR. It has been shown that SRC is a critical mediator of GPCR induced MAPK/ERKs activation (Luttrell, 1996) and it is also known that the SRC family of cytoplasmic tyrosine kinases are involved in prolongation of ERK1/2 phosphorylation (Sorenson, 2002). Therefore the effect of selective inhibitor of the SRC kinase, PP2 on radiation induced ERK1/2 phosphorylation was analysed. 10 µM PP2 completely blocked radiation induced P-ERK1/2 in A549 cells (Fig. 3-17). Pretreatment of cells with 10 µM PI3 kinase inhibitor LY294002 did not modulate ERK1/2 activation indicating PI3K independent IR induced ERK1/2 phosphorylation in A549 cells.

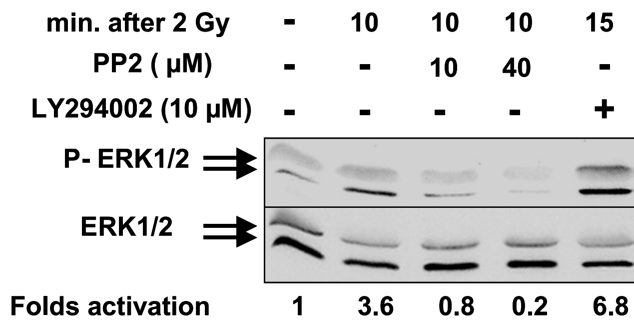


Fig. 3-17: Radiation induces ERK1/2 phosphorylation in A549 cells via cytoplasmic SRC family of tyrosine kinases. 24 h serum-starved A549 cells were incubated with or without PP2 or LY294002 for 30 min and irradiated with a single dose of 2 Gy. At indicated times cells were lysed, subjected to SDS-PAGE and the blot was incubated with antibody against phospho-ERK1/2. Subsequently the blot was stripped and re-blotted against with total ERK1/2. Based on densitometry ratios of P-ERK1/2:ERK1/2 (normalized to 1.0 control) blockage of radiation induced P-ERK1/2 was apparent in cells pretreated with 10 µM of SRC inhibitor PP2.

To exclude unspecific inhibitory effect of PP2 on EGFR tyrosine kinase activity, the pattern of EGFR phosphorylation following EGF (100 ng/ml) treatment in the presence and absence of 20 µM PP2 was analyzed. As shown in Fig. 3-18, PP2 even at higher concentration (20 µM) did not interact with EGFR autophosphorylation.

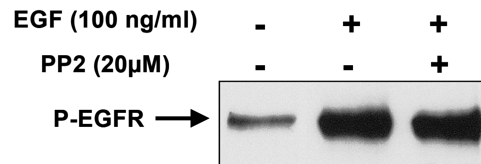


Fig. 3-18: SRC tyrosine kinase inhibitor, PP2 does not block EGFR autophosphorylation. Serum-depleted A549 cells were pre-treated or not with PP2 (20 µM) 30 min before stimulation. 5 min after EGF treatment cells were lysed, immunoprecipitation of EGFR was done and blot was incubated with phosphotyrosine antibody.

3.3 JAK- STATs PATHWAY

The JAK-STAT pathway originally was discovered through the study of interferon induced intracellular signal transduction (Heim, 1999). Signal transducers and activators of transcription factors (STATs) mediate many of the cellular responses that occur following cytokine, growth factor, and hormone signaling. A total of seven different STAT family members (STAT 1-4, STAT 5a and b and STAT 6) encoded in distinct genes have been identified in mammalian cells. Activation of STATs leads to tyrosine phosphorylation, dimerization and translocation of activated proteins from cytoplasm to nucleus, which normally occurs as a tightly regulated process (Shi and Kehrl, 2004). With respect to EGFR mediated modulation of STATs activity, phosphopeptide competition analysis revealed multiple tyrosine residues within the C-tail domain of receptor that can act as docking sites for both STAT1 and STAT3 (Xia *et al.,* 2002). In addition EGFR dependent activation of these two STATs has been reported in a wide variety of human cancer cells (Berclaz *et al.,* 2001).

To gain insight into EGFR dependent functional role of STAT1 and STAT3 in proliferation or post-irradiation survival of A549 and FaDu cells, the pattern of phosphorylation following EGF treatment (100 ng/ml) and irradiation (2 Gy) in the presence and absence of BIBX1382BS (5µM) was analyzed.

EGF induced pronounced phosphorylation of STAT1 in serum starved A549 cells in comparison to weak activation in FaDu cells (Fig. 3-19A). In both cell lines level of total protein was not affected by EGF treatment. To investigate whether the pattern of STAT1 complex formation with EGFR is different in these two cell lines, immunoprecipitation of STAT1 was performed and probed with EGFR antibody. The results indicate co-immunoprecipitation of EGFR to STAT1 in A549 but not in FaDu cells.

In spite of strong radiation induced phosphorylation of EGFR in FaDu cells (Fig. 3-4), activation of STAT1 in this cell lines is not modulated via IR (Fig. 3-19B) which may be because of lack of complex formation of EGFR with STAT1 in this cell line.

Radiation did not activate STAT1 in A549 cells as well. Differential effect of EGF and IR on phosphorylation of this protein in A549 may indicate phosphorylation of different tyrosine sites induced by natural ligands and ionizing radiation.

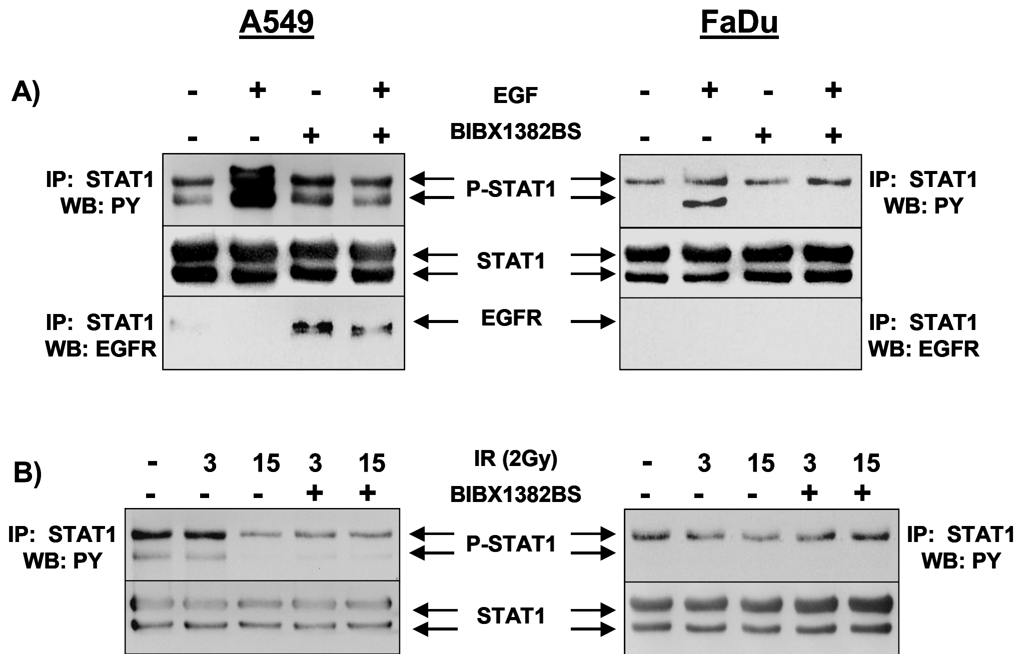


Fig. 3-19: Investigation of STAT1 phosphorylation and co-immunoprecipitation of EGFR following stimulation by EGF or IR in A549 and FaDu cells. A) 24 h serum-depleted A549 and FaDu cells were pre-treated or not with BIBX1382BS (5 µM) and stimulated with 100 ng/ml EGF for 5 min. Total lysate (2 mg protein) was immunoprecipitated with STAT1 antibody and immunoblotted with anti-phosphotyrosine antibody. To show complex formation of EGFR with STAT1, The blot was incubated with anti-EGFR antibody. B) Serum-depleted A549 and FaDu cells were pre-treated or not with BIBX1382BS (5 µM) and irradiated (2 Gy). At indicated time points cells were lysed and P-STAT1 was detected as explained above. Subsequently blots were stripped and re-incubated with antibody against total STAT1. IP: immunoprecipitaion WB: immunoblotting

In the context of STAT3 activation, P-STAT3 analysis after EGF treatment in A549 and FaDu cells indicated a stronger activation in FaDu as compared to A549 cells. Pretreatment with BIBX1382BS reduced this activation to the level of unstimulated control in both cell lines (Fig. 3-20A). In both cell lines co-immunoprecipitation of EGFR to STAT3 could be demonstrated. Although, EGF stimulated STAT3, radiation did not phosphotylate this protein either in A549 or in FaDu cells (Fig. 3-20B).

Fig. 3-20: Investigation of STAT3 phosphorylation and co-immunoprecipitation of EGFR fallowing stimulation by EGF or IR in A549 and FaDu cells. A) 24 h serum-depleted A549 and FaDu cells were pre-treated or not with BIBX1382BS (5 µM) and stimulated with 100 ng/ml EGF for 5 min. Total lysate (2 mg protein) was immunoprecipitated with STAT3 antibody and immunoblotted with anti-phosphotyrosine antibody. To show complex formation of EGFR with STAT3, the blot was incubated with anti-EGFR antibody. B) Serum-depleted A549 and FaDu cells were pre-treated or not with BIBX1382BS (5 µM) and irradiated 2 Gy. At indicated time points cells were lysed and P-STAT3 was detected as explained above. Subsequently blots were stripped and re-blotted with antibody against whole protein. IP: immunoprecipitaion WB: immunoblotting

Taken together, in spite of a strong activation of EGFR via EGF treatment or irradiation in A549 and FaDu cells and BIBX1382BS-dependent abrogation of P-EGFR below the level of the unstimulated control especially in A549 cells , activation of STAT1 and STAT3 was only slightly modulated by EGF or IR. Pretreatment with BIBX1382BS blocked up-regulation of P-STATs but did not affect basal level of activation. Thus it seems that STAT1 and STAT3 activation in A549 and FaDu cells is not stringently dependent on EGFR and may potentially be regulated via other pathways. Consequently, concerning the radiosensitization of A549 following BIBX1382BS treatment, STAT1 and STAT3 can be ruled out as probable mediator of this effect.

3.4 PI3 KINASE-AKT PATHWAY

As addressed, EGFR dependent PI3 kinase AKT pathway is the most important cellular survival pathway. Activated AKT with many effective targets plays a central role in cellular survival strategies, especially after exposure to exogenous stressors, like radiation (Nicholson & Anderson, 2002; Fahy *et al*., 2003). Therefore, the importance of EGFR dependent PI3K-AKT pathway as a probable target for BIBX1382BS induced radiosensitization in different cell lines under the study was analyzed.

3.4.1 Blockage of radiation-induced AKT phosphorylation in A549 and FaDu cells by BIBX1382BS and LY294002

To investigate activation of the AKT pathway as a function of radiation-induced EGFR activity, phosphorylation of AKT on Ser-472/3 was analyzed in A549 and FaDu cells under various conditions. Pre-treatment with BIBX1382BS 30 minutes before irradiation completely blocked basal level of P-AKT and radiation induced AKT activation in A459 cells. In contrast, in FaDu-cells presenting low basal level of P-AKT, BIBX1382BS blocked radiation-induced AKT-phosphorylation to the level of unirradiated control (Fig. 3-21). To test whether the specific radiosensitization effect of BIBX1382BS is via blockage of PI3K-AKT pathway, level of P-AKT in A549 and FaDu cells in the presence and absence of specific inhibitor, LY294002 (Gupta *et al*., 2003) was analyzed. Like BIBX1382BS, LY294002 (10µM) completely blocked the high basal level of P-AKT as well as radiation-induced AKT phosporylation in A549 cells analyzed 5 min after radiation exposure. However, in LY294002 treated FaDu cells radiation-induced AKT phosporylation could only be reduced to the level of unirradiated controls (Fig. 3-21). As tested on the basis of clonogenic survival, LY294002 significantly decreased the surviving fraction of A549 cells irradiated with a single dose of 2 or 4 Gy but did not affect post-irradiation survival of FaDu cells (Fig. 3-22).

To investigate whether the radiosensitizing effect of BIBX1382BS in the other two K-*RAS* mutated human tumor cells, MDA-MB-231 (Fig. 3-5) and PC-3 (Fig. 3-6) is via blockage of PI3 kinase pathway, cells were irradiated with single dose of 2 and 4 Gy alone or in combination with LY294002 (10 µM) and standard clonogenic assay was performed. As shown in Fig. 3-22, PI3 kinase inhibitor enhanced radiation toxicity in both cell lines.

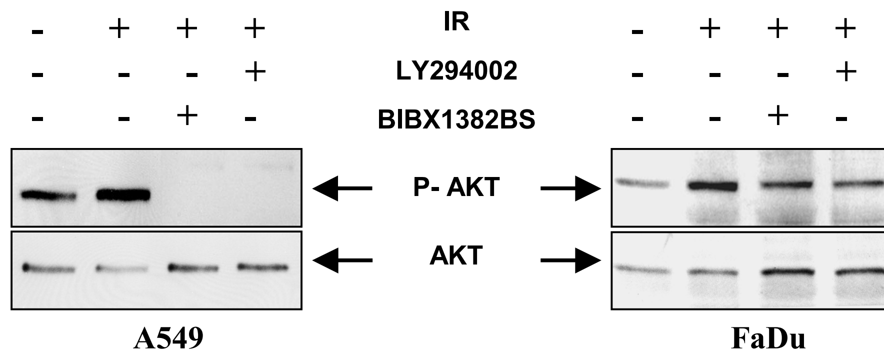


Fig. 3-21: Differential sensitivity of AKT phosphorylation to BIBX1382BS and LY294002 in A549 and FaDu cells. 24 h serum-starved cells were pretreated with EGFR antagonist BIBX1382BS (5 µM) or with PI3 kinase inhibitor LY294002 (10 µM) 30 min before irradiation (2 Gy). Cells were lysed 5 min after irradiation and subjected to SDS-PAGE and immunoblotted applying a specific P-AKT antibody. Loading was controlled using an antibody against total AKT.

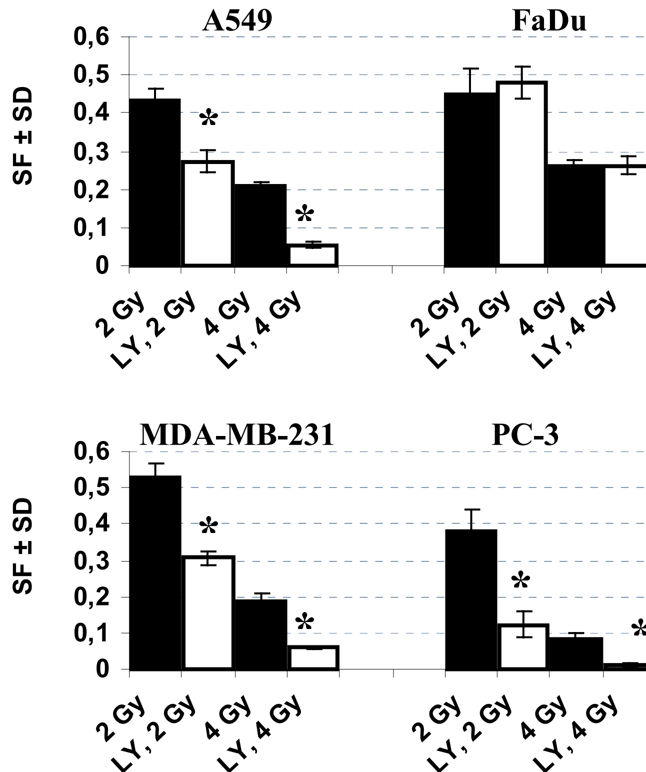


Fig. 3-22: Survival fractions of irradiated A549, FaDu, MDA-MB-231 and PC-3 cells as a function of LY294002 treatment. Log-phase cells (A549, FaDu, MDA-MB-231, PC-3) were irradiated with a single dose of 2 and 4 Gy with or without 30 min pre-treatment with LY294002 (10 µM). 6 h after irradiation cells were plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Asterisks indicate statistically significance difference (Student´s t-test/ $p<0.05$) between surviving fraction of irradiated control and irradiated cells pretreated with PI3 kinase inhibitor LY294002.

3.4.2 Basal level of PI3 kinase activity as probable target for radiosensitization

The results presented so far indicate that for K-*RAS* mutated tumor cells PI3K-AKT pathway is a key target for modulation of radiation response. The described results for FaDu cells (Fig. 3-4, Fig. 3-5 and Fig. 3-9) and results from the following experiments explain in more detail whether targeting of IR induced EGFR phosphorylation is a predictor for enhancement of radiation sensitivity by BIBX1382BS or the radiosensitization effect depends most likely on potential of this inhibitor in blockage of basal level of EGFR dependent PI3 kinase pathway.

The observed results from irradiated PC-3 cells indicated that IR differentially activates EGFR and downstream pathway, *i.e.* PI3 kinase as target for radiosensitization. As shown in Fig. 3-23A IR 2 Gy strongly induces autophosphorylation of EGFR in this cell line and pretreatment with BIBX1382BS completely blocks this phosphorylation. In contrast, analyzing AKT phosphorylation indicates that pattern of PI3 kinase activity in this cell line is different to autophosphorylation of EGF receptor. In spite of strong IR induced EGFR phosphorylation, the level of P-AKT is not affected by irradiation. It seems that both EGFR and PI3 kinase inhibitors effectively block basal level of P-AKT (Fig. 3-23B, 3-23C).

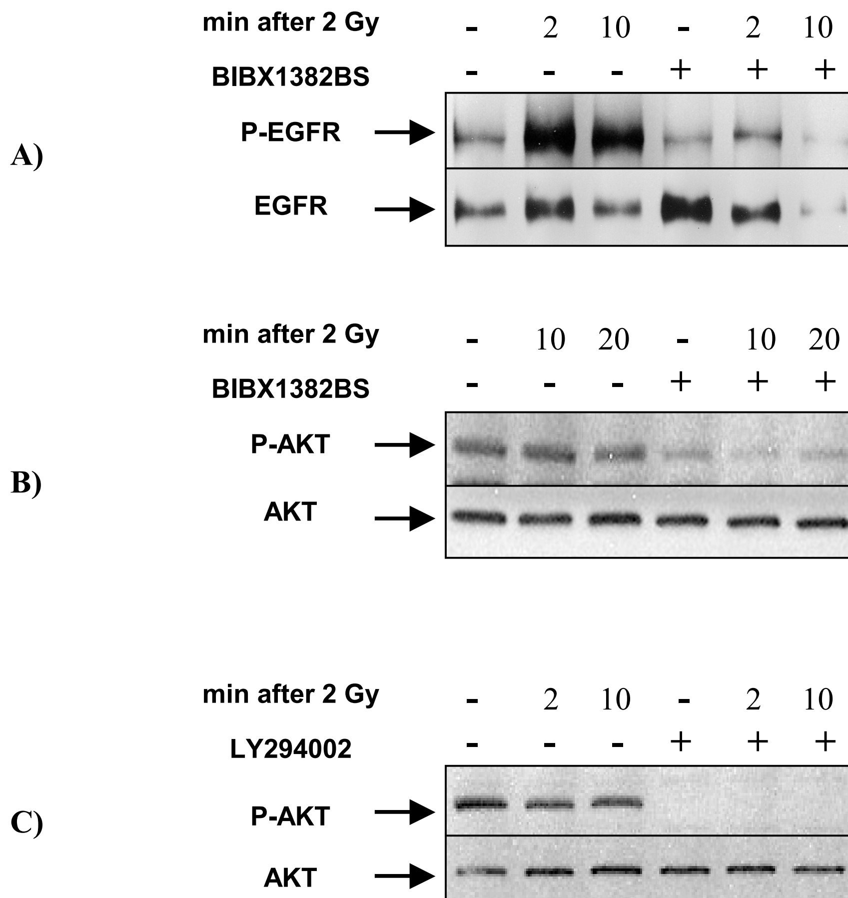


Fig. 3-23: Radiation differentially modulates EGFR and PI3 kinase activity in PC-3 cells. 24 h serum-starved PC-3 cells were incubated with or without BIBX1382BS, LY294002 or an equal volume of (vehicle) DMSO for 30 min and irradiated with a single dose irradiation 2 Gy. At indicated time points cells were lysed. A) To analyze autophosphorylation of EGFR two mg protein was immunoprecipitated with EGFR antibody and tyrosine phosphorylation was detected by immunoblotting with phosphotyrosin antibody. B and C) To detect P-AKT, 100 µg protein was subjected to SDS-PAGE and the blot was detected with P-AKT antibody. To evaluate protein loading the blots were stripped and re-probed with antibody against whole EGFR or AKT.

In order to extend the observed results from PC-3 cell lines regarding to importance of upregulated PI3 kinase pathway in post-irradiation survival of human tumor cells, differential effects of BIBX1382BS and LY294002 in modulation of radiation response of HTB-35 cells with attention to the effects of these inhibitors on basal level of P-AKT was analysed.

PI3 kinase inhibitor, LY294002 enhances radiation effect in HTB-35 cells presenting normal *RAS* as well (Fig. 3-25). We analyzed P-AKT status as readout of PI3 kinase activity in this cell line following IR in the presence or absence of inhibitors. As shown in Fig. 3-24 P-AKT is not strongly enhanced following IR 2 Gy in HTB-35 cells in comparison to non-irradiated control. Pretreatment with 10 µM of LY294002 completely blocks high basal level of AKT

phosphorylation in this cell line (Fig. 3-24 lower part) which may explain enhancement of radiation toxicity by this inhibitor (Fig. 3-25 right part). The importance of P-AKT as target for radiosensitization is also confirmed via BIBX1382BS treatment. Inability of BIBX1382BS in effective blockage of P-AKT (Fig. 3-24 upper part) in HTB-35 cells may explain the fail of is inhibitor in modulation of radiation response in combination with single dose (Fig. 3-25 left part) or fractionated dose IR as already shown in Fig. 3-4.

Above results implicate that blockage of basal level of PI3 kinase AKT activity rather than inhibition of radiation induced EGFR autophosphorylation can be as a valuable predictor for radiosensitization.

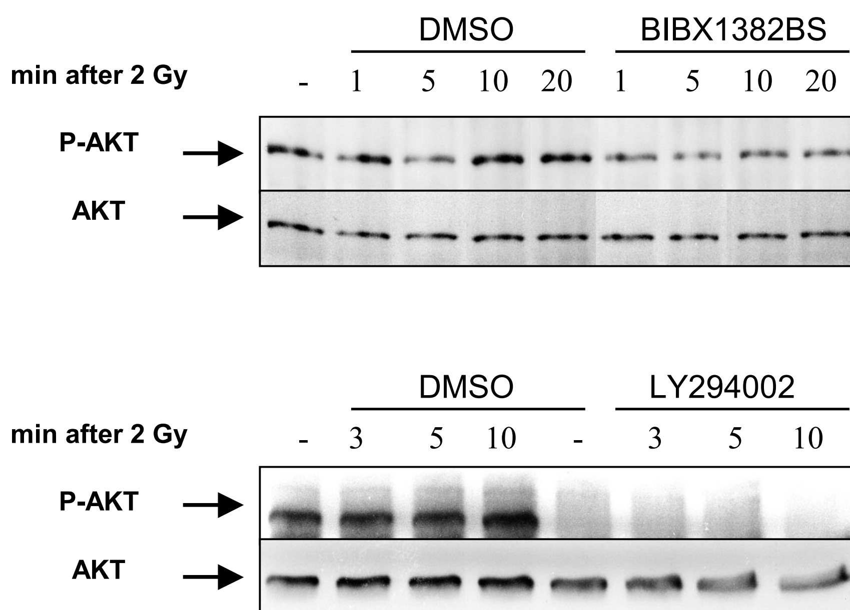


Fig. 3-24: Differential effect of BIBX1382BS and LY294002 on basal level of P-AKT in HTB-35 cells. 24 h serum-starved HTB-35 cells were incubated with or without BIBX1382BS (5 µM), LY294002 (10 µM) or an equal volume of (vehicle) DMSO for 30 min and irradiated with a single dose of 2 Gy. At indicated time points cells were lysed. Proteins were separated by SDS-PAGE and immunblotted with antibody against P-AKT. To evaluate protein loading the blots were stripped and re-probed with antibody against whole AKT.

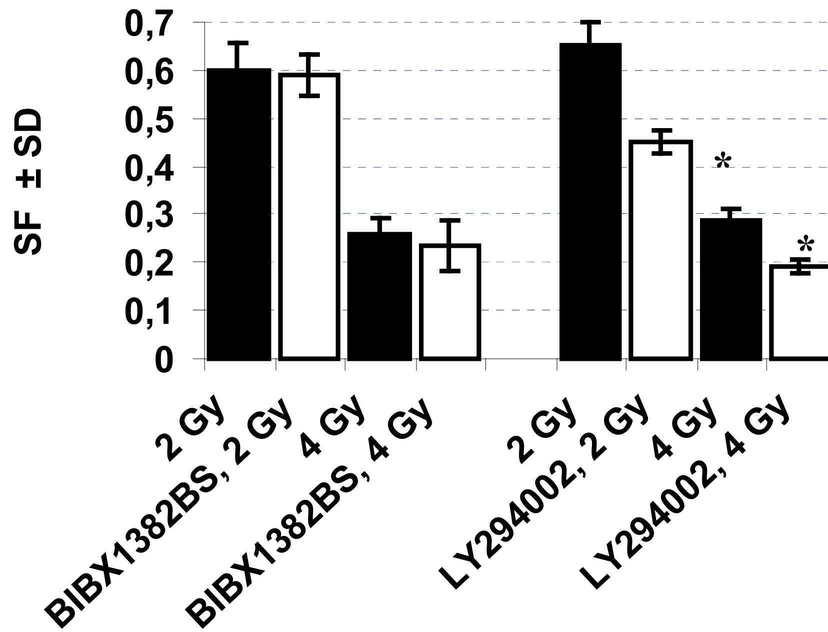


Fig. 3-25: Post-irradiation survival of HTB-35 cells as a function of BIBX1382BS and LY294002 treatment. Log-phase cells were irradiated with a single dose of 2 and 4 Gy with or without 30 min pre-treatment with BIBX1382BS (5 µM left part), LY294002(10 µM right part) or equal volume of (vehicle) DMSO for 30 min. 6 h after irradiation cells were plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Asterisks indicate statistically significance significant difference (Student´s t-test/ $p<0.05$) between irradiated controls and irradiated cells treated with PI3 kinase inhibitor LY294002.

3.5 AUTOCRINE/PARACRINE ACTIVATION OF EGFR IN K-*RAS* MUTATED CELLS

Permanent activation of PI3K-AKT pathway due to EGFR mutation (EGFRvIII) has been reported to promote cellular radioresistance which, could be inhibited by the PI3K-antagonist LY294002 (Li *et al*., 2004; Luwor *et al.,* 2004; Nakamura *et al.,* 2005). Furthermore, Brognard *et al* reporting on the role of constitutive active AKT in chemo-/radioresistance of non-small cell lung cancer (*e.g.* the *RAS*-mutated cell line A549) proposed that EGFR dependent PI3K pathway may selectively be stimulated by autocrine production and binding of EGFR ligand (Brognard *et al.,* 2001). This assumption is substantiated by several reports presenting quantitative immunohistochemical data indicating a significant correlation between EGF receptor ligands and EGFR activation in *RAS*-mutated human tumors (Rubin-Grandis *et al*., 1998). The production of the potent EGFR ligand, TGFα is increased in H-*RAS* mutated tumor models (Grana *et al.,* 2003) and ionizing radiation dependent releasing of this ligand from tumor xenografts has been reported as well (Hagan *et al.,* 2004). Thus, the following experiments examine the hypothesis whether EGFR activity and its downstream PI3K-AKT survival pathway is upregulated in K-*RAS* mutated tumor cells via autocrine production and secretion of EGFR ligands and thus mediates radioresistance.

3.5.1 Modulation of AKT phosphorylation and radiation sensitivity of A549 and FaDu cells by TGFα neutralizing antibody

It was investigated whether the difference in BIBX1382BS-mediated radiosensitization between cells presenting mutated or normal *RAS* could be related to enhanced autocrine production of EGFR ligands, *e.g.* TGFα, and subsequent activation of EGFR. To address this question, AKT phosphorylation in A549 and FaDu cells in the presence or absence of TGFα neutralizing antibody 10 min after radiation exposure was analyzed. As indicated in Fig. 3-26 in the presence of IR, AKT phosphorylation in A549 cells was efficiently inhibited (2.5-fold) by TGFα-neutralization, but not in FaDu cells. Accordingly, clonogenic survival of A549, but not of FaDu cells pretreated with TGFα neutralizing antibody and subsequently irradiated with a single dose of 6 Gy was significantly decreased by about 35 % (Fig. 3-27).

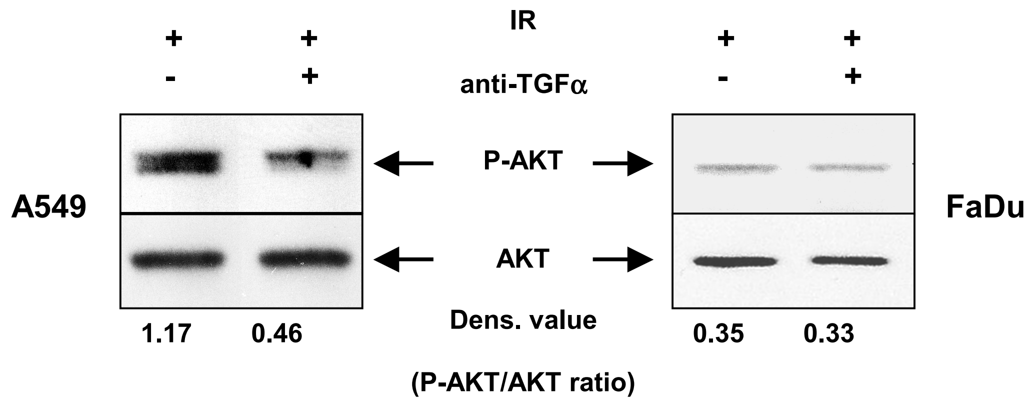


Fig. 3-26: Effect of TGFα neutralizing antibody on AKT phosphorylation in irradiated A549 (left) and FaDu (right) cells. 24 h serum-starved cells were pre-treated for 2 h with or without TGFα neutralizing antibody (10 µg/ml) and subsequently irradiated with a single dose of 2 Gy. 10 min after irradiation cells were lysed and subjected to SDS-PAGE and immunoblotted applying a specific P-AKT antibody. Ratios of P-AKT:AKT under the different treatment conditions were calculated based on densitometry values.

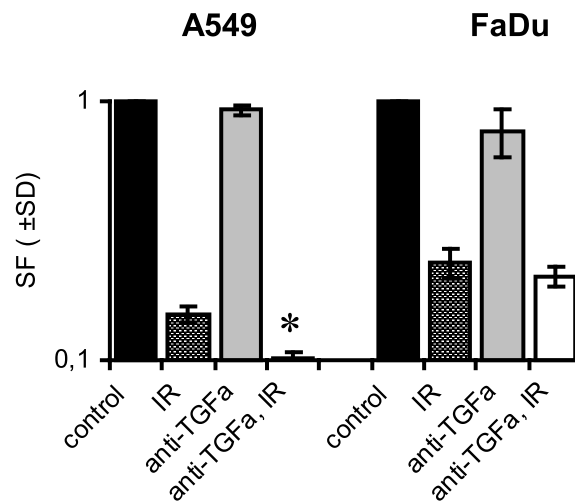


Fig. 3-27: Survival fractions of irradiated A549 and FaDu cells as a function of anti- TGFα treatment. Log-phase cells (A549, FaDu) were irradiated with a single dose of 6 Gy with or without 2 h pre-treatment with TGFα neutralizing antibody (10 µg/ml). 6 h after irradiation cells were plated for colony formation and incubated for 10 days. Based on the colony counts and plating efficiency the surviving fraction was calculated. Data represent the mean ± SD of 6 parallel experiments. Asterisk indicates statistically significance difference (Student´s t-test/ $p<0.05$) between irradiated controls and irradiated cells treated with anti-TGFα.

3.5.2 TGFα and AREG modulate radiation sensitivity.

It was shown that a neutralizing antibody directed against TGFα is able to sensitize *RAS*-mutated human tumor cells to ionizing radiation. Consequently, this experiment addresses more specifically the question, whether TGFα and AREG, as natural EGFR-ligands are able to modulate radiation sensitivity of normal *RAS* presenting human tumor cells *in vitro*. Therefore, FaDu and HTB-35 cells were treated with indicated concentrations of TGFα and

AREG 15 min prior to radiation exposure. Fig. 3-28 shows that treatment with both EGFR ligands resulted in a significantly enhanced clonogenic survival after irradiation with a single dose of 2 Gy.

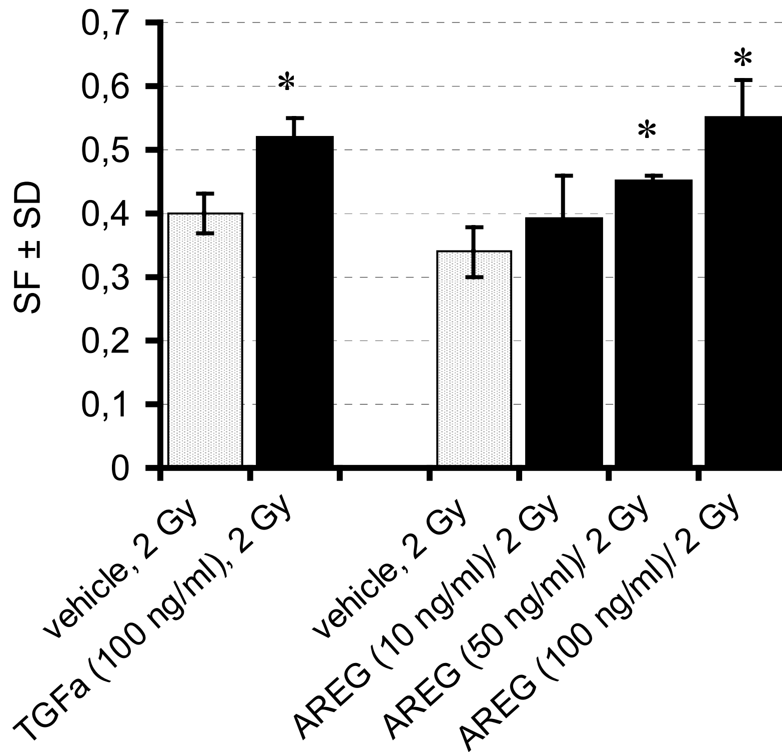
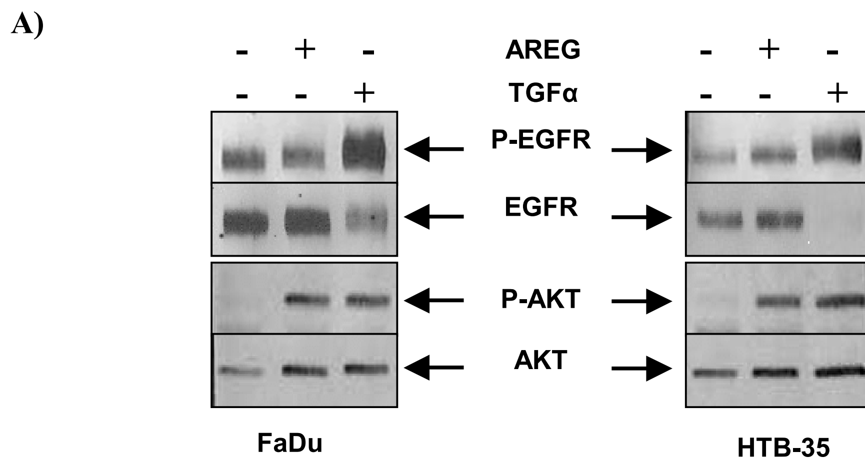


Fig. 3-28: EGF receptor ligands AREG and TGFα enhance radiation resistance in HTB-35 cells. Log-phase HTB-35 cells were pretreated for 15 min with TGFα or AREG (both 100 ng/ml). Thereafter, cells were irradiated with single dose of 2 Gy. After 6 hours cells were plated for colony formation. 10 days later, surviving fraction was calculated on the basis of plating efficiency under the different culture conditions. Data bars shown represent the mean surviving fraction ± SD of 6 parallel experiments. Asterisks indicate statistically significant enhancement ($p < 0.05$/ Student´s t-test) of post-irradiation survival of HTB-35 cells pretreated with TGFα or AREG in comparison with irradiated control pretreated with PBS as vehicle.

Likewise, both ligands led to an immediate phosphorylation and phsophorylation- dependent band shift of EGFR and its downstream signaling component AKT in RAS_{wt}-cells (HTB-35 and FaDu, Fig. 3-29A). Pretreatment of HTB-35 cells with EGFR-TK-specific inhibitors, i.e. BIBX13282BS and PD153035, repressed phosphorylation of AKT in cells stimulated with the EGFR-ligands AREG and TGFα (Fig. 3-29B).

A)



B)

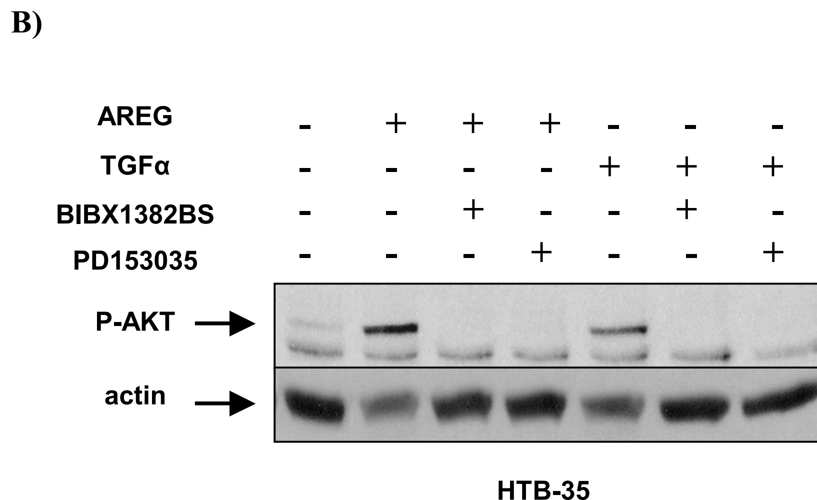


Fig. 3-29: EGFR-dependent stimulation of PI3K-AKT pathway in response to AREG and TGFα treatment. A) FaDu and HTB-35 cells serum-depleted for 24 h were treated with 100 ng/ml AREG and TGFα for additional 5 minutes. Following detection of P-EGFR, and P-AKT, blots were stripped and re-probed with antibodies directed against total EGFR and AKT to control protein loading. B) HTB-35 cells were stimulated with AREG or TGFα (both 100 ng/ml) for 5 min with or without pretreatment (30 min) with EGFR-TK-inhibitors BIBX1382BS (5 µM) or PD153035 (500 pM). Actin was detected as described in *Methods*.

To test whether RAS_{wt}-and RAS_{mt}-cells exert differences in gene expression of TGFα and AREG, quantitative RT-PCR analysis was performed under basal culture conditions. As indicated in Fig. 3-30, both cell lines expressed TGFα at comparable levels. However, expression of AREG was app. 2-fold upregulated in RAS_{mt}-cells as compared to RAS_{wt}-cells. Furthermore, the data indicate that independent of the *RAS*-status AREG mRNA expression is appr. 8-13 folds higher as compared to TGFα.

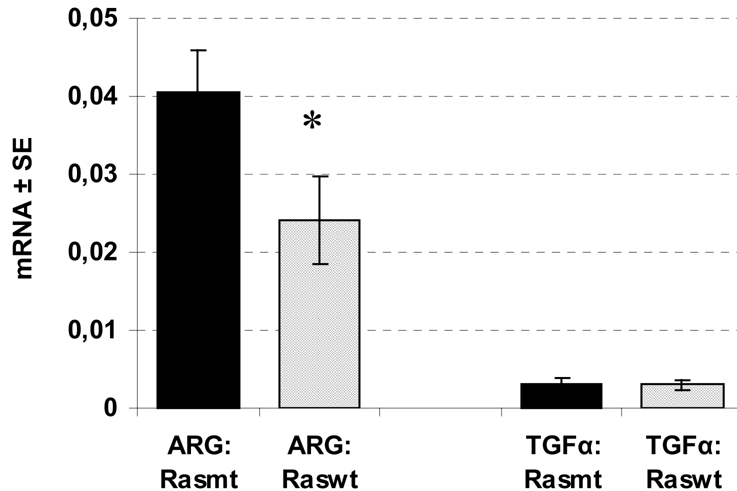


Fig. 30: AREG and TGFα mRNA expression in mutated *RAS* and normal *RAS* presenting cells. Applying real time-RT-PCR, the expression level of *AREG* and *TGFA* was analyzed in *RAS* mutated cells (A549 and PC-3) as well as normal *RAS* presenting cells (FaDu and HTB-35). Relative mRNA expression for the two EGFR-ligands was quantified on the basis of GAPDH mRNA expression. The data shown represent the mean ± SE of 4 independent experiments. Asterisk indicates statistically significant ($p < 0.05$/ Student´s t-test) difference of AREG mRNA expression between RAS_{mt} (A549, PC-3) and RAS_{wt} (FaDu, HTB-35) cells.

3.5.3 RAS_{mt}-CM promotes radioresistance of RAS_{wt}-cells.

To determine whether autocrine or paracrine secretion of EGF receptor ligands is involved in EGFR signaling resulting in altered radiation responses, the potential of conditioned media from *RAS* mutated-cells (RAS_{mt}-CM) to mediate radiation resistance in RAS_{wt}-cells was investigated. As shown in Fig. 3-31, RAS_{mt}-CM (from A549 cells) significantly enhanced clonogenic survival of RAS_{wt}-cells (FaDu and HTB-35) irradiated with a single dose of 2 Gy.

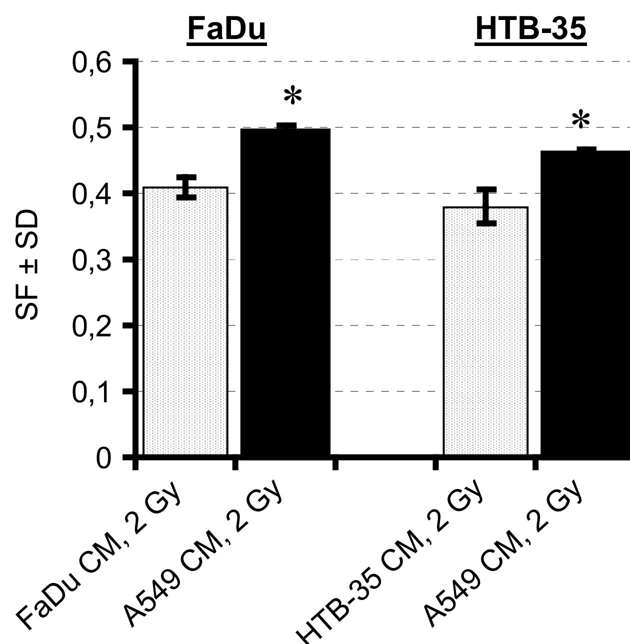


Fig. 3-31: *RAS*$_{mt}$-CM enhances radiation resistance of *RAS*$_{wt}$ cells. Log-phase *RAS*$_{wt}$ cells (HTB-35 and FaDu) were pretreated for 15 min with *RAS*$_{mt}$-CM (from A549 cells) or with medium conditioned by parallel cultures of HTB-35 or FaDu cells (autologous-CM). Thereafter, cells were irradiated with a single dose of 2 Gy. After 6 hours cells were plated for colony formation and incubated for 10 days and surviving fraction was calculated as described in *Materials and Methods*. The data represent the mean of surviving fractions ± SD of 6 parallel experiments. Asterisks indicate statistically significant enhancement ($p < 0.05$/ Student´s t-test/ n=12) in post-irradiation survival of *RAS*$_{wt}$ cells treated with *RAS*$_{mt}$-CM.

3.5.4 RAS$_{mt}$-CM stimulates EGFR-phosphorylation, EGFR-tyrosine kinase activity and AKT phosphorylation in RAS$_{wt}$-cells.

To test whether the observed radiation response of *RAS*$_{wt}$-cells fed with *RAS*$_{mt}$- CM shown in Fig. 3-31 is reflected by a modulation of EGFR phosphorylation and EGFR kinase activity, Western blot analyses and kinase assays after immuno-precipitation of EGFR were performed. As shown in Fig. 3-32A, treatment of *RAS*$_{wt}$-cells (FaDu, HTB-35) with *RAS*$_{mt}$-CM led to a stimulation of EGFR phosphorylation in *RAS*$_{wt}$-cells. In contrast, when *RAS*$_{mt}$-cells (A549 and MDA-MB231) were treated with *RAS*$_{wt}$-CM (FaDu and HTB-35) no change in the phosphorylation pattern of EGFR occurred (Fig. 3-32B).

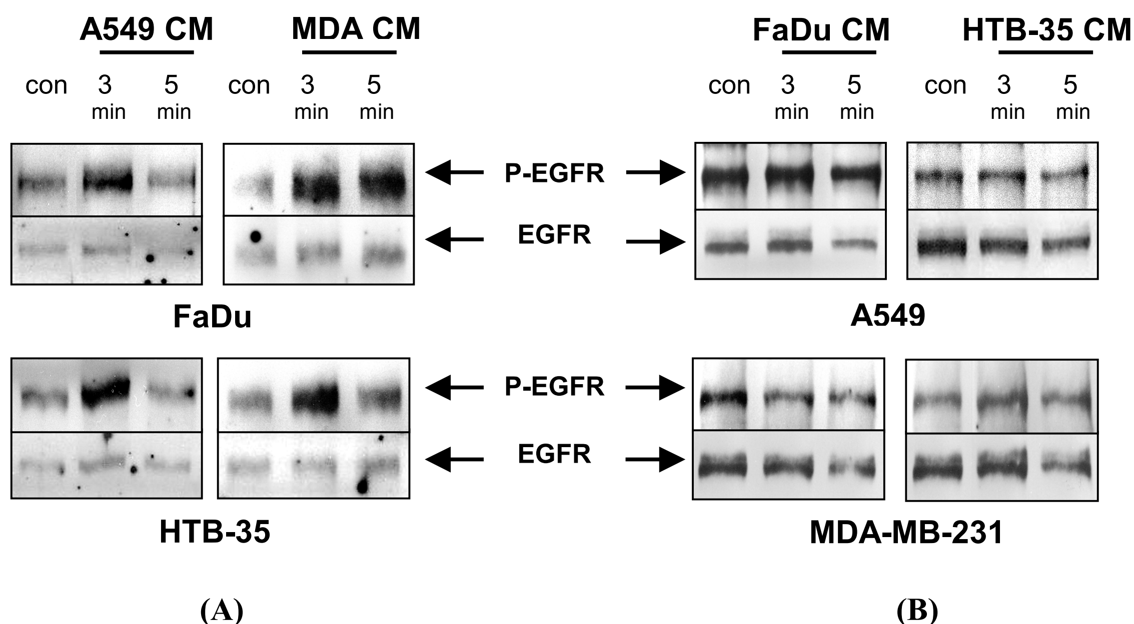


Fig. 3-32: Autophosphorylation of EGFR in response to either *RAS*$_{mt}$- or *RAS*$_{wt}$-CM. A) *RAS*$_{wt}$-cells (FaDu and HTB-35) were serum starved for 48 hours and treated with *RAS*$_{mt}$-CM from A549 and MDA-MB-231 cells. For controls FaDu and HTB-35 cells were treated with autologous-CM for 3 min. B) *RAS*$_{mt}$- cells (A549 and MDA-MB-231) cells were serum starved for 48 hours and treated with either autologous-CM for 3 min or with *RAS*$_{wt}$-CM derived from either FaDu (left part) or HTB-35 cells (right part). To determine EGFR phosphorylation in response to either CMs, cells were lysed at indicated time points and 2 mg of protein was immuno-precipitated with an anti-EGFR antibody and immunobloting was performed by an anti-phosphotyrosine antibody. Blots were stripped and reprobed with an antibody against total EGFR.

These results are supported by the *in vitro* EGFR kinase assay shown in Fig 3-33. *RAS*$_{mt}$- CM led to an increased EGFR-tyrosine kinase activity in *RAS*$_{wt}$-cells (left part), whereas *RAS*$_{wt}$-CM did not alter the activity of EGFR-tyrosine kinase in *RAS*$_{mt}$- A549 cells (right part).

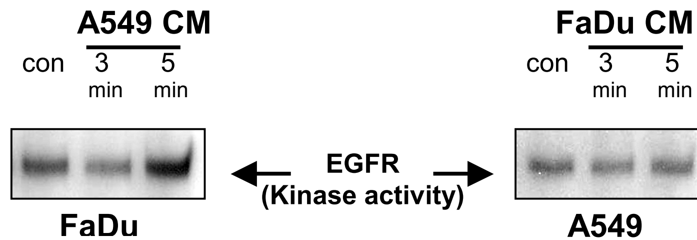


Fig. 3-33: Modulation of EGFR kinase activity in *RAS*$_{wt}$- or *RAS*$_{mt}$-cells as function of treatment with *RAS*$_{mt}$- or *RAS*$_{wt}$-CM. *RAS*$_{wt}$-FaDu cells (left part) or *RAS*$_{mt}$-A549 cells (right part) were treated with *RAS*$_{mt}$- or *RAS*$_{wt}$ CM. After cell lysis and protein quantification, immuno-precipitation of EGFR and EGFR *in vitro* Kinase Assay was performed as described in *Methods*.

This data indicate that media from *RAS*$_{mt}$-cells but not media from *RAS*$_{wt}$-cells contain factors which mediate stimulation of EGFR signal transduction in an autocrine/paracrine manner.

To analyze the potential of *RAS*$_{mt}$-CM to activate EGFR-dependent signal transduction pathways, phosphorylation of AKT as readout of PI3 kinase activity was tested in *RAS*$_{wt}$-FaDu and HTB-35 cells stimulated with CM from *RAS*$_{mt}$-A549, MDA-MB-231 cells. At indicated time points following stimulation, cell lysates were prepared, subjected to SDS-PAGE and immunoblotted with antibody against P-AKT. The Results indicate a strong AKT phosphorylation (Ser-472/3) in FaDu cells detectable as early as 5 min after treatment and stable until 15 min post stimulation (Fig. 3-34A). Similar results were obtained when HTB-35 or normal fibroblast HSF-7 cells were treated with CM from *RAS* mutated cell (data not shown). In contrast to AKT phosphorylation induced by *RAS*$_{mt}$-CM in *RAS*$_{wt}$-cells, treatment of *RAS*$_{mt}$-cells with *RAS*$_{wt}$-CM resulted in strong time dependent reduction of basal level of P-AKT in these cell lines (Fig. 3-34B).

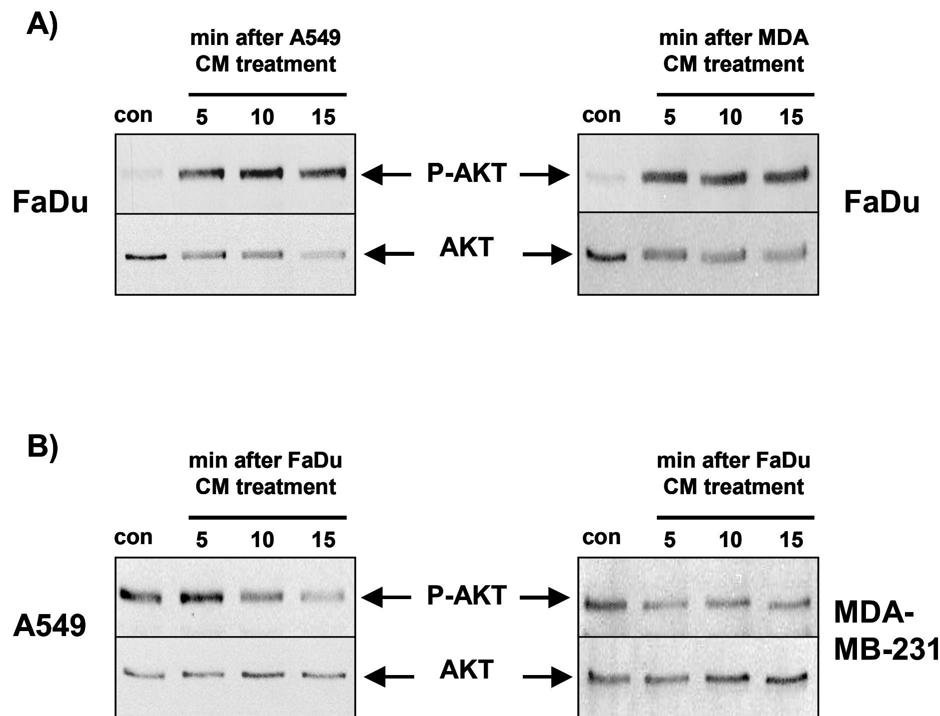


Fig. 3-34: Differential pattern of AKT phosphorylation via conditioned media from *RAS*$_{mt}$ and *RAS*$_{wt}$-cells. A) Serum starved *RAS*$_{wt}$- FaDu cells were incubated in *RAS*$_{mt}$- CM. At indicated times cells were lysed and P-AKT as well as total AKT were assessed by immunoblotting with specific antibodies. Control cells were incubated in autologous-CM and processed accordingly. B) Serum starved *RAS*$_{mt}$- A549 and MDA-MB-231 cells were incubated in *RAS*$_{wt}$- CM. Level of P-AKT and total AKT were analyzed as explained in part A.

To estimate EGFR ligand concentration in CM of *RAS* mutated cells, FaDu cells were treated by 1:2 serial dilution of A549 CM. As a read-out, the level of P-AKT was compared with AKT phosphorylation induced by exogenous TGFα as known EGFR ligand. As compared to the control level in FaDu cells fed with autologous CM, undiluted A549 CM induced AKT markedly (app. 40-fold) (Fig. 3-35). Elevated P-AKT signals were observable up to a 1:32 dilution of the *RAS*$_{mt}$-CM. In comparison, treatment of *RAS*$_{wt}$-FaDu cells with 10 ng/ml of TGFα led only to a 9-fold increase in AKT-phosphorylation.

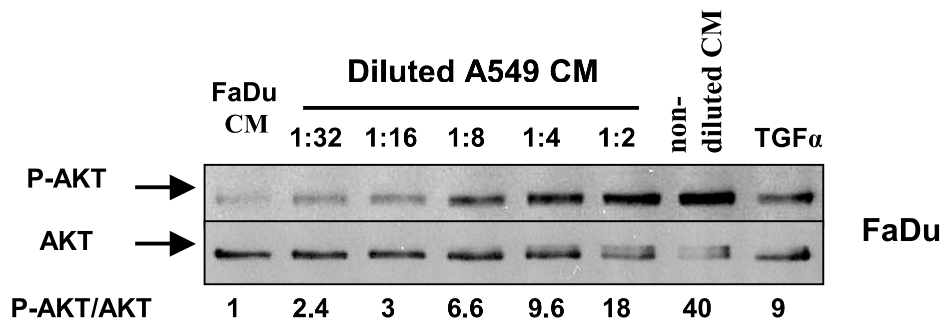


Fig. 3-35: Phosphorylation of AKT in RAS_{wt}-cells as a function of treatment with serial dilutions of RAS_{mt}-CM. RAS_{wt}-cells (FaDu) were treated with 1:2 serial dilution RAS_{mt}-CM (from A549) as well as autologous CM (FaDu CM) for 15 min. As positive control, FaDu cells were treated with 10 ng/ml TGFα for the same time. After stimulation cells were lysed and electrophoresis of samples was performed. Based on densitometry ratios of P-AKT : AKT (normalized to 1.0 in FaDu CM control), the level of P-AKT was determined for the serial dilution steps. As compared to TGFα, undiluted A549 CM stimulated P-AKT appr. 4-fold.

To accumulate more evidences for the role of EGFR ligands in the upregulation of PI3 kinase pathway in K-*RAS* mutated cells neutralizing antibodies directed against the EGFR ligands TGFα and AREG were used. Neutralization of TGFα and AREG clearly inhibited the stimulatory effect of RAS_{mt}-CM (A549) on AKT-phosphorylation in the two RAS_{wt}-cells FaDu and HTB-35 (Fig. 3-36).

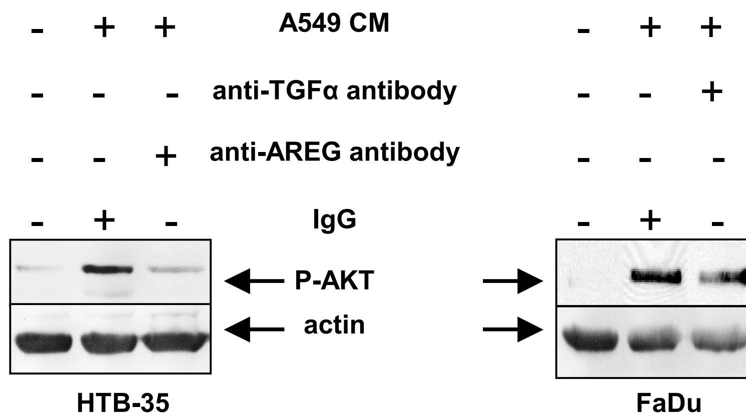


Fig. 3-36: Effect of neutralization of AREG and TGFα on level of P-AKT. 48 h serum-starved RAS_{wt}-cells (FaDu and HTB-35) were treated for 15 min with serum free RAS_{mt}-CM (from A549 cells), which was either pre-incubated with IgG (10 µg/ml), anti- AREG antibody (anti- AREG) or anti-TGFα antibody (10 µg/ml for both) for 2 h. Cells were then lysed and samples were prepared for Western blotting. Western blots were probed for P-AKT and actin.

3.5.5 Phosphorylation of AKT and ERK1/2 in RAS_{wt}-cells is stimulated by RAS_{mt}–CM

EGFR dependent phosphorylation of AKT and ERK1/2 following treatment of RAS_{wt}-cells (FaDu and HTB-35) with RAS_{mt}-CM (from A549, MDA-MB-231 and PC-3 cells) was investigated in the presence or absence of different pharmacological kinase inhibitors. As shown in Fig. 3-37 lanes 2, RAS_{mt}-CM prepared by either of the RAS_{mt}-cell lines induced a strong phosphorylation of both AKT and ERK1/2 in RAS_{wt} cells (Fig. 3-37; lanes 2).

Blockage of PI3 kinase activity by the specific inhibitor LY294002 (10 µM) markedly reduced the appearance of P-AKT, while no effect on P-ERK1/2 was observed (Fig. 3-37; lanes 3). PD98059 (20µM), a specific MEK inhibitor, blocked P-ERK1/2 without any effect on the level of P-AKT (lane 4). Furthermore, the two specific EGFR tyrosine kinase inhibitors BIBX1382BS (5µM) and PD153035 (500 pM) resulted in a pronounced blockage of both P-AKT and P-ERK1/2 induced by RAS_{mt}-CM (Fig. 3-37; lanes 5 and 6). These results indicate that EGFR activation by RAS_{mt}-CM can stimulate both, the PI3K and the MAPK pathway required for phosphorylation of either AKT (via PI3K) or ERK1/2 (via MEK).

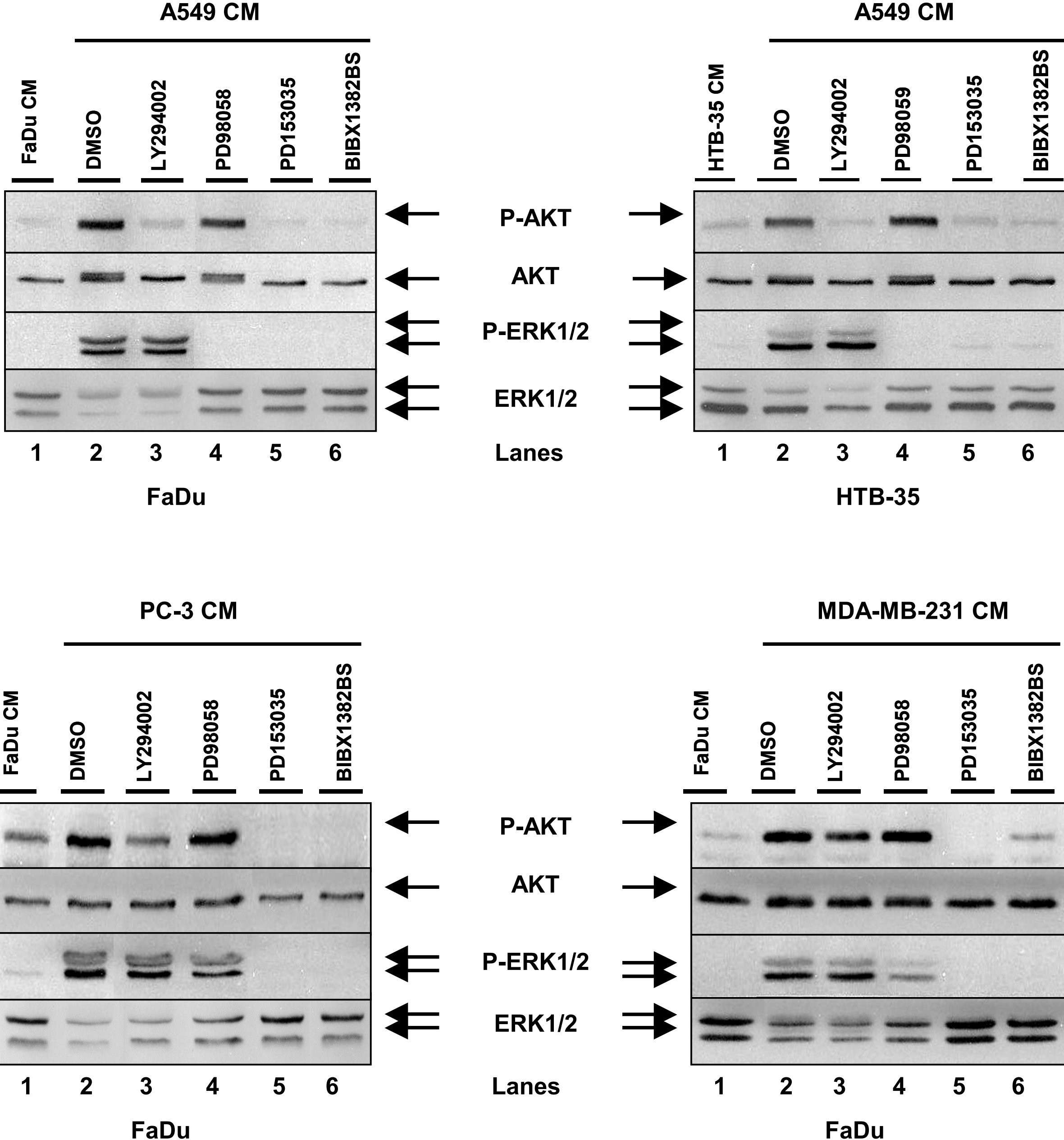


Fig. 3-37: EGFR dependent AKT and ERK1/2 phosphorylation induced in RAS_{wt}-cells by RAS_{mt}- CM. Serum starved RAS_{wt}-cells (FaDu and HTB-35) were pretreated either with 0.01% DMSO (lane 2), 10 µM LY294002 (lane 3), 20 µM PD98059 (lane 4), 500 pM PD153035 (lane 5) or 5 µM BIBX1382BS (lane 6) for 30

min. Cells were incubated in RAS_{mt}- CM (derived from A549, PC-3, or MDA-MB-23; lanes 2-6). 15 min later cells were lysed and P-AKT, P-ERK1/2, as well as total AKT or ERK1/2-proteins were assessed by immunoblotting with specific antibodies. As controls (lane 1) untreated cells (FaDu or HTB-35) were incubated in autologous-CM and processed accordingly.

3.5.6 RAS_{mt}-CM promotes ^{3}H-thymidine incorporation and cell proliferation

The data presented above indicated that secretion of EGFR ligands by RAS_{mt} cells can stimulate the EGF receptor and activate the downstream Ras/MAPK pathway regulating proliferation. Therefore, we investigated the potential of RAS_{mt}-CM to stimulate proliferation in RAS_{wt}-cells by determination of ^{3}H-thymidine incorporation. As indicated in Fig. 3-38, a significant nearly 2-fold enhancement of ^{3}H-thymidine incorporation was observed for the RAS_{wt}-cells (HTB-35 cells) stimulated with RAS_{mt}-CM. This effect was not apparent when RAS_{mt}-A549 cells were stimulated with RAS_{wt}-CM derived from HTB-35 cells.

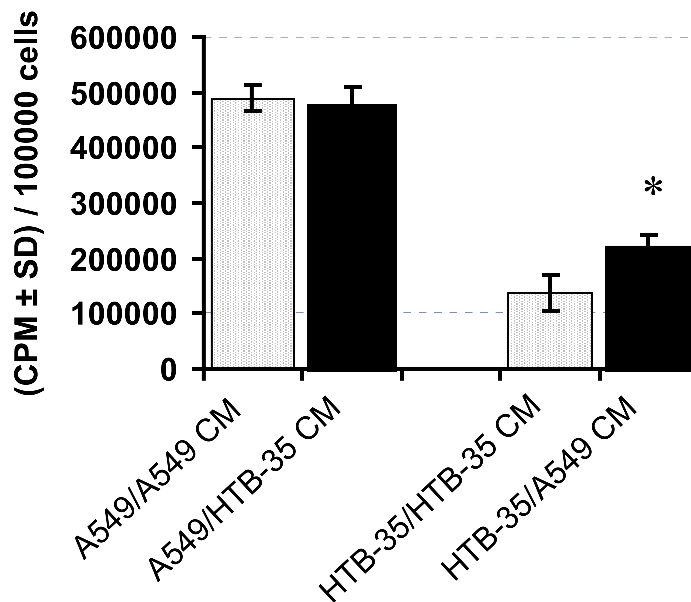


Fig. 3-38: ^{3}H-thymidine incorporation of RAS_{mt}- or RAS_{wt}-cells treated with either RAS_{wt}- CM or RAS_{mt}-CM. RAS_{mt}- (A549) and RAS_{wt}- (HTB-35) cells were seeded in 6 well plates at a constant density of 5×10^{4} /well and incubated in medium containing 10% FCS for 24 h. Then medium was removed, cells were washed with PBS (37°C, 2x) and incubated with serum free medium for additional 24 h. Serum free medium was then replaced with either RAS_{wt}- (HTB-35 CM) or RAS_{mt}-CM (A549 CM) containing 1 µCi/ml of ^{3}H-thymidine and cells were incubated for additional 10 hours. After sample preparation incorporation of ^{3}H-thymidine was quantified by means of scintillation counting using a β-counter. The data represent the means ± SD of 6 parallel experiments. Asterisk indicates statistically significance ($p < 0.05$/ Student´s t-test) enhancement of ^{3}H-thymidine incorporation in HTB-35 cells incubated with A549 CM.

The effect of conditioned media from the different cell lines under study on modulation of proliferation was also tested. Likewise as shown in Fig. 3-39 a significant stimulation of cell proliferation documented by an increase in cell number was apparent when CM from RAS_{mt} cells A549 was administrated to culture of RAS_{wt} HTB-35 cells.

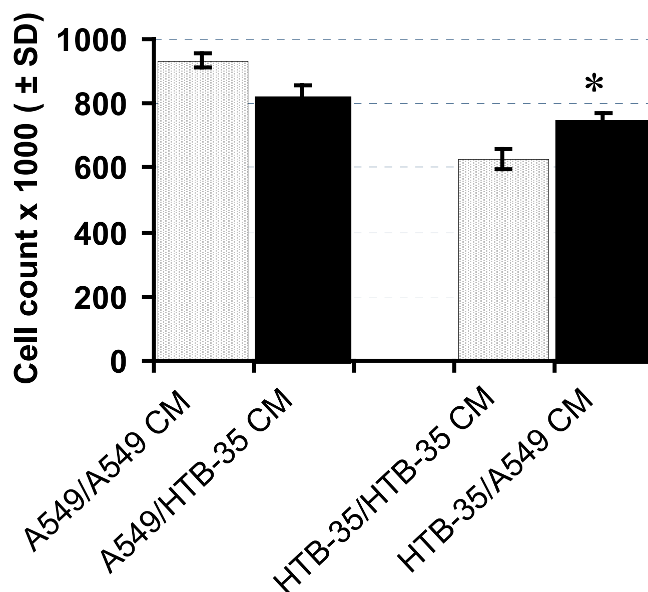


Fig. 3-39: Administration of *RAS*$_{mt}$-CM to culture of *RAS*$_{wt}$ cells enhances proliferation. 10 ^{5} cells were seeded in 60 mm tissue culture dishes, after 24 hours medium was changed with serum free medium. 24 hours after starvation cells were administrated by different CM as indicated. Cell population was determined 36 hours later using Cell Analyser System. Data represent the mean of 6 parallel experiments. Asterisk statistically significant enhancement of cell population in HTB-35 cells treated with CM from A549 (HTB-35/A549 CM) in comparison to control cells treated with native CM (HTB-35/HTB-35 CM) (Student´s t-test/ $p<0.05$).

3.5.7 K-RAS-siRNA inhibits autocrine/paracrine secretion of EGFR ligands in K-RAS mutated cells and mediates radiosensitization

To investigate whether the autocrine activation of EGFR dependent PI3 kinase pathway in A549 and MDA-MB-231 cells is mediated through mutated K-*RAS*, these cells were transfected with K-*RAS* siRNA. Fig. 3-40 indicates the functionality of this approach as expression of K-*RAS* protein which, is markedly reduced in K-*RAS*-siRNA transfected A549 and MDA-MB-231 cells in comparison to control-siRNA transfected cells. As tested with normal *RAS* presenting HTB-35 and FaDu cells, treatment with conditioned media from both K-*RAS*-siRNA transfected A549 and MDA-MB-231 cells markedly repressed phosphorylation of AKT and ERK1/2. Transfection of A549 or MDA-MB-231 cells with control siRNA, however, did not result in repression of the secreted activity in *RAS*$_{mt}$-CM stimulating P-AKT in HTB-35 and FaDu cells.

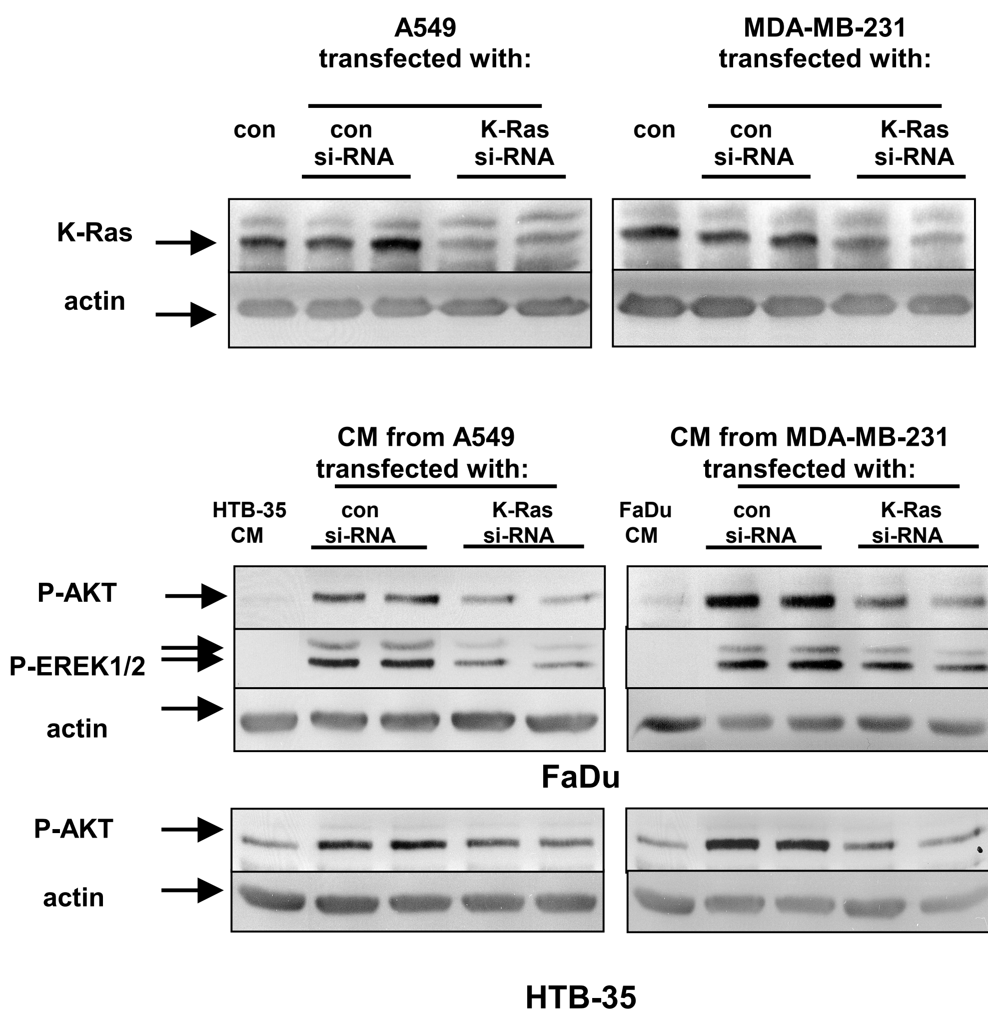


Fig. 3-40: siRNA targeting of K-*RAS* in *RAS*$_{mt}$ A549 and MDA-MB-231 cells blocks the autocrine secretion of EGFR ligands and mediates radiation sensitivity. A549 and MDA-MB-231 cells were transfected with 50 nM negative control siRNA or specific siRNA against K-*RAS*. 4 days later cells were lysed and SDS-PAGE as well as immunoblotting to detect K-*RAS* protein and actin was performed. 48 hours after transfection of A549 and MDA-MB-231 cells medium was replaced with serum free medium and cells were starved for 48 hours to prepare *RAS*$_{mt}$-CM. *RAS*$_{wt}$ cells (FaDu, HTB-35) were treated with *RAS*$_{mt}$-CM for 15 min. Cell lysates were subjected to SDS-PAGE and detection of P-AKT and actin was performed by immunoblotting.

As a consequence of siRNA-dependent downregulation of K-*RAS*, A549 cells transfected with K-*RAS*-siRNA presented significant enhanced radiation sensitivity when exposed to single dose of 3 Gy (fig. 3-41).

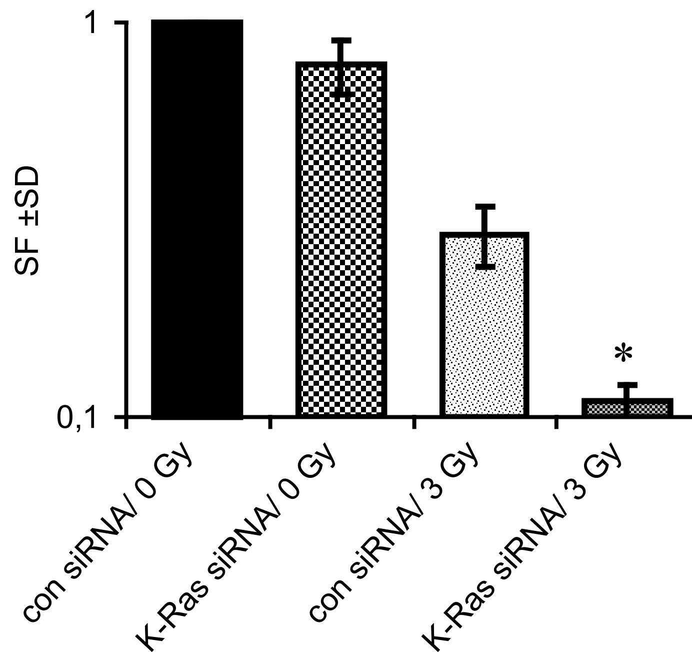


Fig. 3-41: siRNA targeting of K-*RAS* in RAS_{mt} A549 enhances radiation sensitivity. Radiation sensitivity of A549 cells transfected with control siRNA or K-*RAS*-siRNA was tested 3 days after transfection with siRNA by the colony formation assay performed 24 h after irradiation (single dose 3 Gy). Data bars shown represent the mean surviving fraction ± SD of 6 parallel experiments. Asterisk indicates statistical significant difference between irradiated cells transfected with con-siRNA and K-*RAS*-siRNA, respectively ($p < 0.05$; n=6, Student's t-test).

3.5.8 P-ERK1/2 dependent autocrine/paracrine secretion of EGFR ligands in K-RAS mutated tumor cells

It is known that mutations of *RAS* lead to constitutive activation (phosphorylation) of the mitogen-activated protein kinase (MAPK), also known as extracellular signal regulated protein kinase (ERK1/2) (Hsu, 2004). To address the question whether autocrine/paracrine secretion of EGFR ligands in K-*RAS* mutated human tumor cells is mediated through activation of MAPK and downstream cellular targets, *RAS* mutated A549 and MDA-MB-231 cells were serum depleted and incubated for 48 h with PD98059, a specific MEK inhibitor or appropriate concentration of vehicle, DMSO. Thereafter, CM from either inhibitor or vehicle treated cells were collected and the potential of each CM in activation of AKT was investigated in RAS_{wt} cells. As shown in Fig. 3-42 in comparison to the effect of CM from cells incubated to DMSO (vehicle) incubation of either A549 or MDA-MB-231 cells with indicated concentration of MEK inhibitor reduces level of P-AKT in FaDu and HTB-35 cells.

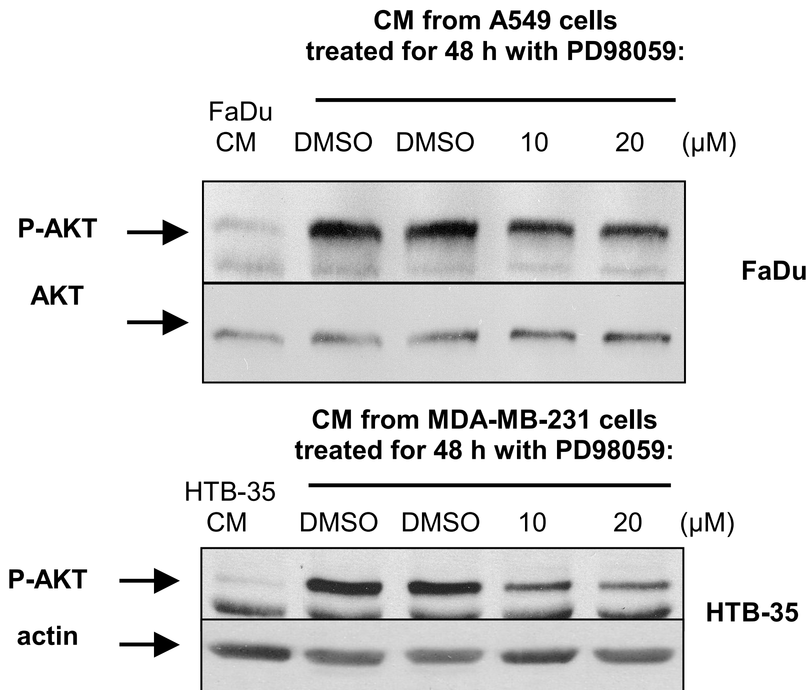


Fig. 3-42: P-ERK1/2 dependent autocrine secretion of EGFR ligands in K-*RAS* mutated human tumor cells. 24 h serum-depleted FaDu and HTB-35 cells were treated with CM from A549 or MDA-MB-31 cells preincubated with 10 or 20 µM MEK inhibitor, PD98059 during 48 h starvation. 15 min after administration of CM cell lysates were prepared and subjected to SDS-PAGE. Applying specific antibodies detection of P-AKT, AKT and actin was performed by immunoblotting.

3.5.9 Autocrine secretion of EGFR ligands and exogenous growth factors in K-RAS mutated cells activate MAPK/ERK as well as PI3K-AKT pathways in a manner independent of Ras activity.

In order to address the question whether phosphorylation of AKT is independent of Ras activity, RAS_{mt}-CM was fed to serum-starved RAS_{wt}- cells (HTB-35) for 15 min. As shown in Fig. 3-43A phosphorylation of AKT is increased 8.5-fold by RAS_{mt}-CM when compared to controls stimulated with autologous CM. However, RAS_{mt}-CM did not stimulate Ras-GTP in HTB-35 cells, indicating that phosphorylation of AKT is independent of Ras-GTP activity but dependent on PI3K activity. This assumption is further supported by the fact that in K-*RAS* mutated A549 cells (Fig. 3-43B) the selective EGFR-TK-inhibitor BIBX1382BS completely abolished the basal level of P-AKT, although Ras-GTP was not affected by this inhibitor.

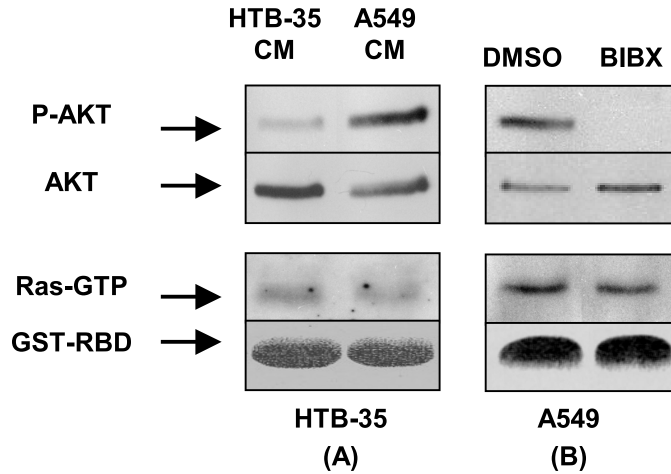
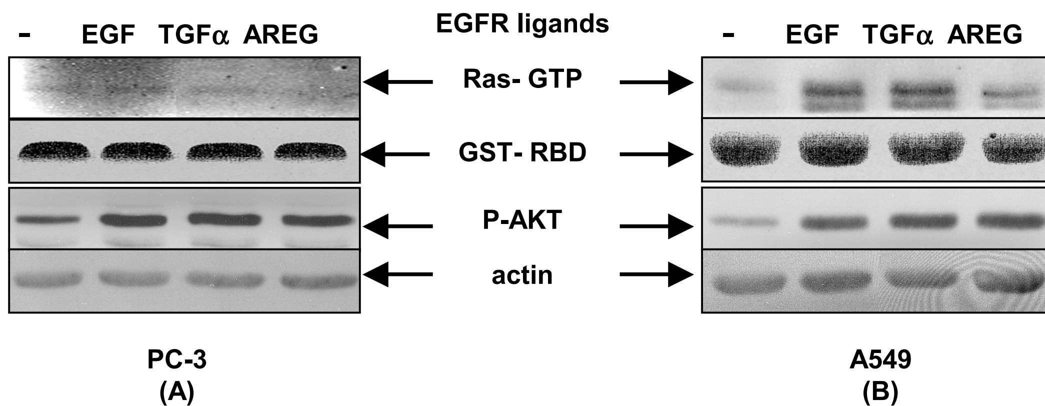


Fig. 3-43: Phosphorylation of AKT is independent of Ras activity. A) 48 h serum-starved RAS_{wt}-cells (HTB-35) were incubated with autologous-CM or RAS_{mt}-CM (from A549 cells) for 15 min. B) 48 h serum-starved RAS_{mt}-cells (A549) were treated either with DMSO (final concentration 0.1 %) or with 5 µM BIBX1382BS for 30 min. Thereafter cells were lysed and GTP bound active $p21^{ras}$ was isolated from lysates as described in *Methods*. 100 µg protein (from samples which were analysed for Ras-GTP) was subjected to SDS-PAGE and P-AKT as well as total AKT protein were assessed using specific antibodies.

In second approach we examined the response pattern of K-*RAS* mutated human tumor cells lines A549, MDA-MB-231 and PC-3 to stimulation with EGF, AREG and TGFα. As shown in Fig. 3-44A, stimulation of PC-3 cells with EGF resulted in only a slight enhancement of Ras-GTP while TGFα and AREG did not stimulate Ras-GTP. However, ligands stimulation resulted in a strong phosphorylation of AKT as a read out of the PI3K activity. In A549 cells, although stimulation with EGF and TGFα in contrast to AREG resulted in an enhancement of Ras-GTP, no difference on AKT phosphorylation by different ligand was observed (Fig. 3-44B).



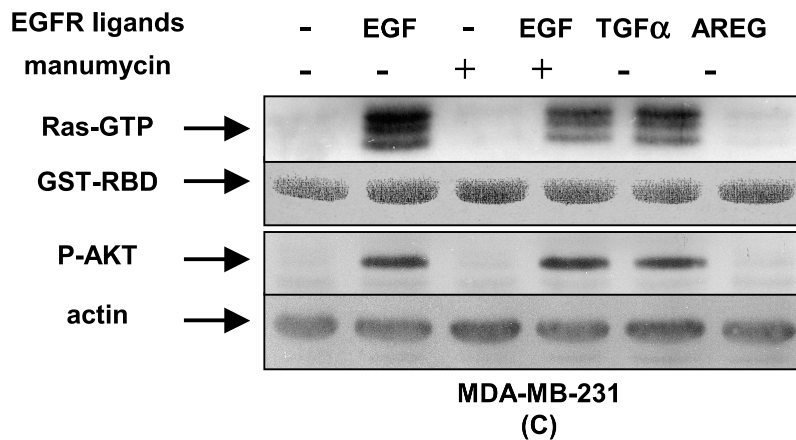


Fig. 3-45: Pattern of Ras-GTP and AKT activity following stimulation with EGF, TGFα and AREG. 48 h serum-starved K-RAS_{mt} PC-3, A549 and MDA-MB-231 cells were treated with 100 ng/ml EGF, TGFα or AREG for 5 min. Thereafter cells were lysed and GTP bound active $p21^{ras}$ was isolated from lysates as described in *Methods*. 100 µg protein (from samples analysed for Ras-GTP) was subjected to SDS-PAGE and P-AKT as well as actin proteins were assessed using specific antibodies.

To further proof Ras-GTP independent PI3K-AKT activation, MDA-MB-231 cells were treated with same concentration of EGF, TGFα and AREG and tested for Ras and AKT activity. As shown in Fig. 3-45C, AREG did not activate Ras and AKT in MDA-MB-231 cells. In contrast, EGF stimulated Ras in a higher level than the effect of TGFα but level of AKT activity via both ligands is same. Likewise, in MDA-MB-231 cells pretreated with 5 µM Manumycin as Farnesyl Transferase Inhibitor (FTI) for 2 hours and stimulated with EGF showed indicate that FTI reduces level of Ras-GTP to 50% without any effect on P-AKT (Fig. 3-45C).

Collectively presented results showed that there is a significant quantitative difference at the level of Ras-GTP and phosphorylation of AKT as a read out of PI3 kinase activity.

As a conclusion, these data suggest that activation of AKT/PKB via EGFR ligands in the tumor cell lines tested is directly modulated via EGFR and independent of Ras activity. This conclusion is further supported by the results so far obtained from EGFR ligands (EGF, TGFα, AREG) induced and radiation induced AKT activation in A549, MDA-MB-231, HTB-35 and FaDu cells transfected with siRNA against K-*RAS* and H-*RAS* (data not shown).

3.6 ALTERATION IN REPAIR OF RADIATION DAMAGE TO DNA, PROBABLE MECHANISM OF BIBX1382BS INDUCED RADIOSENSITIZATION

An early response to IR is phosphorylation of histone H2AX which is critical for recognition and repair of DNA Double Strand Breaks (DSBs) (Paull *et al.,* 2000; Ward & Chen, 2001). Phosphorylated H2AX can be as a predictor of radiation sensitivity and the target for radiation therapy (Taneja *et al.,* 2004). It is demonstrated that Ataxia-telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA-PK) function to phosphorylate H2AX after exposure to ionizing radiation (Stiff *et al.,* 2004).

With respect to the mechanism of radiosensitization following blockage of EGFR only few studies have focused on DNA damage repair. Huang and Harari suggest the redistribution of DNA-dependent protein kinase from the nucleus to the cytosol by anti-epidermal growth factor receptor monoclonal antibody C225 as a potential mechanism which may influence the cellular response to radiation (Huang and Harari, 2000). Delayed repair of DNA strand breaks after treatment with etoposide combined with Gefitinib (Iressa) as an EGFR TK inhibitor has also been reported (Friedmann *et al.,* 2004).

To provide more evidences concerning to the role of DNA repair in radiosensitizing, the pattern of radiation induced phosphorylation of H2AX, ATM and DNA-PK in the presence and absence of BIBX1382BS and LY294002 in A549 and FaDu cell lines was investigated.

3.6.1 Differential modulation of H2AX phosphorylation by inhibitors of EGFR and PI3 kinase in A549 and FaDu cells

To confirm the tight association between DNA DSBs and H2AX phosphorylation (Nazarov *et al.,* 2003), A549 cells were irradiated with single dose IR 1, 2 and 4 Gy. Level of H2AX phosphorylation in ser 139, the major H2AX residue phosphorylated in response to DNA damage (Rogakou *et al., 1998*) was analysed. As shown in Fig. 3-46 a linear radiation dose dependent phosphorylation of H2AX in A549 cells in clinically relevant doses of radiation (0 to 4 Gy) could be observed.

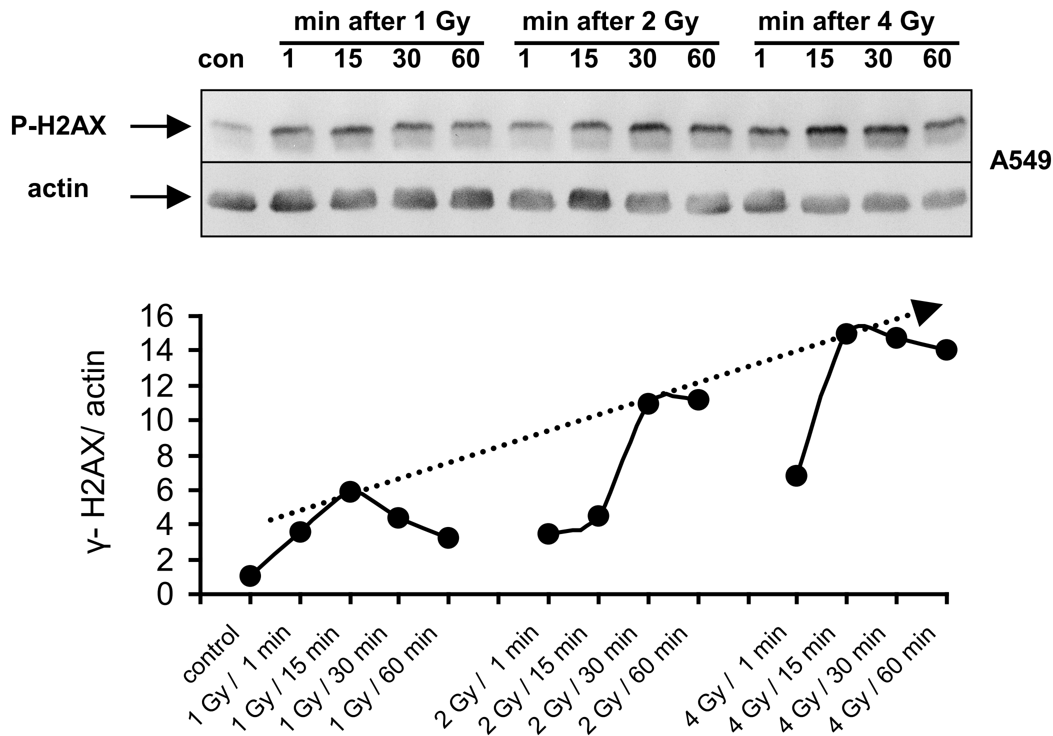


Fig. 3-46: Radiation dose dependent phosphorylation of H2AX in A549 cells. 48 h serum-starved A549 cells were exposed to single dose IR. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against P-H2AX (ser-139) and actin. Based on densitometry ratios of P-H2AX:actin (normalized to 1.0 control) maximum level of H2AX phosphorylation at each time point was calculated which indicate a radiation dose dependent enhancement of phosphorylation.

Based on the observed radiosensitizing effect of BIBX1382BS and LY294002 in A549 but not in FaDu cells, the level of P-H2AX in the presence and absence of both inhibitors was analysed after single dose IR 8 Gy. As shown in Fig. 3-47 in both cells lines H2AX phosphorylation was enhances immediately (1 min) after IR. However the level of phosphorylation in A549 in comparison to FaDu cells is stronger and pre-treatment with both inhibitors blocked the radiation induced H2AX phosphorylation in A549 but not in FaDu cells.

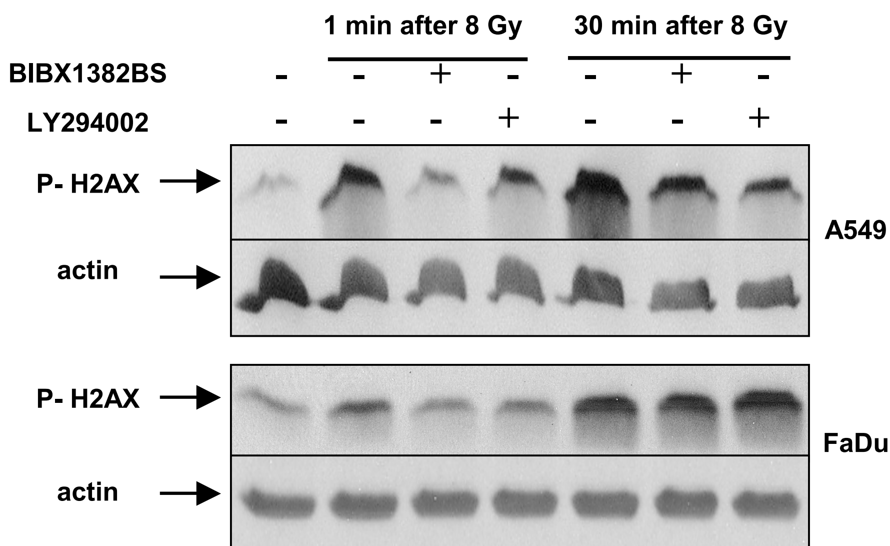


Fig. 3-47: Differential modulation of H2AX phosphorylation by EGFR and PI3 kinase inhibitors in A549 and FaDu cells. 48 h serum-starved A549 and FaDu cells were pretreated for 30 min with BIBX1382BS, LY294002 or DMSO (vehicle) and irradiated with single dose IR of 8 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against P-H2AX (ser-139) and actin.

In a further experiment HTB-35 cells were used since it was shown that EGFR and PI3K inhibitors differentially modulate radiation response in this cell line. As shown in Fig. 3-48 single dose IR (8 Gy) enhanced H2AX phosphorylation as early as 1 min after IR. However, pre-treatment with LY294002 blocked the P-H2AX while no effect of BIBX1382BS could be observed.

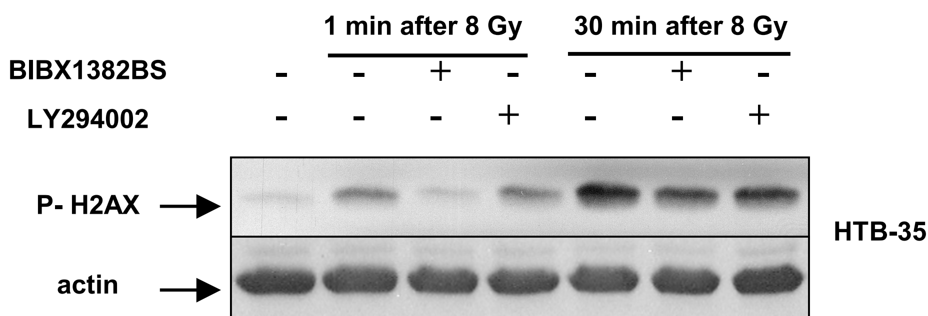


Fig. 3-48: Differential modulation of H2AX phosphorylation by EGFR and PI3 kinase inhibitors in HTB-35 cells. 48 h serum-starved HTB-35 cells were pretreated for 30 min with BIBX1382BS, LY294002 or DMSO (vehicle) and irradiated with single dose IR of 8 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against P-H2AX (ser-139) and actin.

3.6.2 DNA-PK but not ATM may contribute to differential effects of EGFR and PI3K inhibitors on IR induced H2AX phosphorylation

As discussed, it is widely accepted that ATM and DNA-PK are the major player in phosphorylation of H2AX in response to DNA-DSBs. However, it is not clear whether modulation of activation of these two kinases plays a role in the effect of EGFR and PI3K inhibitors on H2AX phosphorylation. To indicate that differential modulation of H2AX phosphorylation via the inhibitors in *RAS* mutated and *RAS* wild type cells arises from differential effect of inhibitors on ATM or DNA-PK, activation of both kinases following irradiation in the presence and absence of the antagonists was analysed.

As shown in Fig. 3-49 IR immediately (1 min) enhances ATM phosphorylation (ser1839) in both A549 and FaDu cell lines. However neither in A549 nor in FaDu cells any inhibitory effect of both antagonists was observed.

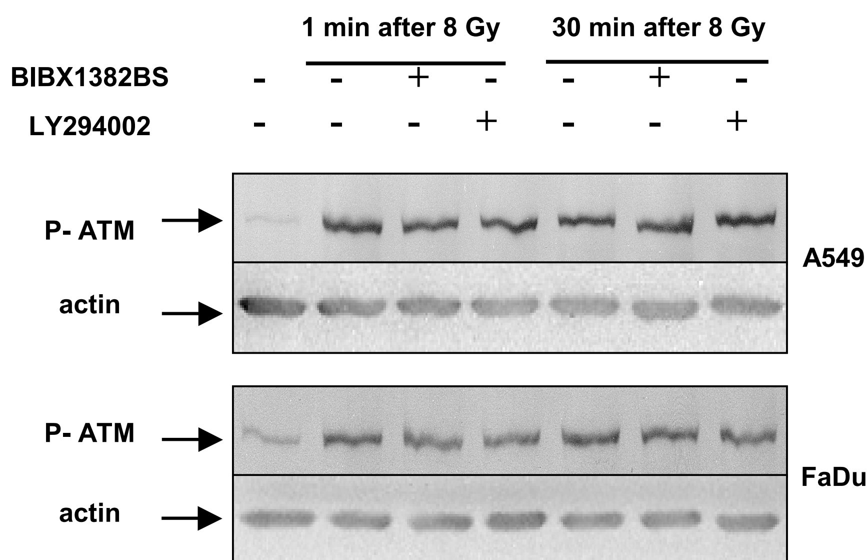


Fig. 3-49: Radiation dose dependent phosphorylation of ATM in A549 and FaDu cells. 48 h serum-starved A549 and FaDu cells were exposed to single dose IR 8 Gy. Cells were lysed at the times indicated, subjected to SDS-PAGE and incubated with antibodies against P-ATM (ser-1981) and actin.

Furthermore in both cell lines DNA-PK activity following irradiation in the presence and absence of BIBX1385BS and LY294002 was examined. As shown in Fig. 3-50 pretreatment with the inhibitors significantly reduced the activity of DNA-PK following IR either in kinase assay or autophosphorylation at Thr2609 only in A549 cells but no antagonistic effect could be observed in FaDu cells.

Fig. 3-50: Differential effect of BIBX1382SB and LY294002 on DNA-PK activity in A549 and FaDu cells. A) 48 h serum-starved A549 and FaDu cells were exposed to single dose IR, 2 Gy and 10 min after IR cells were lysed. Following immunoprecipitation of DNA-PK the immunocomplexes were washed with washing and kinase buffers. Kinase reaction and SDS-PAGE were performed as explained in *methods*. Thereafter gels were dried and phosphorylated GST-TP53 was visualized by autoradiography. B) 200 µg fraction of whole cell lysates used for kinase assay were subjected to SDS-PAGE and incubated with pT2609 antibody. Thereafter blots were stripped and reprobed with total DNA-PKcs antibody.

3.6.3 Cytokinesis-block micronuclei assay

Chromosomal aberrations are a direct consequences of DNA damage following irradiation that can be monitored by different assays, *e.g. metaphase analysis*, *premature chromosome condensation (PCC)*, *sister chromatid exchange (SCE)* and the *micronuclei assay (MNi)*. Micronuclei assay is proposed to be a simple alternative approach to assess chromosomal damages. Furthermore, micronuclei assay enables to reliably measure both chromosome loss and chromosome breakage. As shown in Fig. 3-51, MNi as a reflection of DNA damage contain either chromosomal fragments (acentric fragments) or whole chromosomes unable to migrate to the spindle poles during mitosis.

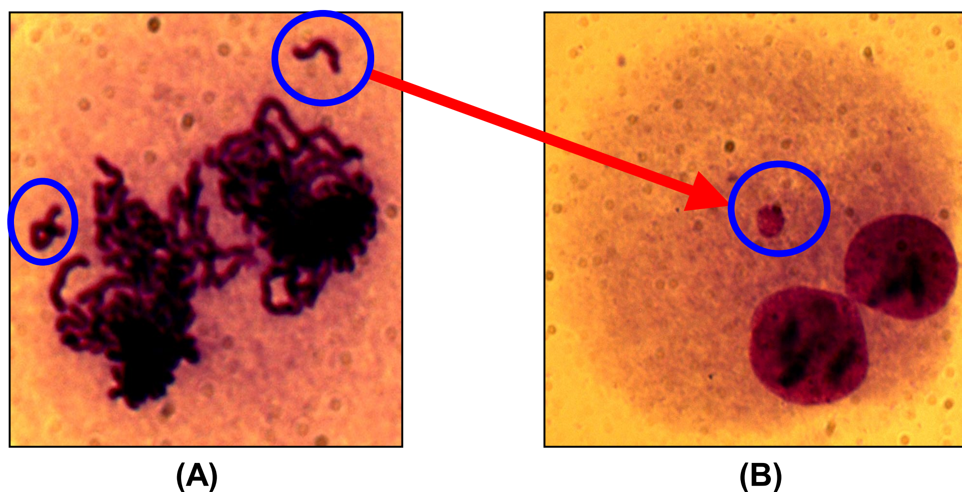


Fig. 3-51: The origin of micronuclei from lagging whole chromosomes or acentric fragments at anaphase (A) and typical appearance as well as relative size of micronuclei in binucleated A549 cells (B). Exponentially growing A549 cells were pretreated with BIBX1382BS for 30 min and irradiated 2Gy. Immediately after irradiation cells were treated with 2 µg/ml cytochalasine-B and after 24 hours slides were prepared as explained in *Methods*.

Therefore, MNi provide a convenient and reliable index of both chromosome breakage and chromosome loss (Fenech, 2000). This assay was initially developed for used with cultured human lymphocytes but has now been adapted to various cells types such as solid tumors as predictive variable for response to treatment (Shidnia *et al.,* 1990) or as an index for radiosensitization in both tumors or normal tissues (Ono *et al.,* 1993).

To provide evidences indicating the prevention of DNA damage repair by BIBX1382BS as partial mechanism in radiosensitization in K-*RAS* mutated cells but not in *RAS* wild type cells, cytokinesis-block micronuclei assay was performed. As shown in Fig. 3-52, BIBX1382BS treatment before IR in A549 cells enhances frequency of radiation induced MNi in binucleate A549 cells while no effect is observed in FaDu cells.

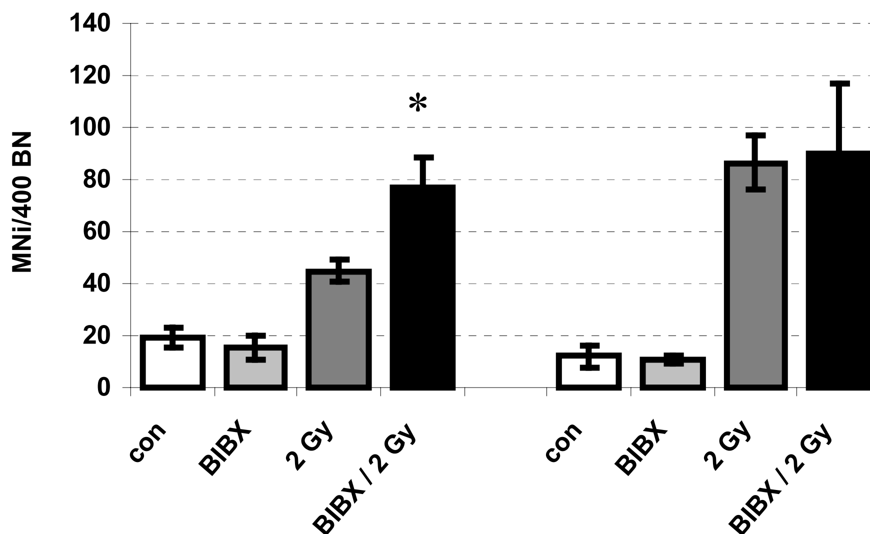


Fig. 3-52: Differential effect of BIBX1382BS on the frequency of radiation induced micronuclei in A549 and FaDu cells . Exponentially growing A549 and FaDu cells were pretreated with BIBX1382BS (5 µM) or DMSO followed with or without 2 Gy irradiation. Immediately after irradiation all conditions were treated with 2 µg/ml cytochalasine-B and after 24 hours slides were prepared as explained in *Methods*. Frequency of micronuclei (MNi) was determined in binucleate (BN) cells based on accepted criteria (Fenech, 2000). Asterisk indicates statistically significant enhancement in frequency of radiation induced micronuclei in A549 cells pretreated with BIBX1382BS (Student´s t-test/ $p<0.05$). Data are average of 4 different experiments. Bars: SD.

4 DISCUSSION

4.1 Targeting of epidermal growth factor receptor in cancer therapy

Increased expression of EGFR has been observed in a wide spectrum of tumors including non-small cell lung cancer (NSCLC) and squamous cell carcinoma (SCC) of head and neck (Grandis *et al.,* 1993; Rusch *et al.,* 1993) and has been correlated to disease progression and poor overall clinical outcome (Nicholson *et al.,* 2001; Buchholz *et al.,* 2005; Azria *et al.,* 2005). Therefore, a panel of inhibitors of the EGFR has been developed and is available for preclinical and in some cases already clinical use. In addition to single agent therapy, these inhibitors when combined with conventional cytotoxic therapies, such as chemotherapy or radiation therapy may improve clinical outcome. Because overexpressed or mutated EGFR can mediate and promote radioresistance and even repopulation of tumor cells during radiation therapy (Kavanagh *et al.,* 1995; Liang *et al.,* 2003; Baumann and Krausse, 2004), EGFR targeting especially during local applicable therapy seems promising. Although the detailed molecular understanding of EGFR antagonists are not elucidated by now, blockage of the EGFR by small molecule tyrosine kinase (TK) inhibitors and antibodies against the extra-cellular domain of the receptor have already been introduced into clinical trials.

Over the recent years preclinical data have accumulated indicating a very heterogeneous response of different tumor cells *in vitro* and tumor entities *in vivo* to EGFR inhibitors, which may reflect cell or tumor type specific differences in response pattern most likely based on the genetic status. Despite of a clear inhibition of EGFR-TK-activity, inability of small molecule TK inhibitors with respect to modulation of radiation response has been reported to various tumors (Baumann *et al.,* 2003; Krause *et al.,* 2004; Giocanti *et al.,* 2004). Thus, a detailed understanding of the molecular requirements for effective use of EGFR inhibitors to enhance radiation sensitivity of tumor cells is missing so far and needs to be established.

Since the expression level of EGFR does not always necessarily reflect its activation status, this may indicate some limitations regarding the use of EGFR expression as a predictor of response to specific inhibitors (Suzuki *et al.,* 2003). With respect to differential antiproliferative or radiosensitizing effects of EGFR inhibitors, this issue becomes more and more complicated and provides the reason to intensify *in vitro* studies to identify molecular and biological predictors of tumor cell responses to the EGFR inhibitors, such as monoclonal

antibodies or TK inhibitors. Introducing such prediction markers will thus help to discriminate pationts as EGFR responders or non-responders. This will enhance treatment efficacy for a variety of cancer types for which radiation therapy is the superior treatment modality.
As addressed above accelerated repopulation of tumor cells during radiation treatment has been well defined as a phenomenon that adversely impacts overall tumor response and thus local control (Petersen *et al.,* 2001; Baumann *et al.,* 2003). A proposed mechanism for this phenomenon involves the capacity of IR to activate EGFR which is linked to several components of mitogenic and proliferative signaling (Schmidt-Ullrich *et al.,* 1997; Dent *et al.,* 1999). This is in agreement with many reports indicating that EGF and its signaling through EGFR can promote cell proliferation and mediate cell survival by suppressing apoptosis when cells are exposed to various death signals (Lan and Wong, 1999; Payne *et al.,* 1999).

For all cell lines tested already a single dose IR of 2 Gy stimulated autophosphorylation of EGFR. Pretreatment of cells with specific EGFR TK inhibitor BIBX1382BS completely blocked this autophosphorylation. As reported by several groups, the autophosphorylation of EGFR induced by ionizing radiation occurs ligand independently (Todd *et al.,* 1999; Liang *et al.,* 2003) and is able to differentially activate EGFR dependent downstream pathways like PI3K-AKT and MAPK/ERKs. This aspect could very well be reproduced in the present study after IR.
In spite of preclinical data indicating radiosensitization of EGFR TK inhibitors in human tumor cells, using a well-established human tumor xenograft model in nude mice, *i.e.* the human head and neck tumor cell line FaDu, conflicting results of the effect of combined radiotherapy and EGFR antagonism have been reported by Baumann *et al.* (Baumann *et al.,* 2003). In this report no beneficial effect of blocking EGFR-tyrosine kinase activity by the small molecule TK inhibitor BIBX1382BS in combination with fractionated radiotherapy using local tumor control as experimental endpoint was observed. Although BIBX1382BS led to an inhibition of radiation-induced autophosphorylation of EGFR and exerted a strong antiproliferative effect in FaDu cells, both *in vitro* and *in vivo*, the results of the accompanying single dose irradiation *in vitro* and fractionated radiotherapy experiments *in vivo* revealed no radiosensitizing effect of EGFR blockage by BIBX1382BS (Baumann *et al.,* 2003; Krause *et al.,* 2004). The results on the response of FaDu-cells to combined BIBX1382BS and fractionated radiation treatment *in vitro* reported in the present work supports the findings by Baumann *et al.* for the same cell line. In both studies no radiosensitization could be observed although these cells presented a strong antiproliferative

effect when treated with BIBX1382BS alone. Furthermore in the present study combination of BIBX1382BS with radiation did not result in enhancement of radiation toxicity in cervix carcinoma cell line (HTB-35) and normal as well as transformed human skin fibroblasts HSF-7 and HH4-ded cells as well. Nevertheless FaDu, HTB-35, HSF-7 and HH4-ded cells, like all other cell lines tested herein, showed a clear radiation-induced EGFR-autophosphorylation, which was effectively inhibited by pretreatment with BIBX1382BS. Since the two tumor cell lines HTB-35 and FaDu cells demonstrate moderate to high level of EGFR protein, the simple quantification of EGFR may thus not guarantee a particular response pattern, *i.e.* radiosensitization induced by EGFR inhibitors. On the other hand, the potential of EGFR TK inhibitors to block radiation induced receptor autophosphorylation may not be a sufficient predictor to combat accelerated repopulation during fractionated radiation therapy.

4.2 BIBX1382BS induces radiosensitization through blockage of EGFR-PI3K-AKT dependent but MAPK/ERKs independent signaling pathway in K-*RAS* mutated tumor cells

Studying the radiosensitizing effect of BIBX1382BS in combination with single and fractionated dose irradiation showed that the bronchial adenocarcinoma cell line A549, the mammary tumor cell line MDA-MB-231 and the prostate carcinoma cell line PC-3 which all harbor a point mutation in K-*RAS* gene codon 12 (A549, PC-3) and codon 13 (MDA-MB-231), could be radiosensitized in response to inhibition of EGFR-autophosphorylation mediated by the inhibitor. To investigate specifically the molecular differences, which may underlie BIBX1382BS-mediated different radiation responses of K-*RAS* mutated (K- RAS_{mt}) and K-*RAS* wildtype (K-RAS_{wt}) cells, experiments were focused on the analysis of signaling cascades down-stream of EGFR in each group of cell lines.

In agreement with the literature, ligand-dependent or independent EGFR-activation stimulated mainly 3 downstream pathways such as the PI3K-AKT, the Ras-Raf-MAPK and the JAK-STAT pathways (Reardon *et al.,* 1999; Hynes *et al.,* 2001; Arteaga, 2002; Dent *et al.,* 2003). These pathways activate specific genes mediating enhanced cell cycle traversal, inhibition of cell death, increased cell adhesion and mobility potential. PI3K-AKT (Tenzer *et al.,* 2001; Fahy *et al.,* 2003) and MAPK/ERKs (Seong *et al.,* 2002) are the best characterized kinases down-stream of EGFR known to promote survival signals following radiation exposure. The importance of these pathways through stimulation of proliferation via MAPK or inhibition of

apoptosis via AKT in the regulation of cell survival following radiation exposure has been reported (Liang *et al.,* 2003). The direct comparison of BIBX1382BS radiosensitized K-RAS_{mt} cells and non-radiosensitized K-RAS_{wt} cells revealed distinct differences in the response profile of the AKT and MAPK pathways. The extent of AKT phosphorylation during radiation exposure was differentially sensitive to BIBX1382BS treatment. A complete inhibition of P-AKT below control levels could be achieved for K-RAS_{mt} cells after BIBX1382BS treatment, but not for *RAS* wild type FaDu and HTB-35 cells. For FaDu cells, BIBX1382BS led only to a reduction of the radiation induced P-AKT to control levels, while in HTB-35 cells in spite of radiation induced EGFR phosphorylation no AKT phosphorylation after IR was observed. BIBX1382BS treatment in this cell line could not reduce basal level of AKT activity presumably indicating an EGFR independent PI3K activity. However, EGF treatment resulted in phosphorylation of AKT in both K-RAS_{mt} and K-RAS_{wt} cell lines and the response was inhibited to the same degree by BIBX1382BS. Regarding to the role of another receptor TK activity mediating resistance to anti-EGFR therapy, Chakravarti *et al.* reported that constitutive IGFR-1 dependent PI3K activity is involved in resistance to EGFR TK inhibitor AG1478. The authors have shown that co-targeting of IGFR-1 and EGFR abrogates resistance to EGFR inhibition and enhances radiation induced apoptosis (Chakravarti *et al.,* 2002). Thus, these data indicate a specific difference in the PI3K-AKT signaling pathway in response to ligand-dependent or irradiation-dependent activation of EGFR.

The assumption that inhibition of EGFR-PI3K-AKT pathway may be involved in BIBX1382BS induced radiosensitization is further supported by the experiments applying the specific PI3K inhibitor, LY294002. Low concentration of this inhibitor (10 µM) which specifically blocks activation of receptor tyrosine kinase dependent PI3K, completely inhibited basal level and radiation induced AKT phosphoprylation in K-RAS_{mt} cells and strongly enhanced radiation response of these tumor cells in combination with single dose of irradiation. LY294002 in concentrations up to 75 µM tested did not influence the radiation induced activity of PI3K family member ATM in A549 cells (data not shown). Together as summarized in Fig. 4-1 the data presented herein indicated that targeting of EGFR-PI3K-AKT pathway enhances radiation sensitivity in K-RAS_{mt} cells.

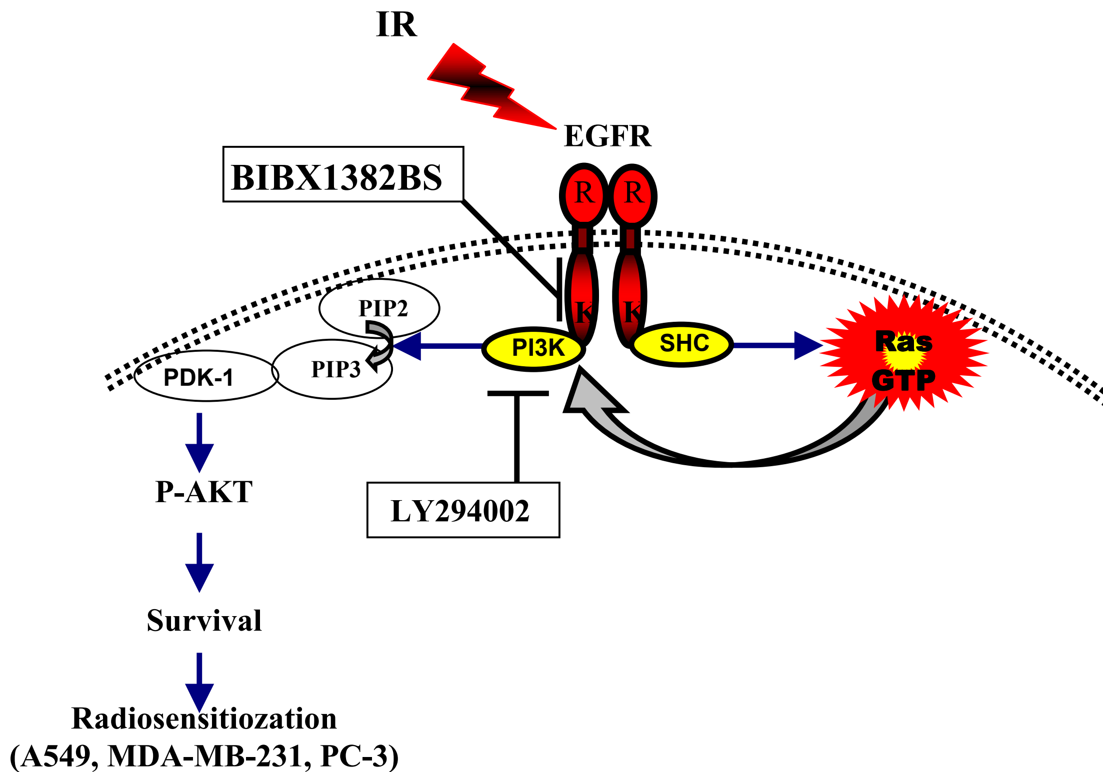


Fig. 4-1: Blockade of EGFR-PI3K-AKT pathway enhances radiation toxicity in K-*RAS*$_{mt}$ tumor cells.

EGFR-PI3K-AKT signaling is an important anti-apoptotic pathway primarily involved in the regulation of tumor cell survival (Bronner *et al.,* 2000; Shelton *et al.,* 2005). Especially for radiation-induced apoptosis there is clear evidence that this process does not play a major role in the inactivation of human solid tumor cells by ionizing radiation both *in vitro* and *in vivo* (Brown and Wouters, 1999; Bromfield *et al.,* 2003; Brown and Attardi, 2005). The data on a significant K-*RAS*-mutation specific radiosensitizing effect of the EGFR-TK inhibitor BIBX1382BS is in complete agreement with this view. In this study only slight enhancement of apoptotic cell death could be observed which does not account for the increased cellular radiation sensitivity. Preliminary data from ongoing studies in our laboratory further support this assumption. Although wildtype p53 is an essential component of the pathway leading from DNA damage to apoptosis (Lin *et al.,* 2002) no alteration in the radiation dose response curve of A549 presenting wildtype p53 and A549 cells expressing mutated p53 is apparent after BIBX1382BS treatment and subsequent single or fractionated dose irradiation. This results further indicate that radiosensitization by BIBX1382BS is independent of p53-mediated apoptosis.

It is very likely that most advanced solid tumor cells derive their growth advantage from more than one aberrant molecular growth pathway. However, phosphorylation of ERK1/2 which is mediated through ligand-dependent or ligand-independent EGFR phosphorylation (Chakravarti *et al.,* 2002) could be a hallmark of MAPK activation and reliable predictor of cell proliferation (El Sheikh *et al.,* 2004). As tested herein for different cell lines, blockage of EGFR autophosphorylation by BIBX1382BS resulted in a differential antiproliferative effect. The complete inhibition of this mitogenic pathway in FaDu cells under normal control conditions could be one reason for the highest antiproliferative efficacy of BIBX1382BS in these cells. Furthermore under the same conditions, basal P-ERK1/2 could only be reduced in A549 cells to a certain degree but not blocked completely, like in FaDu cells. This indicates that lack of the antiproliferative effect of BIBX1382BS in A549 cells was not due to inability of P-EGFR inhibition but rather due to a lack of inhibition of P-ERK1/2. This assumption is in agreement with a negative correlation between expression of P-ERK1/2 and response to EGFR TK inhibitor, gefitinib in NSCLC reported by Han *et al* (Han *et al.,* 2005).

As shown, FaDu cells responded with the most sensitivity to BIBX1382BS treatment as tested by the inhibition of ERK1/2 phosphorylation following radiation exposure. In comparison to FaDu cells, irradiation-induced ERK1/2 phosphorylation in serum starved A549 cells was not exclusively sensitive to BIBX1382BS treatment. The assumption that ERK1/2 activation is independent of EGFR in A549 cells is supported by the data sets summarized as follows:

1. The similar pattern of SHC phosphorylation and co-immunoprecipitation of Grb2 to SHC in BIBX1382BS responsive FaDu cells and non-responsive A549 cells which is corresponding to EGFR phosphorylation time points
2. Immediate activation of EGFR in A549 cells but delayed phosphorylation of ERK1/2 following IR
3. Inhibition of EGF induced ERK1/2 activation by BIBX1382BS in A549 cells

Based on these results and the data observed for the other cell lines under the study it can be concluded that ERK1/2 phosphorylation induced via IR is mainly independent of EGF receptor activity and probably dependent on another member of cytoplasmic (*e.g.* src) or membrane bound receptor tyrosine kinases.

As discussed above, the level of radiation induced P-ERK1/2 in A549 cells was not affected by BIBX1382BS treatment but could completely be abolished by treatment with the specific MEK inhibitor PD98059. Nevertheless, radiation sensitivity of A549 cells was not affected by

PD98059 treatment. Insensitivity of the clonogenic survival of irradiated cells to the MEK inhibitor PD98059 indicates a MAPK/ERKs independent enhancement of radiation toxicity by BIBX1382BS. This conclusion is further supported by studies indicating lack of radiosensitization in a number of RAS_{wt} and RAS_{mt} cell lines following blockage of MAPK/ERKS pathway (Gupta *et al.,* 2001; Grana *et al.,* 2002; Kim *et al.,* 2005).

Based on the data discussed so far and summarized in Fig. 4-2 it can be concluded that an effective approach to predict any response of tumor cell to EGFR tyrosine kinase inhibitors would be to measure the inhibitory effect on key signal transduction pathways downstream of EGFR, *i.e.* MAPK/ERKs (antiproliferative effect) and PI3K-AKT (radiosensitizing effect) rather than just inhibition of receptor phosphorylation.

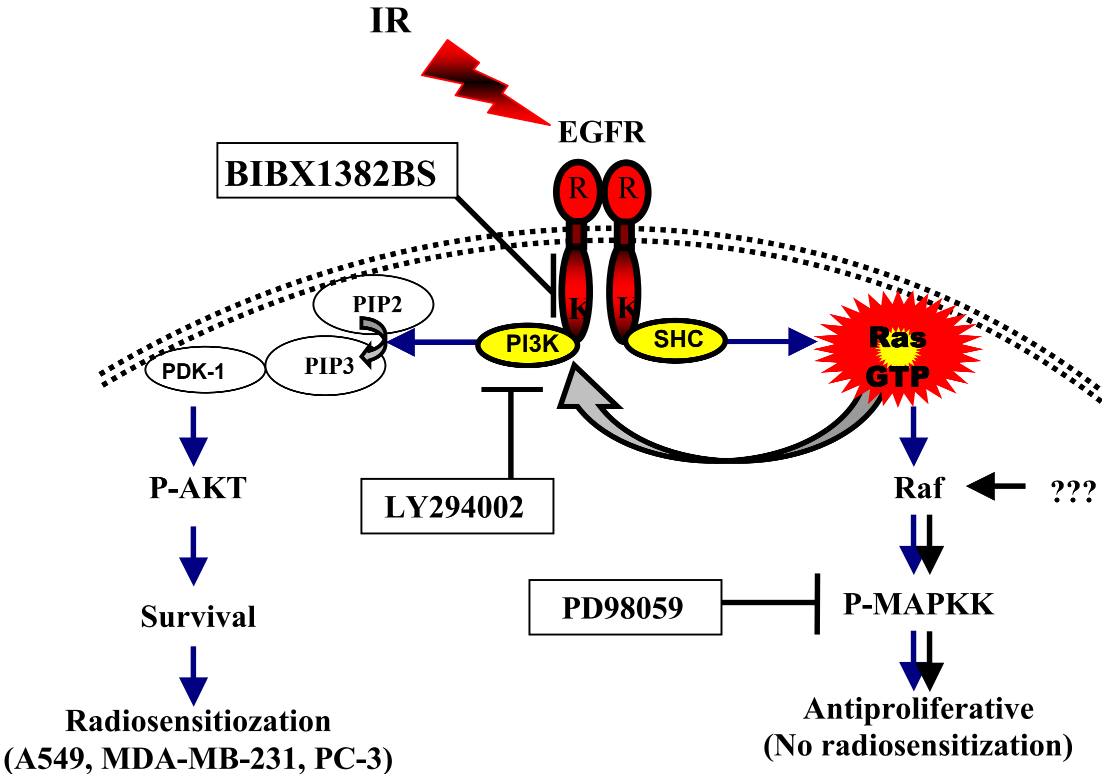


Fig. 4-2: Targeting of different EGFR downstream pathways results in differential effects on proliferation and survival.

4.3 Autocrine secretion of EGFR ligands in K-*RAS* mutated tumor cells enhances radioresistance via EGFR-PI3K-AKT pathway

Cell proliferation, differentiation and survival are generally regulated by a variety of growth factors and cytokines. In cancer cells dysregulated cellular signaling and proliferation may occur through overexpression or mutation of proto-oncogenes. In this context the proto-oncogene *RAS* plays a major role in controlling the differentiation or proliferation . In human cells, three potentially oncogenic *RAS* genes (H-*RAS*, K-*RAS*, N-*RAS*) exist which code for highly related but distinct proteins. Mutations are usually limited to only one of the *RAS* genes and are generally dependent on tissue and tumor type. K-*RAS* mutations occur frequently in NSCLC, colorectal and pancreatic carcinomas; H- *RAS* mutations are common in bladder, kidney and thyroid carcinomas; N-*RAS* mutations are primarily found in melanoma, hepatocellular carcinoma and hematologic malignancies. Approximately 30% of all human neoplasms harbor a mutation in *RAS* genes. Mutations occur most frequently in K-*RAS* and less often in H-*RAS* (Adjei, 2001). Ras activation via mutation has been reported to confer radioresistance (Bernhard *et al.,* 1996; Sklar, 1988; Grana *et al.,* 2002; Grana *et al.,* 2003) indicating activation of survival pathways by the mutations and interaction of ionizing radiation with these pathways. Consistent with this hypothesis are reports indicating the role of PI3K pathway in Ras mediated radioresistance (Bernhard *et al.,* 2000; Gupta *et al.,* 2001; Grana *et al.,* 2002; Caron *et al.,* 2005) and conversely, radiation sensitivity by inhibition of activated Ras *e.g.* by farnesyl transferase inhibitors (Bernhard *et al.,* 2000; Brunner *et al.,* 2003; Brunner *et al.,* 2004).

As addressed, while studying the radiosensitizing effect of blockage of EGFR TK activity in K-*RAS*-mutated human tumor cells experiments were performed with the PI3K inhibitor, LY294002. Radiation sensitivity in K-RAS_{mt} cells was enhanced with the PI3K inhibitor as well. AKT phosphorylation analysed as downstream component of PI3K activity indicated that except in A549, IR did not modulate AKT activity in PC-3 and MDA-MB-231 cells. In contrast, all three cell lines presented high basal level of P-AKT in nonstimulated condition. Therefore it might be concluded that constitutive activation of EGFR-PI3K-AKT pathway is the target of BIBX1382BS and LY294002 for radiosensitization. This conclusion is supported by the radiosensitizing effect of pan erbB tyrosine kinase inhibitor, CI-1033 *in vitro* and *in vivo* only in tumor cells which express permanent activated EGFR and erbB2 (Nyati *et al.,* 2004).

EGFR overexpression, EGFRvIII and autocrine secretion of EGFR ligands are the mechanisms that may contribute to constitutive activation of the PI3K-AKT pathway (Liang *el al.,* 2003; Grana *el al.,* 2003; Li *el al.,* 2004). EGFRvIII is a mutated EGFR characterized by the deletion of exons 2 to 7, resulting in a sense mutation that has a truncated extracellular domain with ligand-independent constitutive kinase activity and non-responsive to EGFR ligands EGF and TGFα (Ekstrand *et al.,* 1994). The results presented herein indicate that K-*RAS*$_{mt}$ tumor cells in comparison to *RAS*$_{wt}$ tumor cells or normal fibroblast do not generally overexpress EGFR. Likewise, the potential of EGFR ligands in receptor activation and downstream pathways in K-*RAS*$_{mt}$ cells and the radiosensitizing effect of C225, an EGFR neutralizing antibody, in A549 and MDA-MB-231 cells (Dittmann *et al.,* 2005) indicate the absence of EGFRvIII in K-*RAS* mutated cells. Therefore autocrine/paracrine secretion of EGFR ligands seems to be the potential mechanism for constitutive activation of PI3K-AKT pathway in K-*RAS*$_{mt}$ human tumor cells.

In this context, Grana *et al.* investigated whether conditioned media from cells transformed with oncogenic H-*RAS* or administration of TGFα can induce radioresistance (Grana *et al.,* 2003). It was shown that radioresistance was dependent on EGFR-activation mediated by either TGFα or conditioned medium from H-*RAS*-transformed cells and subsequent stimulation of the downstream survival pathway PI3K-AKT. Resistance to radiation was abrogated when cells were treated with the EGFR/erbB2-kinase inhibitor GW57206. These results are in good agreement with the data from the present study showing that conditioned medium from K-*RAS*$_{mt}$ human tumor cells is effective in stimulating EGFR phosphorylation and activating especially the P3K-AKT survival pathway in normal *RAS* presenting human tumor cell lines. In the present investigation although exogenously added TGFα-mediated radioresistance in K-*RAS*$_{wt}$ cells, and neutralizing antibody against this ligand induced radiosensitization only in K-*RAS*$_{mt}$ cells, differences in basal production of TGFα at least at the mRNA level could not be demonstrated between K- *RAS*$_{mt}$- and *RAS*$_{wt}$- tumor cells. At the current state the mechanism of differential radiosensitization effect of TGFα neutralizing antibody in K-*RAS*$_{mt}$- and K-*RAS*$_{wt}$ tumor cells remains unclear.

However, in comparison to *RAS*$_{wt}$-cells, K-*RAS*$_{mt}$- tumor cells presented an app. 2-fold increase in amphiregulin (AREG) mRNA expression. This EGFR ligand led to a significant radioresistance of normal *RAS* presenting cells and its neutralization by a specific antibody markedly inhibited the activation of PI3K-AKT survival pathway and enhanced radiation sensitivity of K-*RAS*$_{mt}$ cells. These results present for the first time evidence that AREG not only is able to stimulate radioresistance, but is likely a prominent autocrine factor in

mediating resistance of K-*RAS*-mutated human tumor cells to IR through a selective stimulation of the EGFR-dependent PI3K-AKT survival pathway. This data are in line with reports on radiosensitization of A431 squamous carcinoma cells by C225 (Milas *et al.,* 2000; Nasu *et al.,* 2001) presumably due to inhibition of autostimulation by EGFR ligands production in culture medium (Van de Vijver *et al.,* 1991).

Although very attractive, this hypothesis does not rule out the possibility that unknown genotypic differences between these different tumor cells presenting K-*RAS* mutation might be involved in the autocrine secretion of EGFR ligands as well. For further support of the hypothesis siRNA transfection experiments were thus performed. A pronounced inhibition of AKT phosphorylation was observed when RAS_{wt} cells were fed with conditioned media (CM) from K-RAS_{mt} cells transfected with K-*RAS* siRNA. This data provided direct evidence for the role of K-*RAS* in autocrine/paracrine secretion of EGFR ligands in human tumor cells presenting K-*RAS* mutation. In addition, using clonogenicity as an end point, it was shown that radiation sensitivity of K-*RAS*-siRNA transfected A549 cells is enhanced. This results are in line with radiosensitizing effect of farnesyl transferase inhibitor, FTI-277 for K-RAS_{mt} cell lines (Bernhard *et al.,* 1998). FTIs block the farnesylation of the Ras protein necessary for membrane anchoring (Brunner *et al.,* 2003). Thus the report by Bernhard *et al.* and the data presented herein strongly support the role of K-*RAS* mutation in mediating radioresistance. Furthermore, based on our data on the effect of CM from K-RAS_{mt} cells it can be proposed that radioresistance of tumor cells presenting K-*RAS* mutations is mediated through an autocrine and constitutively EGFR activating mechanism, which involves mutated K-*RAS* dependent overproduction of EGFR ligands, such as AREG.

With respect to stimulation of the pro-proliferative Raf/MAPK/ERKs pathway, mutated *RAS* signaling is mainly independent of upstream EGFR activation (Blaker *et al.,* 2004). The exact mechanism involved in the enhanced autocrine secretion of EGFR ligands in K-RAS_{mt} cells is not understood so far. In H-*RAS* mutations it has been proposed that downstream activation of MAPK/ERKs might lead to production of EGFR ligands and could stimulate EGFR in an autocrine fashion (Hamilton and Wolfmann, 1998; Martinez-Lacaci *et al.,* 2000). As shown here, incubation of serum starved A549 and MDA-MB-231 cells with specific MEK inhibitor, PD98059 resulted in complete blockage of P-ERK1/2 in both cell lines. Furthermore when RAS_{wt} cells were fed with CM from these cells, pronounced inhibition of AKT phosphorylation was observed. This might indicate a prominent role of upregulated P-ERK1/2 in K-*RAS* mutated tumor cells for stimulated autocrine secretion of EGFR ligands.

4.4 Ras-GTP independent PI3K-AKT activation following EGFR stimulation

As shown in Fig. 4-2 PI3K-AKT survival pathway induced through EGFR-signaling is thought to be dependent on Ras-GTP activity (Dent *et al.,* 2003). This assumption is supported mainly by studies using normal cells transformed with oncogenic *RAS* (Grana *et al.,* 2002; Jin *et al.,* 2003). However, evidence is accumulating that EGFR-mediated activation of PI3K-AKT can occur independent of Ras as described for cells presenting *EGFR* muttation (Moscatello *et al.,* 1998; Li *et al.,* 2004; Luwor *et al.,* 2004). Regarding to blockage of P-EGFR by TK inhibitor BIBX1382BS, treatment of non-stimulated A549 cells by the inhibitor resulted in a complete blockage of P-AKT. However, basal Ras-GTP was not modulated in this cell line at all. Likewise, the strong activation of AKT in HTB-35 cells treated with A549 CM without any effect on Ras-GTP supported the assumption that stimulation of EGFR in *RAS* mutated cells predominantly activates the PI3K-AKT survival pathway in response to EGFR stimulation independent of Ras-activity. Furthermore data so far obtained from ongoing studies with K-*RAS* and H-*RAS* siRNA transfection in both K-RAS_{mt} (A549, MDA-MB-231) and RAS_{wt} (FaDu, HTB-35) cells is supporting this assumption. In these experiments although K-*RAS* and H-*RAS* expression were completely suppressed by siRNA but the level of AKT activity induced by EGFR ligands (EGF, AREG, TGFα) and IR was not affected. Data by Bhat-Nakshatri *et al.* showing a direct activation of PI3K through tyrosine kinase activity of EGFR are in line with these findings (Bhat-Nakshatri *et al.,* 2002). The discussed RAS-GTP independent autocrine activation of EGFR-PI3K-AKT pathway in K-RAS_{mt} cells is summarized in Fig. 4-3.

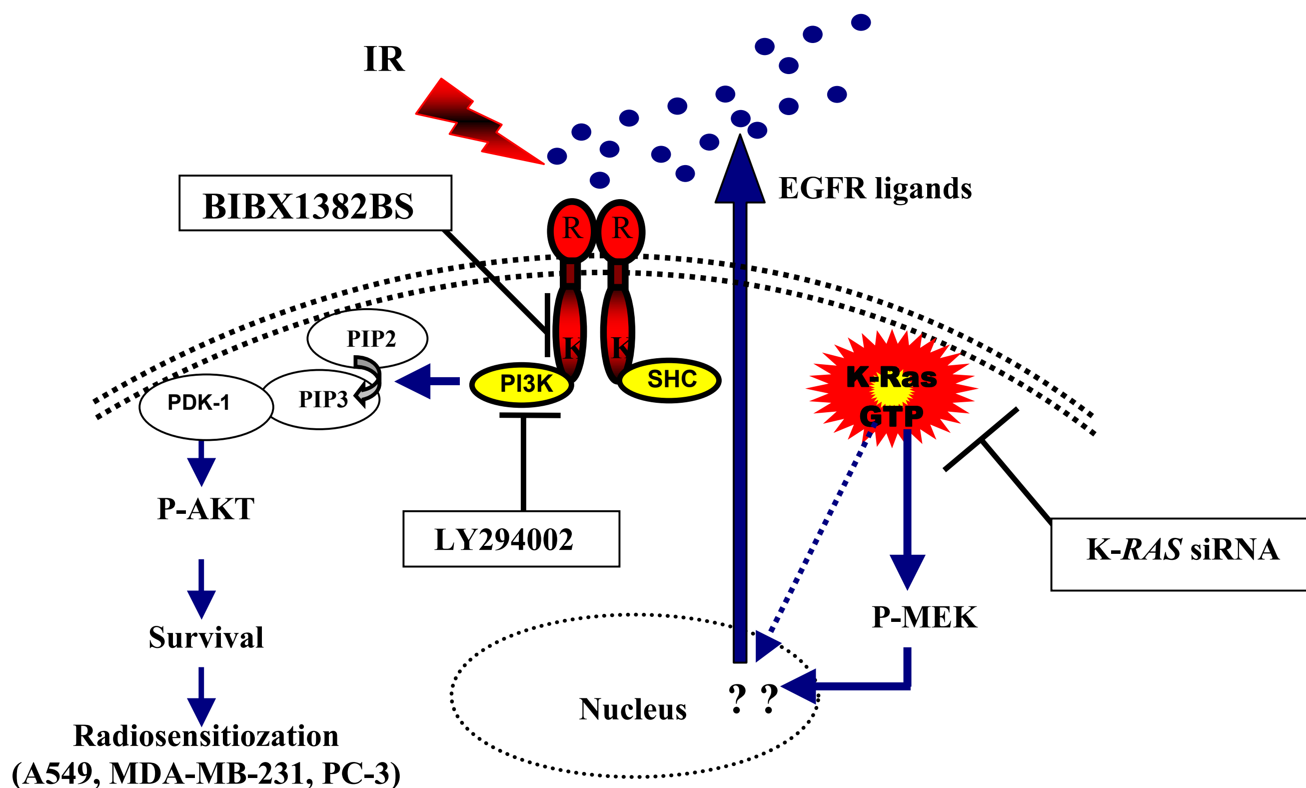


Fig. 4-3: Role of constitutively active Ras-GTP in the activation of EGFR-PI3K-AKT pathway in K-*RAS*$_{mt}$ cells via autocrine secretion of EGFR ligands.

4.5 Blockage of EGFR-PI3K-AKT signaling increases radiation sensitivity of K-*RAS* mutated human tumor cells *in vitro* by affecting DNA repair

In addition to the already addressed, *i.e.* cell cycle progression and apoptosis, the radiosensitivity by EGFR inhibitors may also be consequent of DNA repair process affected by the inhibitor treatment. Various studies investigating the chemo- or radiosensitizing effects of EGFR antagonists (TK-inhibitors as well as EGFR-specific antibodies, *i.e.* C225) reported that besides marked effects on proliferation and cell survival inhibition of EGFR signaling may also affect DNA-damage repair potentially through downregulation of DNA-PK (Huang and Harari, 2000; Friedmann *et al.,* 2004; Dittmann *et al.,* 2005). Therefore in the present study DNA repair as a function of inhibition of EGFR-PI3K-AKT pathway was investigated.

It is generally assumed that efficient DNA repair in actively growing cells requires growth factor signaling. This assumption is supported by the chemosensitizing effect of anti-EGFR monoclonal antibodies and the enhanced sensitivity to cytotoxic drugs in tumor cells lacking EGFR (Park *et al.,* 2005). In the context of ionizing radiation, DNA double strand breaks (DNA-DSB) are the most important DNA lesions leading to radiation-induced cell kill (Khana and Jackson, 2001). Two processes are primarily involved in repair of DNA-DSB, *i.e.* non-homologous endjoining (NHEJ) and homologous recombination (HR) (Iliakis *et al.,* 2004). Profound knowledge exists that NHEJ is the major process of DNA-DSB repair responsible for cell survival after exposure to ionizing radiation (Pastwa and Blasiak, 2003) and the DNA-PKcs is the critical protein in this process.

Detailed analyses into the interaction of EGFR with DNA-PK and the specific role of EGFR in regulating the activity profile of DNA-PK from our laboratory indicated that internalization and translocation of activated EGFR into the nucleus seems to be an important step in the activation of DNA-PK (Dittmann *et al.,* 2005). It could clearly be shown that the monoclonal EGFR-antibody C225 not only can sensitize *in vitro* cultured K-RAS_{mt} cells (A549 and MDA-MB231) to ionizing radiation but also does abolish nuclear translocation of radiation-induced EGFR which leads to a significant inhibition of DNA-PK activity as well as repair of DNA-DSB and decreased clonogenic cell survival (Dittmann *et al.,* 2005). Although these results imply that protein-protein interactions of internalized and nuclear translocated EGFR is necessary for the observed effects on DNA-repair, these experiments do not rule out the potential role of the EGFR-PI3K-AKT signaling cascade in the regulation of DNA-PK and control of DNA-repair. The EGFR tyrosine kinase inhibitor BIBX1382BS and the PI3K inhibitor LY294002 both abrogated radiation-induced kinase activity of DNA-PKcs and its autophosphorylation at Thr-2609 which is indicative for the activated nuclear repair enzyme (Chan *et al.,* 2002*)* only in K- RAS_{mt} A549 but not K-RAS_{wt} FaDu cells The differential inhibitor effect was not due to a potential effect of BIBX1382BS and LY294002 on the expression of total DNA-PK protein in the two cell lines, since it did not alter the content of nuclear DNA-PK. Recently Shintani *et al.* reported that inhibition of EGFR-TK-inhibitor by gefitinib/iressa (ZD1839) leads to a reduction of the nuclear fraction of DNA-PKcs protein (Shintani *et al.,* 2003) which correlates with an enhanced radiation sensitivity of human oral cancer cell mouse xenografts. Without giving any detailed information at the functional level of DNA-PK (*e.g.* Thr-2609 phosphorylation or kinase assay data as well as DNA-damage repair measurements), the authors discuss that this result suggests a mechanism whereby the repair of DNA-DSB after radiation exposure is impaired. Reduction of nuclear fraction of

DNA-PK at the total protein level has also been described by Huang and Harari after EGFR blockage by C225 (Huang and Harari, 2000). How these results obtained with gefitinib and C225 compare to our results at the functional level of DNA-PK presented herein for the EGFR-inhibitor BIBX1382BS is currently not clear. Nevertheless our data of reduced DNA-PK activity measured at the level of Thr-2609 phosphorylation clearly correlates with the impaired DNA-damage repair activity determined at the level of micronuclei formation 24 h post irradiation. Furthermore, the presented BIBX1382BS- as well as LY294002-mediated blockage of IR induced DNA-PKcs activity and its effect on the modulation of proteins involved in the recognition of DNA-DSB and execution of damage repair, clearly indicates the functional requirement of the EGFR-PI3K-AKT-pathway in regulating DNA-repair machinery after exposure to ionizing radiation.
In contrast to radiation induced H2AX and DNA-PK phosphorylation, ATM phosphorylation was not affected by the treatment of either cell line with the EGFR and PI3K inhibitors. However, the ATM protein has been reported to be the major kinase responsible for histone H2AX phosphoryaltion in response to DNA-DSBs induced by ionising radiation (Burma *et al.,* 2001). In the view of this assumption our data might indicate that DNA-PKcs but not ATM is at least in part activated via radiation induced EGFR-PI3K-AKT signaling to modulate H2AX phosphorylation. In this context further investigations to elucidate the role of other kinases, *i.e.* ATR, in mediating phosphorylation of H2AX downstream of EGFR are necessary. So far, however, on the basis of the inhibitor experiments described the data presented in this report provide the first direct evidence that EGFR-dependent stimulation of the PI3K-AKT pathway can directly regulate the activation profile of DNA-PK as well as removal of radiation-induced DNA-damage, mainly DNA-double strand breaks in K-RAS_{mt} human tumor cells.

4.6 Concluding discussion

Taking all information from the literature and the data presented together, the pathways and mechanisms involved in the regulation of radiation resistance in K-RAS_{mt} human tumor cells as well as the principles of molecular targeting approaches to inactive EGFR-signaling by the EGFR-specific tyrosine kinase inhibitor BIBX1382BS can be proposed as outlined in Fig. 4-4. According to these mechanisms radioresisitance of K-RAS_{mt} human tumor cells presenting constitutively active K-Ras-GTP activity is mediated through upregulated production and secretion of the EGFR ligand AREG. In an autocrine loop AREG can then bind to EGFR and

stimulate EGFR-signaling. In this model PI3K-AKT activation is directly triggered by EGFR and is not mediated via Ras-GTP. Targeting of each component of the K-Ras-AREG-EGFR-PI3K-AKT pathway via different strategies enhances radiation toxicity in K-RAS_{mt} human tumor cells but not in RAS_{wt} cells. With respect to the functional endpoint radiosensitization, *i.e.* reduced cell survival, blockage of DNA repair via impaired induction of DNA-PKcs through EGFR-PI3K-AKT pathway is presumably the important mechanism. Thus, impaired DNA-repair will lead to the accumulation of unrepaired, residual DNA damage (*i.e.* micronuclei) which ultimately result in cell death via the mitotic catastrophy (Brown and Wouters, 1995; Gudkov and Komarova 2003; Brown and Attardi, 2005) rather than apoptotic cell death.

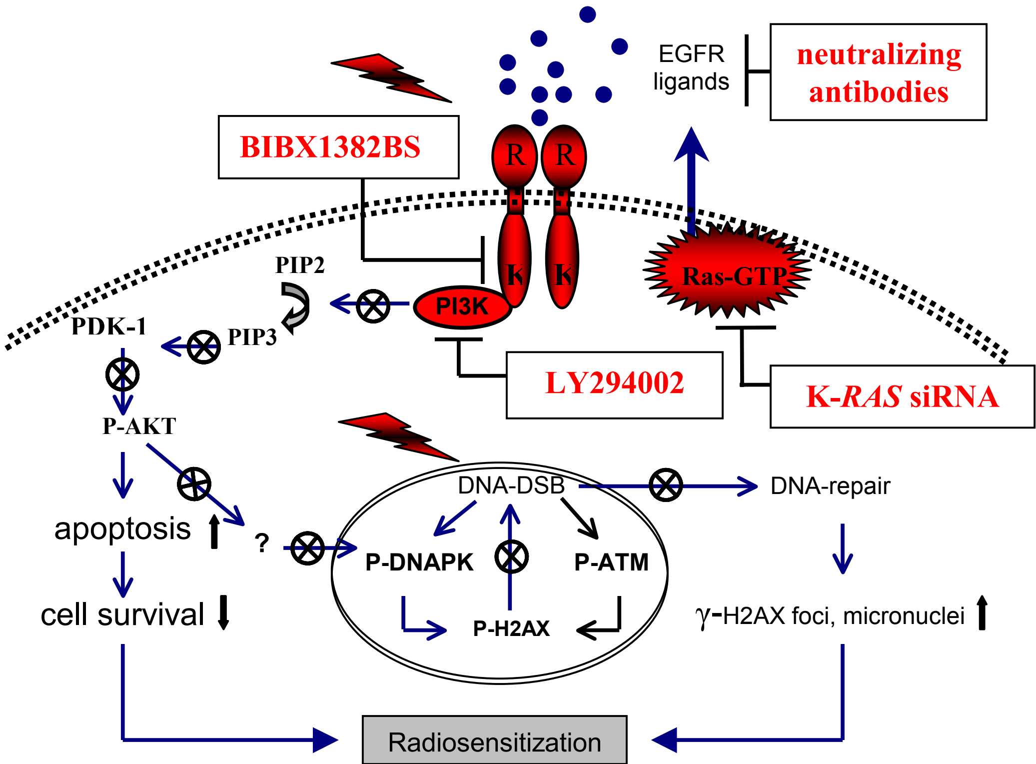


Fig. 4-4: Schematic illustration of the pathways involved in radiosensitization of K-RAS_{mt} human tumor cells.

OUTLOOK

On the basis of the data presented herein the following questions need to be addressed in consecutive study to further elucidate the mechanisms proposed.

- Does BIBX1382BS radiosensitize K-RAS_{mt} tumor xenografts in nude mice?
- Do other selective inhibitors of EGFR-TK-activity mediate the same molecular and cellular responses in K-RAS_{mt} human tumor cells *in vitro* and tumor xenograft models *in vivo*?
- Does activated AKT directly mediate induction of nuclear DNA-PKcs through phosphorylation at Thr-2609?
- Are other DNA repair processes than NHEJ, like base excision repair (BER) or homologous recombination (HR) also affected by EGFR-TK-inhibitors?

Answering these questions will help to completely understand the complex mechanisms underlying the processes of radiosensitization of human tumor cells via EGFR-targeting approaches. Thus, by taking into account the molecular and genetic principles of radiorestistance/radiosensitization specific strategies for the effective clinical use of EGFR-antagonists in radiation oncology can be developed to improve therapy outcome of patients with EGFR overexpressing tumors.

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Radioresistance of K-RAS mutated human tumor cells is mediated through EGFR- dependent but Ras-independent activation of PI3K-AKT pathway.

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