Doctoral Thesis

Relevance of the PI3K/Akt survival signaling in the cellular stress response to ionizing radiation

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Relevance of the PI3K/Akt Survival Signaling in the Cellular Stress Response to Ionizing Radiation

A dissertation submitted to the
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ABSTRACT

Cancer is still a major cause of death. Thus, an important interest of cancer research is to find and improve new treatment strategies. Ionizing radiation (IR) belongs with surgery and chemotherapy to the three major cancer treatment modalities. IR can be applied as single therapy, as combined therapy with chemotherapy or as palliative therapy, but it is also often applied as a pre- or postoperative adjuvant therapy to prevent the growth of new tumor tissue from residual sites (recidive).

A benefit of ionizing radiation as a therapeutic tool is the possibility to apply it locoregionally, thereby preventing systemic toxicity. However, like chemotherapeutics, IR does affect all the afflicted cells eventually leading to severe side effects after therapy. In addition, there are a large number of human malignant tumor cells showing a very poor response to IR. Furthermore, the applied dose to the tumor cannot be increased like needed because of the neighboring tissue and organs located in the radiation field. Thus, a major goal of cancer research is to improve the knowledge on the molecular processes induced by radiotherapy to optimize therapies and to overcome treatment failures.

The degree of tumor cell radioresistance is not only dependent on its own genetic background but is also influenced by its microenvironment. Thus, characterization of the intracellular as well as intercellular communicative signals associated with IR is of major importance to improve molecular targeted chemo-radiotherapy.

Lesions in the DNA (single and double strands breaks) constitute the central cellular damages induced by ionizing radiation. As reaction a multitude of molecular and cellular processes is activated which can result in cell cycle arrest, induction of the DNA-repair machinery (to restore damage of DNA) or in cell death (post mitosis or by apoptosis). However, IR also initiates a complex network of signaling processes independent of DNA-damage. These signal transduction cascades represent interesting targets for anticancer treatment modalities to combine ionizing radiation with molecular defined pharmacological compounds. Modulating specific key-entities of these signal transduction cascades potently sensitizes for radiation induced cell death.

In tumor cells, IR activates ErbB-receptors and downstream signaling via the phosphatidylinositol 3'-kinase (PI3K)/Akt survival pathway that protects against apoptotic stress stimuli. Additionally, this pathway is overexpressed in many human carcinomas and has been linked to tumorigenesis, tumor progression, invasion, metastasis and angiogenesis as well as poor treatment outcome and resistance to different cytotoxic drugs and IR. Therefore, compounds that downregulate this pathway are of clinical interest for single and combined anticancer treatment modalities.

The aim of this work was to analyse the relevance of the PI3K/Akt-pathway for the radiosensitizing potential of the two recently developed clinically relevant kinase inhibitors, PKC412 and PKI166. We further investigated the mechanism(s) leading to the activation of this signaling cascade upon radiation stimulation in different cell types (tumor and endothelial cells) by using the inhibitors of the ErbB1/2-class receptors and the VEGF-receptor, PKI166 and PTK787, respectively.

We demonstrated that the PI3K/Akt-pathway is a target for the antiproliferative and cytotoxic effects mediated by the radiosensitizer PKC412 (a staurosporine derivative protein kinase C (PKC)-inhibitor). Artificial expression – using a retroviral-based cell infection system – of the constitutive-active form of Akt in H-ras-oncogene-transformed mouse fibroblasts abrogated the antiproliferative and cytotoxic effects of PKC412. We further identified the tumor suppressor PTEN as a critical component for the PKC412-mediated inhibition of the PI3K/Akt-pathway, in contrary to the direct PI3K-inhibitor LY294002. This correlated with
the requirement of an intact PTEN for PKC412 to exert its antiproliferative and cytotoxic effects. Further, we analyzed the mechanism of action and radiosensitizing potential of the novel dual ErbB1/2-receptor inhibitor PKI166. PKI166 sensitized ErbB2-overexpressing cells to IR-induced apoptosis. However, cotreatment with PKI166 did not result in any enhancement of the radiation-induced antiproliferative effect in these cells. In contrary, PKI166 clearly enhanced the antiproliferative and cytotoxic effects of irradiation in two ErbB1-overexpressing cell lines. A connection between the ErbB2-receptor and PI3K/Akt-signaling was identified and PKI166 was shown to inhibit the activation of the PI3K/Akt-survival pathway in the ErbB2-overexpressing cells investigated.

In analogy to tumor cells, IR also activates the PI3K/Akt-survival cascade in endothelial cells, contributing to an increase in radioresistance. We showed that endothelial cells possess intact EGF- and VEGF-ligand-induced signaling from their corresponding receptors to Akt. Furthermore, receptor-selective signaling-inhibition was achieved by both receptor tyrosine kinase inhibitors PKI166 and PTK787, respectively. Importantly, we could detect direct VEGF-receptor-activation by IR in endothelial cells. Finally, using both inhibitors PKI166 and PTK787 we demonstrated that IR could induce the PI3K/Akt-survival pathway in both tumor and endothelial cells; however, this process occurred in a differential cell type and receptor dependent pattern, via ErbB-receptors in tumor cells and VEGF-receptors in endothelial cells.

In conclusion, the different mode of action between both PKC412 (PTEN-dependent) and LY294002 (PTEN-independent) on the PI3K/Akt-survival pathway indicates the importance of characterizing the pharmacogenetic profile to improve treatment specificity and efficacy. Moreover, we demonstrate that this signaling cascade is also targeted by PKI166 in ErbB2-overexpressing cells. However, the exact mechanism of action of this receptor kinase inhibitor still remains unclear and needs further investigations. Nevertheless, overall these studies demonstrate that inhibiting the PI3K/Akt-pathway represents a promising approach to enhance the cytotoxicity of ionizing radiation. In this work, we also characterize an additional mechanism induced by radiation in endothelial cells, which is important for tumor radioresistance linked to the tumor microenvironment. At last, this might help to refine and elaborate new treatment strategies combining radiation with small molecular defined compounds.
ZUSAMMENFASSUNG


Auf molekularer Ebene aktivieren ionisierende Strahlen in Tumorzellen die ErbB-Rezeptoren und damit die Phosphatidylinositol 3'-Kinase (PI3K)/Akt-Signalübermittlungskaskade, welche die Zellen gegen apoptotische Stressfaktoren schützt. Diese Kaskade ist in vielen humanen Tumoren überexprimiert und ist verantwortlich für aggressiveres Wachstum, Infiltration, Angiogenesis und Metastasierung, was letztendlich zur Resistenz gegen verschiedene zelltoxische Substanzen und Strahlung führt. Aus diesen Gründen sind Substanzen, die diesen spezifischen Weg hemmen, von grossem klinischen Interesse für die kombinierte Krebstherapie.

Das Ziel dieser Arbeit war die Relevanz der PI3K/Akt-Signalübermittlungskaskade für die radiosensibilisierende Wirkung der beiden neuen klinisch relevanten Kinasen-Inhibitoren PKC412 (PKC) und PKI166 (ErbB1/2) zu analysieren. Zudem, wurde der Mechanismus der Aktivierung dieser Kaskade nach Bestrahlung in Zellen unterschiedlicher Herkunft untersucht (Tumor- und Endothelialzellen). Dazu wurden zwei Rezeptor-Tyrosinkinase-Inhibitoren der ErbB1/2-Rezeptor Familie (PKI166) und des VEGF-Receptors (PTK787) verwendet.


Abschliessend kann gesagt werden, dass die unterschiedliche Wirkungsweise zwischen PKC412 (PTEN-abhängig) und LY294002 (PTEN-unabhängig) über die PI3K/Akt-Übermittlungskaskade darauf hindeuten, dass es wichtig ist, das pharmakogenetische Profil einer Substanz zu charakterisieren, um Behandlungsspezifität und -wirksamkeit zu verbessern. Überdies zeigten wir in ErbB2-überexprimierenden Zellen, dass diese Kaskade eine Zielstruktur von PKI166 ist. Der exakte Wirkmechanismus dieses Inhibitors bleibt aber unklar und braucht weitere Untersuchungen. Insgesamt zeigt diese Studie, dass die Inhibition dieser PI3K/Akt-Signalüberlebenskaskade einen vielversprechenden Ansatz darstellen, um die Strahlen-Zytotoxizität auf Tumorzellen zu erhöhen. In dieser Arbeit wurde ausserdem ein zusätzlicher strahleninduzierter Mechanismus in Endothelialzellen beschrieben, welcher für die Tumor-Radioreisistenz und gekoppelte Tumor-Mikro-Umgebung wichtig ist. Solche Untersuchungen könnten helfen, neue Behandlungsstrategien, welche Bestrahlung und kleine molekular definierten Substanzen kombinieren, zu verbessern.
RÉSUMÉ

Le cancer est encore de nos jours une cause majeure de décès. C’est pourquoi, un intérêt primordial de la recherche sur le cancer consiste à trouver et à améliorer de nouvelles stratégies de traitement. Les radiations ionisantes (RI) font partie avec la chirurgie et la chimiothérapie des trois traitements principaux du cancer. La radiothérapie peut être appliquée seule, en combinaison avec la chimiothérapie ou en tant que thérapie palliative. Elle est également souvent utilisée comme thérapie adjuvante pré- ou postopératoire, afin de prévenir le développement de nouveaux tissus tumoraux à partir de sites résiduels (récidive). La radiothérapie possède l’avantage de pouvoir être appliquée de façon très ciblée prévenant ainsi une toxicité systémique. Cependant, comme les substances chimiothérapeutiques, les radiations affectent toutes les cellules qu’elles atteignent, pouvant provoquer des effets secondaires sévères. De surcroît, de nombreuses tumeurs malignes ne répondent aux radiations que très faiblement. Enfin, la dose appliquable à une certaine tumeur est limitée par la sensibilité des tissus et organes avoisinants se trouvant dans le champ des radiations. Pour ces différentes raisons, un défi majeur de la recherche sur le cancer consiste à approfondir les connaissances des processus moléculaires induits par les radiations, afin d’améliorer les thérapies et de surmonter les défaillances de traitements.

Le degré de résistance aux radiations (radiorésistance) des cellules tumorales est non seulement dépendant de leur patrimoine génétique, mais est également influencé par le microenvironnement dans lequel elles se trouvent. C’est pourquoi, la caractérisation des signaux de communication, tant intra- que intercellulaires, associés aux radiations se révèle être primordiale afin d’améliorer les chimio/radiothérapies moléculaires ciblées.

Les lésions de l’ADN (simples et doubles ruptures de filaments) représentent les dommages cellulaires centraux induits par les radiations ionisantes. En réaction, une multitude de processus moléculaires et cellulaires est activée pouvant conduire à l’arrêt du cycle cellulaire, à l’induction de l’appareil de réparation de l’ADN ou encore à la mort cellulaire (post-mitotique ou par apoptose). Cependant, les radiations induisent également un réseau complexe de signaux indépendants des dégâts produits sur l’ADN. Ces cascades de signaux représentent des cibles très intéressantes pour les traitements anticancéreux combinant les radiations ionisantes à des substances pharmacologiques bien définies au niveau moléculaire. La modulation d’entités centrales et spécifiques de telles cascades de signaux sensibilise très efficacement à la mort cellulaire induite par les radiations.

Dans les cellules tumorales, les radiations ionisantes activent les récepteurs ErbB et, en aval de ceux-ci, la cascade phosphatidylinositol 3'-kinase (PI3K)/Akt favorisant la survie cellulaire et protégeant contre les stimulations de stress apoptotiques. De plus, cette cascade est surexprimée dans de nombreux carcinomes humains et est étroitement liée à la tumorigénèse, à la progression et à l’invasion des tumeurs, aux métastases, à l’angiogénèse ainsi qu’à une faible réponse aux traitements et à une résistance à différents médicaments et aux radiations. Des composés dérégulant cette cascade sont par conséquent d’un intérêt clinique particulier pour les traitements anticancéreux simples et combinés.

Ce travail a pour objectif l’analyse de l’importance de la cascade PI3K/Akt en rapport avec le potentiel radiosensibilisateur des deux inhibiteurs de kinases récemment développés et de relevance clinique, PKC412 et PKI166. Nous avons également étudié le(s) mécanisme(s) aboutissant à l’activation de cette cascade après stimulation par les radiations dans différents types cellulaires (cellules tumorales et endothéliales). Pour ce faire, nous avons utilisé les inhibiteurs de tyrosine kinase PKI166 et PTK787 des récepteurs des catégories respectives ErbB1/2 et VEGFR.
Nous avons démontré que la cascade cellulaire PI3K/Akt représente une cible pour l’effet antiprolifératif et cytotoxique induit par le radiosensibilisateur PKC412 (un inhibiteur de la protéine kinase C (PKC) dérivé de la staurosporine). L’expression artificielle d’une forme constitutivement active de Akt – au moyen d’un système d’infection rétrovirale de cellules – dans des fibroblastes de souris transformés par l’oncogène H-ras neutralise l’effet antiprolifératif et cytotoxique de PKC412. Nous avons identifié le suppresseur de tumeurs PTEN comme un composant critique pour l’inhibition de la cascade PI3K/Akt par PKC412, au contraire de LY294002 un inhibiteur direct de PI3K. De plus, la présence de PTEN sous sa forme intacte a été nécessaire pour que PKC412 puisse exercer ces effets antiprolifératifs et cytotoxiques.

Nous avons également analysé le mécanisme d’action et le potentiel de radiosensibilisation de PKI166, un nouvel inhibiteur double des récepteurs ErbB1 et ErbB2. PKI166 a sensibilisé des cellules surexprimant ErbB2 à l’apoptose induite par les radiations, mais n’a pas augmenté l’effet antiprolifératif induit par les radiations sur ces cellules. PKI166 a par contre accru les effets antiprolifératifs et cytotoxiques des radiations sur deux lignées cellulaires surexprimant ErbB1. Une relation entre le récepteur ErbB2 et la cascade PI3K/Akt a été identifiée et il a pu être démontré que PKI166 empêchait l’activation de la cascade PI3K/Akt dans les cellules testées surexprimant ErbB2.

De façon analogue aux cellules tumorales, les radiations activent également la cascade PI3K/Akt dans les cellules endothéliales, contribuant ainsi à un accroissement de la radiorésistance. Nous avons démontré que les cellules endothéliales possèdent des signaux intacts induits par les ligands EGF et VEGF à partir de leurs récepteurs respectifs à Akt. Ces signaux ont été sélectivement inhibés par les deux inhibiteurs correspondants, PKI166 et PTK787. Finalement, nous avons détecté d’une façon directe l’activation des récepteurs VEGFR par les radiations dans les cellules endothéliales. Par ces études, nous avons démontré que les radiations induisent la cascade de survie PI3K/Akt, aussi bien dans les cellules tumorales qu’endothéliales. Pourtant ce processus se produit de façon différentielle selon le type de cellule et dépend des récepteurs (ErbB dans les cellules tumorales et VEGFR dans les cellules endothéliales).

En conclusion, les modes d’action différents des deux composés PKC412 (dépendant de PTEN) et LY294002 (indépendant de PTEN) sur la cascade de survie PI3K/Akt indiquent l’importance de caractériser le profil pharmacogénétique d’une substance afin d’améliorer la spécificité et l’efficacité d’un traitement. De plus, nous démontrons que cette cascade est déréglée par PKI166 dans des cellules surexprimant ErbB2. Le profil pharmacogénétique exact de cette substance reste cependant lacunaire et nécessite des investigations supplémentaires. Ces études démontrent néanmoins que l’inhibition de la cascade de survie PI3K/Akt représente une approche prometteuse afin d’améliorer la cytotoxicité des radiations ionisantes. Dans ce travail, nous avons également caractérisé un mécanisme supplémentaire induit par les radiations dans les cellules endothéliales, lequel est important pour la radiorésistance des tumeurs par rapport à leur microenvironnement. Finalement, ces résultats peuvent contribuer à affiner et élaborer de nouvelles stratégies de traitement combinant les radiations ionisantes à des composés bien définis et de petit poids moléculaire.
Il cancro è, ancora oggi, una delle maggiori cause di decesso. Per questo motivo un interesse primordiale della ricerca sul cancro consiste nel trovare e migliorare delle nuove strategie di trattamento. Le radiazioni ionizzanti (RI) costituiscono, con la chirurgia e la chemioterapia, i tre trattamenti principali del cancro. La radioterapia può essere applicata singolarmente, combinata alla chemioterapia o come terapia palliativa. Essa viene spesso anche applicata come terapia ausiliaria pre- o postoperatoria per impedire lo sviluppo di nuovi tessuti tumorali da luoghi residui (recidività).

La radioterapia possiede il vantaggio di poter essere applicata in maniera molto mirata, evitando quindi una tossicità sistemica. Tuttavia, così come le sostanze chemioterapeutiche, anche le radiazioni colpiscono tutte le cellule che raggiungono, con il pericolo di provocare dei gravi effetti secondari. In aggiunta a questo, molti tumori maligni rispondono solo debolmente alle radiazioni. Infine, la dose applicata ad alcuni tumori è limitata dalla sensibilità di tessuti e organi situati nel campo elettromagnetico della radiazione. Per queste varie ragioni, una sfida principale della ricerca sul cancro consiste nell’approfondire le conoscenze dei processi molecolari indotti dalle radiazioni, allo scopo di migliorare le terapie e di superare le debolezze del trattamento.

Il grado di resistenza alle radiazioni (radioresistenza) delle cellule tumorali non dipende solo dal loro patrimonio genetico, ma anche dall’influenza che ha su di esse il microambiente nel quale si trovano.

Per questa ragione, la caratterizzazione dei segnali di comunicazione, sia intra- che intercellulari, associati alle radiazioni, si rivela essere d’importanza primordiale per migliorare le chemio/radioterapie molecolari mirate.

Le lesioni del DNA (singole e doppie rotture dei filamenti) rappresentano i danni cellulari centrali indotti dalle radiazioni ionizzanti. Come reazione, una moltitudine di processi molecolari e cellulari che possono provocare l’arresto del ciclo cellulare, l’induzione del processo di riparazione del DNA o la morte cellulare (post-mitotica o per apoptosi) viene attivata. Tuttavia, le radiazioni inducono anche una complessa cascata di segnali indipendente dai danni prodotti sul DNA. Queste cascate di segnali rappresentano degli obiettivi molto interessanti per i trattamenti anticancerogeni che combinano le radiazioni ionizzanti a delle sostanze farmacologiche ben definite a livello molecolare. La modulazione delle entità centrali e specifiche di tali cascate di segnale sensibilizza molto efficacemente alla morte cellulare indotta dalle radiazioni.

Nelle cellule tumorali, le radiazioni ionizzanti attivano i recettori ErbB e, a valle di questi, la cascata fosfatidilinositosilo 3'-chinasi (PI3K)/Akt favorendo la sopravvivenza cellulare e proteggendo contro le stimolazioni da stress apoptotico. In più, questa cascata viene sovraespresa in molti carcinomi umani ed è strettamente legata alla tumorigenesi, alla progressione e all’invasione dei tumori, alle metastasi, all’angiogenesi così come ad una debole risposta ai trattamenti ed ad una resistenza a differenti medicamenti ed alle radiazioni. Dei composti che deregolano questa cascata sono, di conseguenza, di particolare interesse clinico per i trattamenti anticancerogeni semplici e combinati.

Questo lavoro aveva come obiettivo di analizzare l’importanza della cascata PI3K/Akt in rapporto al potenziale radiosensibilizzatore di due inibitori di chinasi, recentemente sviluppati e rilevanti clinicamente, PKC412 e PKI166. Abbiamo inoltre studiato dei meccanismi che portano all’attivazione di questa cascata dopo la radiostimolazione in differenti tipi di cellule (cellule tumorali ed endoteliali). Per far questo, abbiamo utilizzato gli inibitori di tirosinasi PKI166 e PTK787 dei recettori delle rispettive categorie ErbB1/2 e VEGFR.

Abbiamo dimostrato che la cascata cellulare PI3K/Akt rappresenta un buon bersaglio per
l’effetto antiproliferativo e citotossico indotto dal radiosensibilizzatore PKC412 (un inibitore della proteina chinasi C (PKC) deriavata dalla staurosporina). L’espressione artificiale di una forma costitutivamente attiva d’Akt – utilizzando un sistema d’infezione retrovirale delle cellule – in fibroblasti di topi trasformati dall’oncogene H-ras, neutralizza l’effetto antiproliferativo e citotossico di PKC412. Abbiamo inoltre scoperto che il soppressore di tumori PTEN è un componente critico per l’inibizione della cascata PI3K/Akt da parte di PKC412, al contrario di LY294002 che è un inibitore diretto di PI3K. Questo correlava con la necessità della presenza di PTEN nella sua forma intatta per permettere che PKC412 potesse esercitare i suoi effetti antiproliferativi e citotossici.

Abbiamo analizzato anche il meccanismo d’azione ed il potenziale di radiosensibilizzazione di PKI166, un nuovo doppio inibitore dei recettori ErbBl ed ErbB2. PKI166 ha sensibilizzato delle cellule che sovraesprimevano ErbB2 all’apoptosi indotta dalle radiazioni, ma non ha aumentato neanche minimanente l’effetto antiproliferativo indotto dalle radiazioni su queste cellule. Al contrario, PKI166 ha aumentato gli effetti antiproliferativi e citotossici delle radiazioni in due linee di cellule sovraesprimenti ErbB1. Una relazione fra il recettore ErbB2 e la cascata PI3K/Akt è stata individuata e si è potuto dimostrare che PKI166 impediva l’attivazione della cascata PI3K/Akt nelle cellule sovraesprimenti ErbB2.

In maniera analoga alle cellule tumorali, le radiazioni attivano la cascata PI3K/Akt anche nelle cellule endoteliali, contribuendo così ad un aumento della radioresistenza. Abbiamo mostrato che le cellule endoteliali possiedono dei segnali intatti indotti dai ligandi EGF e VEGF a partire dai loro rispettivi recettori ad Akt. Questi segnali sono stati selettivamente inibiti dai due rispettivi inibitori, PKI166 e PTK787. Infine, abbiamo potuto rilevare in maniera diretta l’attivazione dei recettori VEGFR dalle radiazioni nelle cellule endoteliali.

Con questi studi, abbiammo dimostrato che le radiazioni inducono la cascata di sopravvivenza PI3K/Akt sia nelle cellule tumorali che in quelle endoteliali. In ogni modo, questo processo occorre in maniera differenziale secondo il tipo di cellule e a dipendenza dei recettori (ErbB nelle cellule tumorali e VEGFR nelle cellule endoteliali).

In conclusione, i diversi modi d’azione dei due composti PKC412 (dipendente da PTEN) e LY294002 (indipendente da PTEN) sulla cascata di sopravvivenza PI3K/Akt indicano l’importanza di caratterizzare il profilo farmacogenetico di una sostanza al fine di migliorarne la specificità e l’efficacia di trattamento. In più, dimostriamo che questa cascata è deregolata da PKI166 nelle cellule sovraesprimenti ErbB2. Il profilo farmacogenetico esatto di questa sostanza rimane comunque ancora poco chiaro e necessita di ulteriori indagini. Non di meno, questi studi dimostrano che l’inibizione della cascata di sopravvivenza PI3K/Akt rappresenta un approccio promettente per migliorare la citotossicità delle radiazioni ionizzanti. In questo lavoro, abbiamo anche caratterizzato un meccanismo supplementare indotto dalle radiazioni nelle cellule endoteliali, il quale è importante per la radioresistenza dei tumori in rapporto al loro microambiente. Per finire, questi risultati possono contribuire a raffinare ed ad elaborare delle nuove strategie di trattamento che combinano le radiazioni ionizzanti con dei composti ben definiti di piccolo peso molecolare.
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## CURRICULUM VITAE

## ACKNOWLEDGEMENTS
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<th>Abbreviation</th>
<th>Term</th>
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<tbody>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>AML</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium persulfate</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BCS</td>
<td>Bovine calf serum</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECGS/H</td>
<td>Endothelial cell growth supplement/heparin</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EGM</td>
<td>Epithelial growth medium</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol bis(β-aminoethyl)ether tetraacetic acid</td>
</tr>
<tr>
<td>EGR</td>
<td>Early growth response</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>Flt-1, -3</td>
<td>Fms-like tyrosine kinase-1, -3</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>HER1/2, ErbB</td>
<td>Human epidermal growth factor receptor</td>
</tr>
<tr>
<td>HIV-TAT</td>
<td>human immunodeficiency virus-transactivator protein</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
</tr>
<tr>
<td>IKKα</td>
<td>IkappaB kinase α</td>
</tr>
<tr>
<td>IMRT</td>
<td>Intensity modulated radiation therapy</td>
</tr>
<tr>
<td>IR</td>
<td>Ionizing radiation</td>
</tr>
<tr>
<td>KI</td>
<td>Kinase inhibitor</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-drug resistance</td>
</tr>
<tr>
<td>MEF</td>
<td>Mouse embryo fibroblast</td>
</tr>
<tr>
<td>MEK</td>
<td>MAPK/ERK</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MyrAkt</td>
<td>Myristoylated Akt</td>
</tr>
<tr>
<td>NBCS</td>
<td>New born calf serum</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κB</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non small cell lung cancer</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF-R</td>
<td>Platelet derived growth factor receptor</td>
</tr>
<tr>
<td>PDK-1, -2</td>
<td>Phosphoinositide-dependent kinase -1, -2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristoyl acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethansulfonsäure fluorid</td>
</tr>
<tr>
<td>pNA</td>
<td>p-nitroanilide</td>
</tr>
<tr>
<td>PTEN</td>
<td>Phosphatase and tensin homolog deleted on chromosome 10</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene Fluoride</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homologous and collagen</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>STP</td>
<td>Staurosporine</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TPA</td>
<td>Tetradecanoyl phorbol acetate</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>VEGFR</td>
<td>Vascular endothelial growth factor receptor</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Cancer and Cancer Treatment

Cancer is still among the top leading causes of death, rivaling with heart diseases for the number one position. This is valid for all age groups. Nevertheless, patterns of incidence and death rates vary with age, sex, race and geographic location. In the United States, the most common cancer expected to occur in women are breast (32%), lung and bronchus (12%), colon and rectum (11%) and uterus (6%), whereas in men cancers of the prostate (33%), lung and bronchus (14%), colon and rectum (11%) and uterus (6%) comprise over 60 percent of all new incident cancers. The lifetime probability of developing cancer is higher for men (43.5%) than for women (38.5%). However, because of breast cancer, women have a slightly higher probability of developing cancer before the age of 60. Lung cancer accounts for the highest mortality by far in men (31%) and women (25%). The cancer problem in children is also growing and except for accidents, cancer is the leading cause of death for children (aged 1 to 14 years) of both sexes (Cancer statistics in the US, (1)).

Ionizing radiation (IR) along with surgery and chemotherapy belongs to the three major cancer treatment modalities. It can be applied as single therapy, as concomitant therapy with chemotherapy, but also often as an adjuvant therapy after surgical removal of a tumor to prevent the growth of new tumor tissue from residual sites (recidive) and lastly as a palliative therapy.

75% of all patients with solid tumors are treated with radiotherapy acting either as single treatment alone or as a combination therapy. Half of these patients receive the radiotherapy as curative therapy, the other half as palliative therapy. Of all cured cancer cases 49% were cured after surgery, 27% after radiotherapy alone, 13% due to surgery followed by radiotherapy and 11% by chemotherapy either alone or as a combination therapy. This shows the high importance of radiotherapy as single and combined treatment modality (USA-statistics out of (2)).

A benefit of ionizing radiation as a therapeutic tool is the possibility to apply it locoregionally. The tumor can be treated as a more specific part and systemic toxicity can be prevented. However, there are some limitations on efficacy of radiation therapy. Like chemotherapeutics, IR does affect all cells of the targeted tissue. This can lead to severe side-effects after therapy. In addition there is a large number of human malignant tumor cells showing a very poor response to IR. However, the applied dose to the tumor can not be increased as needed because of the neighbouring tissue and sensitive organs located in the radiation field. Thus, a major goal of cancer research is gaining more knowledge about the mechanisms of radiotherapy to optimize therapies and to overcome treatment failures. One possibility to overcome treatment resistance is the modulation of cellular processes in a way that (re-)sensitizes tumor cells for ionizing radiation. Radiosensitization is defined as increased radiation sensitivity of cells (tumor or normal tissue) in the absence of significant drug-induced cytotoxicity.

The spectrum of chemical agents increasing the cytotoxic effect of ionizing radiation has been significantly enhanced during the last several years. This is mainly a result of the identification of multiple cellular processes induced by IR. During decades of research agents were developed and tested with the focus on IR and its direct and deleterious damage of DNA. However we now realize that the cellular response to IR is also affected by signaling processes that are not generated at the site of DNA and these are now being investigated with chemical agents derived from a wide range of research fields such as immunosuppression and angiogenesis.
1. Introduction – Ionizing Radiation as Treatment Modality

Figure 1-1 Combined treatment with ionizing radiation and anticancer-agents: On the macro-(whole organism)-level IR can be targeted to the tumor site whereas chemotherapy is restricted by systemic application. On the micro-(cell)-level molecular-defined compounds target specific molecular entities whereas IR induces multiple molecular damages and stress responses. Combined treatment is a promising approach to optimize the modality specific advantages and to enlarge the therapeutic window (adapted from (3)).

1.2 Ionizing Radiation as Treatment Modality

IR can be either of electromagnetic (X-rays and γ-rays) or particulate origin (neutrons, protons, alpha particles, and heavier higher Z particles) but for standard radiotherapy mainly higher energy photons are applied generated by a MV-linear accelerator or from 60 Cobalt-units. The energy of IR is sufficient for the ejection of an orbital electron from the atom or molecule. These electrons in turn cause ionizing effects which are the basis by which high energy photons – classified as secondary IR – cause radiobiological effects. Since over 80% of the cell is composed of water, irradiation results in ionization of H2O and subsequent generation of hydrated e⁻ and hydroxyl radicals. These reactants are the most relevant chemical entities for damaging critical biological targets by IR (4). Since the discovery of X-rays by W.C. Roentgen late in 1895 and their early diagnostic and therapeutic application during the first decades of the last century the quality of radiotherapy steadily increased with the use of new energy sources, better diagnostic localization of solid tumors and better restriction of applied radiation to the tumor site. This concept culminates nowadays in the development of Intensity Modulated Radiation Therapy (IMRT) by tailoring many small beams of radiation to fit the anatomy of the patient. IMRT delivers a more targeted dose to the tumor than conventional irradiation, thereby sparing surrounding healthy tissue. Thus a major advantage of radiation therapy using IR is the targeted, locoregional application of the toxic insult close to the tumor site avoiding the often restricting side effects of systemic chemotherapy (5, 6).
1.3 The Cellular Response to Ionizing Radiation

In parallel to the improvement of clinical radiotherapy during the last century radiobiological concepts were developed to understand the clinical response to radiation on the cellular and molecular level. Simple clonogenic cell survival assays were optimized to describe the cellular response to irradiation and to correlate a dose response with increasing DNA-damage and cytotoxicity. IR-induced cell death is not a fast process and irradiated cells may even be able to struggle through one or two mitoses before they loose their reproductive integrity. In contrast a radiobiological clonogenic “survivor” cell proliferates indefinitely to produce a large colony. Different mathematical equations were formulated to describe the shape of survival curves for mammalian cells after treatment with increasing doses of IR and under different treatment conditions. With these in vitro analyses earlier observations like increased radioresistance in absence of oxygen could be reproduced in a laboratory set-up and still valid radiobiological concepts were developed up to 50 years ago. Variation of cellular radiosensitivity could be linked to the different cell cycle stages a cell is passing through, showing a high sensitivity at the G2-M-transition, intermediate sensitivity in G1 and early S-phase and rather low sensitivity especially after low dose treatment in late S-phase. An apparent increase of radioresistance after treatment with a multiple irradiation regimen could also be explained by the phenomenon of repopulation (see below). Further, specific stages of cell repair were defined as repair of sublethal and potentially lethal damage and were used to define models for the intrinsic radiosensitivity of target cells (for a detailed description of these concepts, see (7)). Current research in radiobiology is challenged to understand these phenomenological data and concepts on the molecular level. At the same time we use these overwhelming amounts of information to develop new therapeutic tools for combined treatment modalities with IR applying novel molecular biological techniques and chemical synthetic approaches.

Research during the last two decades has focused on two major classes of pharmacological agents for radiosensitization. Cellular radiosensitivity can be enhanced by halogenated pyrimidines (IUdR, BrUdR) though this sensitization-process highly depends on the amount of DNA-incorporation which can be upregulated by the concomitant application of 5-FU. Likewise ribonucleotide reductase inhibitors such as 2’,2’-difluoro-2’ deoxycytidine (Gemcitabine) also increase the lethal effect of IR by the depletion of deoxynucleoside triphosphates required for DNA-synthesis (8-12). The other class of compounds targets a major tumor-related aspect. In general the response of cells to IR is strongly dependent on oxygen. Hypoxia confers radioresistance to a wide range of cells irrespective of the genetic background (13-16). The mechanism behind this phenomenon is that oxygen chemically interacts with IR-induced DNA-radicals and thereby “fixes” these lesions and renders them irreparable. In a hypoxic environment these DNA-radicals can be restored by cellular abundant molecular hydrogen donators. Many chemical approaches were developed during the last two decades to overcome hypoxia by the replacement of oxygen with other electron-affinic compounds that react with the free electron of the IR-induced DNA-radical. Mono-functional 2-nitroimidazole and bi-functional alkylating derivatives thereof were tested but these approaches often failed due to unspecific neurotoxicity or lack of a significant overall benefit. Nevertheless these approaches resulted in the new field of bioreductive drugs such as tirapazamine that do not try to “re-oxygenate” hypoxic tumor areas but rather exploit hypoxia as a selective environment for the generation of hypoxic cytotoxins. Thus combined treatment with ionizing radiation will have a suprapradditive tumor killing effect due to complementary cytotoxicity (see (16) and references therein).
1.4 New Molecular Targets

DNA is not the only cellular target of IR and we realize that IR induces a complex network of signaling processes independent of DNA-lesions, like proliferation stop, protein expression, activation of the DNA-repair machinery and apoptosis. The status of these signal transduction cascades determines the cellular response to radiation treatment what renders them to be interesting targets for radiosensitization of tumor cells (17-19). Further, depending on cell type, differentiation stage and genetic background of the tumor cell the reaction pattern to radiation changes. The radiosensitivity of a three-dimensional tumor is also influenced by its microenvironment and therefore exogenous factors such as composition of the extracellular matrix, oxygen pressure or nutrition by new built blood vessels play an important role (20).

The goal of modern applied radiobiology is the understanding and modulation of these processes to overcome an intrinsic radioresistance of aggressive tumors. We are only beginning to understand which molecular entities and signaling pathways as well as microenvironmental factors are interesting targets for radiosensitization (20-23). Many approaches are performed with molecular-defined pharmacological compounds that were originally designed as anti-cancer agents for single modality treatment.

In our laboratory we are investigating such anti-cancer agents in collaboration with Novartis Pharma Inc.. Small molecular compounds that are already tested in vitro and in vivo for their cytotoxic profile alone – some of them are already under clinical investigations – are screened for their radiosensitizing effect. Promising substances showing radiosensitization in vitro are further investigated in vivo or followed by mechanistic studies to elucidate the network of signal transduction cascades as important targets for radiosensitization. In this work we focused on three of them, the protein kinase C inhibitor PKC412, the epithelial growth factor receptor inhibitor PKI166 and the vascular epithelial growth factor receptor inhibitor PTK787.

1.4.1 Protein Kinase C Inhibitors as Radiosensitizers

The transduction of mitogenic and stress-related stimuli is governed by a network of multiple signaling cascades from the plasma membrane to the nucleus and multiple signaling entities serve as suitable targets for the development of antineoplastic agents. Members of the PKC-family function as transducers for various lipid second messengers in the regulation, transduction and propagation of cell proliferative stimuli and thus are interesting targets for antiproliferative cancer treatments (24, 25). The different PKC isoforms can be subdivided into three classes. PKC-α,β,γ belong to the “conventional” calcium-dependent PKC-subfamily (cPKC), the “novel” calcium-independent PKC-family (nPKC) comprise e.g. PKC-δ,ε,ζ and the “atypical” aPKC-isoforms ζ,λ,α are – in contrast to c- and nPKC-isoforms – not activated by phorbol esters and diacylglycerol. The function of PKCs is altered in different tumor malignancies showing enhanced activity for example in some breast, lung and brain tumors when compared with normal adjacent tissue. This could be due to aberrant expression of PKC itself but also of upstream oncogenic entities in PKC-linked growth promoting signaling pathways (26). Previous studies have indicated that these serine/threonine kinases are interesting targets not only for a single treatment-modality but also in combination with additional chemotherapeutic agents and IR. PKC-inhibitors are potent inducers of apoptosis but also sensitize tumor cells to anti-metabolites or cytotoxic and DNA-damaging agents. On the other hand, PKC-stimulation by phorbol esters can rescue different cell types from glucocorticoid- and growth factor withdrawal-induced cell death (27-29).

The identification of protein kinase C (PKC) as target for radiosensitization goes back to the
observations that PKC mediates the radiation-inducibility of tumor necrosis factor (TNF) and of transcription factors such as EGR-1, c-jun and c-fos, mainly investigated in tumor cells of hematopoietic origin. These immediate early genes are important in the adaptation of cells to oxidative stress. PKC-activation emerges as rapidly as 30 s following irradiation, is required for IR-induced transcripational expression of these factors, and downregulation or inhibition of PKC abrogates their expression (30-33). Different investigators also reported that radiation induces the formation of diacylglycerol, inositoltriphosphate (IP3) or arachidonic acid resulting in Ca\textsuperscript{2+}-release from the endoplasmic reticulum and subsequent PKC-activation. Thus these data indicate that irradiation results in either receptor tyrosine kinase activation or oxidative activation of phospholipases (PLC\textgamma or PLA2) and argue for the generation of intracellular signaling from the site of DNA damage in addition to and independent of signaling induced from the site of damaged DNA (34-36).

A new twist on PKC and IR-generated signaling pathways at the plasma-membrane originated from the intriguing investigations by Haimovitz-Friedman and colleagues on the release of the apoptotic second messenger ceramide after irradiation. They observed that IR initiates sphingomyelin hydrolysis to ceramide and subsequent apoptosis induction. The sphingomyelin pathway is an important apoptotic cell death pathway that is generated at the cell membrane by various triggers e.g. H\textsubscript{2}O\textsubscript{2}, TNF-\alpha and Fas/Apo-1 (37, 38). This pathway is initiated by phosphodiester-bond-hydrolysis of sphingomyelin (N-acylsphingosin-1-phosphocholine) by specific phospholipases (sphingomyelinases) to generate the intracellular apoptotic second messenger ceramide. Most important, ceramide is also generated after irradiation of DNA/nuclei-free membrane preparations clearly supporting the novel concept that apoptotic signaling is also induced by IR and independent of DNA-damage. Interestingly PKC-activation by TPA or bFGF abrogates IR-induced sphingomyelin-hydrolysis and radiation-induced apoptosis but apoptosis is restored by synthetic ceramide analogues. Thus IR-induced apoptosis might be controlled by a delicate homeostasis between ceramide-generation and IR- or growth factor-mediated PKC-activation and offers a new regulatory target to lower a treatment threshold. However this mechanism for direct IR-induced apoptosis might be relevant only in some cells, while in others ceramide may only serve as a co-signal or does not play a role in the death response at all (39-43).

1.4.1.1 N-Benzoyl-Staurosporine (PKC412) as Radiosensitizer

Various natural products from different origins have been identified to be potent and specific PKC-inhibitors. The indolocarbazole staurosporine was originally isolated from the culture broth of Streptomyces sp. (44, 45).

PKC412 – a synthetic N-benzoylated staurosporine derivative – was developed by Ciba Geigy/Novartis, and was formerly called CGP-41251.

PKC412 is a competitive inhibitor of the ATP binding site of protein kinase C. The additional N-benzoyl group renders this derivative more specific against specific PKC-isoforms. The classical PKC-isoforms \(\alpha,\beta,\gamma\) are potently inhibited with a low nanomolar IC\textsubscript{50}. For inhibition of the Ca\textsuperscript{2+}-independent PKC-isoforms \(\delta,\varepsilon\) higher concentrations are necessary and the atypical PKC\(\zeta\)-isoform is not inhibited.

Whereas its mother-compound staurosporine is more unspecific and therefore too toxic for \textit{in vivo} application, PKC412 is more specific due to the benzoyl-group and is well tolerated \textit{in vivo}. PKC412 shows a broad antiproliferative activity and tumor growth control effect against different tumor cell lines \textit{in vitro} and \textit{in vivo}. The tumor growth control effect \textit{in vivo} is further enhanced by inhibition of ligand-induced autophosphorylation of the VEGF receptor tyrosine kinase resulting in an additional anti-angiogenic effect by PKC412 (46, 47).

PKC412 displays potent antitumor activity against various tumor types, but more important
PKC412 also significantly enhances the antitumoral activity of 5-FU, cis- and carboplatinum, doxorubicin, vinblastine and taxol against solid tumors that do not show a response against single treatment with PKC412 alone. Interestingly PKC412 has also the potential to revert the multi-drug-resistance-phenotype in cancer cells and thus makes it an interesting candidate for combined treatment modalities e.g. reversing the cytotoxic drug resistance towards adriamycin. Furthermore, it was recently reported that PKC412 directly inhibits the Flt3-tyrosine kinase (IC50=500nM, in vitro-kinase assay) and induces G1 arrest and apoptosis in Ba/F3-Flt3 cells (IC50=10nM in vivo). Since Flt3 is mutated in about 30% of all acute myelogenous leukemia (AML)-patients PKC412 would be an interesting candidate for testing as an anti-leukemia agent in these patients (48).

However, it is neither the anti-angiogenic nor the MDR-reversing feature of PKC412 that is responsible for the in vitro and in vivo observed supraadditive effect when PKC412 is combined with IR. Depending on the cellular genetic background and the treatment conditions PKC412 exerts different cellular responses and mechanisms for radiosensitization. Genetically defined tumor cells are an effective tool to investigate the mechanism of radiosensitization on the molecular level. Our own group investigated a p53-dependent mode of cell death in vitro and in vivo when PKC412 is applied in combination with IR. PKC412 sensitized both p53-wildtype and p53-deficient E1A/ras-oncogene transformed mouse embryo fibroblasts (MEFs) for treatment with IR. Figure 1-3 illustrates the radiosensitizing potential of PKC412 in p53-deficient E1A/ras-transformed MEFs in vitro and in vivo. In p53-wildtype cells though, combined treatment drastically induced apoptotic cell death whereas no apoptosis induction could be observed in p53-deficient cells in vitro and in histological tumor sections (49, 50). Further in p53-wildtype cells combined treatment resulted in cooperative cytochrome C-release drastically activating the caspase-9/caspase-3 apoptotic pathway but activation of this apoptotic pathway was completely absent in p53-deficient isogenic tumor cells. A strict requirement for this specific apoptotic pathway by PKC412/IR-induced cell kill was revealed by comparing oncogene-transformed caspase-9-wildtype and caspase-9-deficient embryo fibroblasts (51). Biochemical studies identified the PI3K/Akt-pathway as potential mediator for the PKC412 induced apoptosis in these cells (52). In this work we analyzed the PI3K/Akt-pathway dependence of the PKC412 mediated effect using a genetical
1. INTRODUCTION – Protein Kinase C Inhibitors as Radiosensitizers

approach.

Figure 1-3 Radiosensitizing effect of PKC412 in p53-deficient E1A/ras-transformed mouse embryo fibroblasts in vitro (A) and in vivo (B). A, clonogenic survival of cells treated with increasing doses of PKC412 and radiation alone or in combination. Single seeded cells were allowed to grow 8-10 days after the start of treatment. B, effect of PKC412 and radiation alone or combined on the growth of p53-deficient MEFs in nude mice. Treatment on 4 consecutive days (3 Gy, 100 mg/kg PKC412 alone or combined) was started after tumor reached a minimal volume of 150 mm³ (day 14). For a detailed description of these experiments see (49).

Different strategies in the field of applied cancer research are currently investigated to overcome treatment resistance. The identification of signaling pathways responsible for a high IR-specific apoptotic treatment threshold is important to improve combined treatment modalities with apoptotic radiosensitizers.

1.4.1.2 The PI3-Kinase/Akt Survival Pathway as Target for Radiosensitization

The phosphatidylinositol 3-kinase (PI3K)/Akt signal transduction pathway has been identified as major determinant for the cellular response to apoptotic stress stimuli.
This survival pathway is activated in response to several growth and survival factors and plays an important role in diverse cellular processes (53, 54). After growth factor stimulation PI3K phosphorylates membrane phosphoinositides at the 3D-position of the inositol ring that act as second messengers to mediate the downstream effects of PI3K. This reaction is antagonized by the lipid phosphatase and tumor suppressor PTEN (see below). One of the PI3-kinase downstream targets is the serine-threonine protein kinase Akt also referred to as protein kinase B (PKB or RAC) (55, 56). Activation of Akt involves the binding of PI3K-phosphorylated phosphoinositides to the Akt-pleckstrin domain and its subsequent translocation to the plasma membrane, where Akt is phosphorylated by the PI-dependent kinase PDK1 and an unidentified kinase referred as PDK2. Phosphorylation of the two phosphorylation sites Thr308 by PDK1 and Ser473 is required for its activation (52, 57-59). Several downstream targets of PKB/Akt have been identified e.g. the bcl-2 family member Bad, caspase-9, the transcription factor forkhead, and mdm2 (60-62), all involved in apoptosis regulation, or glycogen synthase kinase 3 (GSK3) regulating glucose transport and glycogen synthesis (63-65). Akt also phosphorylates IKKα leading to the activation of the transcription
factor NF-κB (66). Recent publications demonstrated that Akt may contribute to tumor-cell proliferation by interaction with p27. Akt-mediated phosphorylation of p27, retents this tumor suppressor in the cytoplasm and thereby precludes p27-induced G1-arrest (67, 68). Akt anti-apoptotic and pro-survival capacities were also suggested to be in part mediated by phosphorylation and cytoplasmic retention of p21, another cell cycle inhibitor (69). Akt is also involved in such cell and stress specific processes like hypoxia-induced expression of vascular endothelial growth factor. Thus PKB/Akt is involved in multiple cellular processes, and different crosstalks exist between other growth-promoting and cell survival signaling pathways, but the exact mechanism of their regulation is far from clear (70-76). Constitutively active PI3K or Akt results in an enhanced protection against apoptotic cellular insults such as growth factor deprivation, UV-irradiation or loss of matrix attachment (77, 78). Both elevated PI3K and Akt activities have been identified in various tumor types due to amplified gene copy numbers and this pathway has been linked not only to apoptosis suppression but also to oncogenesis (79-81). Furthermore inactivating mutations of the lipid-phosphatase and tumor suppressor PTEN that acts as a PI3K signaling antagonist also often correlate with an apoptosis-resistant tumor phenotype (82). Different inhibitors of the PI3K/Akt-pathway are known. Growth factor-induced activation is almost completely prevented by pretreatment of cells with the specific PI3K-inhibitors LY294002 and wortmannin or by overexpression of a dominant negative form of PI3K (83, 84). The phosphorylation status of Akt is also modulated in a PI3K-independent way by the phosphatase inhibitors pervanadate and okadaic acid also suggesting that Akt is a target of protein phosphatase 2A (85-87). Though these inhibitors are exceedingly toxic for clinical application. Nevertheless, inhibition of PI3K-activity by LY294002 abrogates a high apoptotic threshold in otherwise treatment resistant cancer cells and sensitizes to chemo- and radiotherapy (88-90). The radiosensitizing potential of wortmannin has also been reported in human cancer cells irrespective of their p53-status (91).

Recently a crosstalk between the PI3K/Akt-pathway and PKC-activity has been observed. Overexpression of PKC stimulated Akt activity and suppressed cytokine-dependent apoptosis. On the other hand the phorbol ester PMA, an activator of PKC, downregulates growth factor-induced Akt activation, and specific isoforms of PKC directly interact as negative regulators of Akt (92-94). Moreover, inhibition of Akt function upon PKCat activation has been demonstrated recently to be a crucial event in triggering the apoptotic response of phorbol esters in LNCaP cells (95). Thus PKC-inhibitors might be potential modulators of this survival pathway.
PTEN (phosphatase and tensin homolog deleted on chromosom ten (96); also known as MMAC-1 or TEP1) is an important regulator of the PI3K/Akt-pathway. Besides its essential role in embryonic development, PTEN is one of the most frequently mutated tumor suppressors in human cancer. Given its multiple and diverse functions in cellular processes such as growth, survival, differentiation, adhesion, migration, invasion and apoptosis it is not surprising to find it related to several human malignancies. PTEN is often mutated or deleted in glioblastomas (70%) or advanced prostate cancers (60%) and in a lesser extend in cancer from other origins (endometrial, breast, lung, kidney, bladder, testis, head and neck, melanoma and lymphoma). Germline mutations of PTEN may also cause hamartomas (benign growths) found in rare human diseases such as Cowden and Bannayan-Zonana syndromes, Lhermitte-Duclos disease and juvenile polyposis coli (97).

PTEN functions primarily as a lipid phosphatase by dephosphorylating several phosphoinositide signaling molecules, specifically removing phosphate from the 3D-position of the inositol ring and thus acts as major antagonist of PI3K (98, 99). In addition, PTEN displays weak tyrosine phosphatase activity, which may downregulate signaling pathways involving focal adhesion kinase (FAK) or Shc (100).

The role of PTEN in cell survival has been attributed to its lipid phosphatase activity. PTEN knockout mouse embryo fibroblasts (PTEN-/- MEFs) were shown to exhibit reduced sensitivity to apoptosis and wild-type PTEN, but not a PTEN mutant with inactivated lipid phosphatase function (G129E mutant), could restore the sensitivity to agonist-induced apoptosis (101). PTEN was able to sensitize cells to a variety of apoptotic stimuli such as death receptors, kinase inhibitors and chemotherapeutic agents. It was demonstrated that PTEN expression sensitized LNCaP prostate cancer cells to apoptosis induced by chemotherapeutic agents such as staurosporine, doxorubicin and vincristine or etoposide and mitoxantrone (102, 103). Furthermore, introduction of PTEN in the chemoresistant U87MG and LN-308 malignant glioma cell lines markedly sensitizes these cells to ionizing radiation and to CD95-ligand induced apoptosis (104).

Mutations in PTEN accompany brain tumors progression which is associated with angiogenesis (105, 106). Since reconstitution of wildtype PTEN but not of the lipid phosphatase inactive mutant G129E in U87MG glioma cells leads to decreased tumor growth in vivo and suppressed angiogenic activity, PTEN and particularly its lipid phosphatase activity was proposed to control tumor-induced angiogenesis (107). This confirmed previous in vitro data suggesting a link between PTEN-dependent PI3K/Akt-mediated VEGF expression downregulation and its potential control of angiogenesis (82).

In this work, the response of the PI3K/Akt-survival pathway to treatment with the PKC-inhibitor PKC412 and the importance of PTEN in this context were investigated especially since downregulation of the PI3K/Akt-survival pathway might be a valid target to overcome an apoptotic threshold.

1.4.2 Epidermal Growth Factor Receptors as Targets for Radiosensitization

IR-induced activation of receptor tyrosine kinases represents a further area of investigation to understand the cellular stress response to IR. Consequently these receptors became a promising target for radiosensitization. In particular inhibitors of the epidermal growth factor receptor (EGFR, ErbB1/HER1) and related isoforms are currently being tested in preclinical and clinical studies as combined treatment modality in combination with IR.

Growth factor receptors are activated by extracellular hormone ligand binding but interestingly irradiation with ultraviolet light or IR also causes receptor dimerization and activation, best investigated with the EGFR (see below). The EGF-receptor is part of the ErbB
family of receptor tyrosine kinases which also include ErbB2/HER2-neu, ErbB3/HER3 and ErbB4/HER4. All members consist of an extracellular ligand-binding domain, a single membrane-spanning region and an intracellular protein tyrosine kinase domain. Ligands to the EGFR include EGF, transforming growth factor α, heparin-binding EGF, amphiregulin and betacellulin (108-112). ErbB family members form homo- or heterodimers after extracellular ligand binding, and dimerization activates the intracellular tyrosine kinase leading to auto/trans-phosphorylation of the specific tyrosine residues within the cytoplasmic subunit. These serve as docking sites for signaling entities of further downstream signal transduction pathways such as ras/MAP kinase, PI3K and STAT-3 (84, 113, 114). However, differences between each ErbB member have been related. ErbB3 has impaired kinase activity and only becomes phosphorylated and functions as a signaling entity when dimerized with another ErbB receptor (115). Furthermore, ErbB2 possesses no known ligand and needs a heterodimerization partner to acquire signaling potential. However, it has a central role in this receptor family since it is the preferred dimerization partner for the other ErbBs (116, 117).

The ErbB1-receptor is overexpressed in many human carcinomas and has been linked to tumorigenesis, tumor progression, invasion and metastasis as well as pro-angiogenic effects like blood vessel recruitment (118, 119). Molecular characterization of ErbB1-signaling in tumor cells described the activation of this autocrine growth pathway to be responsible for disregulated proliferation. ErbB1-receptor overexpression has also been associated with poor treatment outcome and resistance to different cytotoxic drugs and is therefore an important target for therapeutic interventions (109, 110, 120).

Overexpression of the ErbB1-receptor and ligand-induced ErbB1-stimulation also correlate with enhanced radioresistance in human head neck and mammary tumors. Likewise, artificial overexpression of ErbB2 in MCF-7 renders this cell line more radioresistant than the parental cell line. Thus, different mechanisms may lead to increased ErbB1-receptor activation and subsequently cellular responses (activation of DNA-repair machinery, PI3K/Akt-survival-pathway) that result in an enhanced radioresistant cellular phenotype (22, 121-126).

Ionizing radiation itself as well directly mediates cell survival and repopulation ultimately resulting in treatment resistance (127, 128). The phosphorylation and subsequent activation of ErbB-receptors in tumor cells has been shown to be of central importance for this defense mechanism (129). Downstream of ErbB two different but not totally independent pro-survival cascades may be potential mediators of this response: the mitogen-activated protein kinase (MAPK)-pathway and the PI3K/Akt-pathway (130, 131). Recently, it was demonstrated that the PI3K/Akt cascade is the major pathway responsible for radioresistance in cancer cells (89). Furthermore, IR-induced ErbB-receptor activation favors angiogenesis via tumor-cell production of pro-angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) (39, 132). Therefore, the high intrinsic radioresistance in ErbB-overexpressing tumor cells, the inhibition of cellular proliferation and antagonizing IR-induced accelerated repopulation during radiotherapy as well as counteracting angiogenesis represents a major rationale to combine inhibitors of ErbB-signaling during fractionated radiotherapy.

Different pharmacological and biological approaches have been developed for blocking ErbB-activation in cancer cells. The use of monoclonal antibodies (MAbs) against the extracellular domain of ErbB-receptors (essentially of ErbB1 and ErbB2) and the design of low-molecular-weight compounds inhibiting their intracellular tyrosine kinase activity appear the most promising ones. Both approaches resulted in compounds that have entered clinical trials during recent years, alone and in combination with chemotherapy. Combined treatment with locoregional-applied radiotherapy is now intensely investigated. Some of the known differences between low-MW tyrosine kinase inhibitors (KIs) and MAbs that could account for the different responses seen in the clinic are listed in Table 1.
1. INTRODUCTION – Epidermal Growth Factor Receptors as Targets for Radiosensitization

Table 1. Advantages and disadvantages of KIs compared with MAbs

<table>
<thead>
<tr>
<th>Advantage/Detriment</th>
<th>Kinase inhibitors (KIs)</th>
<th>Monoclonal antibodies (MAbs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral administration</td>
<td>KIs are oral; MAbs require intravenous administration.</td>
<td></td>
</tr>
<tr>
<td>Low MW distribution</td>
<td>KIs are low MW and distribute more rapidly and possibly more completely than MAbs.</td>
<td></td>
</tr>
<tr>
<td>Crossact with other kinases</td>
<td>KIs crossact with other kinases; MAbs are truly specific.</td>
<td></td>
</tr>
<tr>
<td>Lack immune functions</td>
<td>KIs lack immune functions that can be mediated by MAbs.</td>
<td></td>
</tr>
<tr>
<td>Dose-limiting toxicities</td>
<td>KIs exhibit dose-limiting systemic toxicities not seen with MAbs.</td>
<td></td>
</tr>
<tr>
<td>Downregulation</td>
<td>KIs do not downregulate EGFR, but MAbs do.</td>
<td></td>
</tr>
</tbody>
</table>

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

1.4.2.1 Inhibitory Antibodies as Radiosensitizers

Monoclonal antibodies (MAbs) that target the extracellular domain of ErbB-receptors have already been tested in combination with IR in advanced clinical trials. Among the various monoclonal antibodies against ErbB-receptors, the radiosensitizing effect of C225 (Cetuximab; Imclone) is best investigated. C225 is a humanized chimeric monoclonal antibody targeting the extracellular domain of ErbB1 and competes with the natural ligands EGF and TNFα for receptor binding. Antibody binding mediates receptor dimerization but without activation of the receptor tyrosine kinase, resulting in receptor internalization an important mechanism for attenuating receptor signaling. Subsequent downregulation of ErbB1 prevents further receptor binding and activation by the respective ligands (133-135). In addition, C225 can elicit antibody-dependent cellular cytotoxicity (136).

The radiosensitizing potential of C225 has been tested in vitro and in vivo against various ErbB1-overexpressing cell lines and xenografts. The mechanism for this cooperative effect in vitro is difficult to elucidate. While enhanced apoptosis induction might lead to tumor infiltration with macrophages and granulocytes, enhanced tumor cell necrosis and inhibition of angiogenesis also contribute to the cooperative tumor growth control effect after combined treatment (137-143). Interestingly, different inhibitors of ErbB-receptor controlled signaling also show an anti-angiogenic effect when tested in vivo. This is most probably linked to the downregulation of ErbB-controlled expression of pro-angiogenic factors like VEGF (144-146). This in turn correlates with the promising new strategy to combine anti-angiogenic approaches with radiotherapy.

Based on these promising preclinical studies combining ErbB-receptor-inhibitor-treatment with IR, clinical trials were initiated. Clinical phase I/II studies of C225 given alone or in combination with radiation have now been completed. In one study escalating doses of C225 were combined with conventional radiation therapy to explore the feasibility of delivering weekly C225 in combination with standard radiotherapy for patients with locally advanced and unresectable squamous cell carcinomas of the head and neck. Among 15 evaluated patients there was a 100% response rate and 13 of 15 patients achieved complete remission (147). Taking into consideration that the expected response rate with radiation alone would have been around 60%, these results were highly encouraging and have lead to the initiation of an international phase III randomized trial examining the impact of C225 on advanced head and neck cancer patients treated with definitive radiation therapy.
Radiosensitizing studies with another clinically investigated monoclonal antibody targeting ErbB2 (trastuzumab) confirm the radiosensitizing potential of ErbB-specific antibodies, and a specific enhancement of tumor sensitivity to IR has been confined to ErbB2-overexpressing cells in vitro and in vivo (126). Since ErbB2 is mainly overexpressed in tumor cells this treatment strategy bears a potentially wide therapeutic window.

1.4.2.2 Low Molecular Weight Tyrosine Kinase Inhibitors as Radiosensitizers

Among low molecular weight compounds two quinazoline derivatives, ZD1839 and CI-1033 are best investigated in combination with irradiation. ZD1839 (Iressa, Gefitinib) is an orally bioavailable competitive inhibitor of the ATP-binding site of the ErbB1-receptor but also inhibits growth of ErbB2-overexpressing breast cancer cells in vitro and in vivo (148). Combined treatment with ZD1839 and IR was tested against different human cancer cell lines and in a colon cancer xenograft model and enhanced the cytotoxicity of both single and fractionated radiotherapy (149-154). Phase III clinical trials with ZD1839 in combination with IR are currently performed in patients with non-small cell lung cancer (152, 155-159).

In combination with single dose and fractionated irradiation CI-1033 has a strong synergistic effect against different ErbB-overexpressing cells (160, 161). This pan-ErbB tyrosine kinase inhibitor and other small molecular tyrosine kinase inhibitors will enter clinical trials in combination with ionizing radiation once their clinical profile will be more advanced as single agent or in combination with chemotherapy. Very recently the newly developed reversible ErbB1-receptor tyrosine kinase inhibitor Erlotinib (Tarceva, OSI-774) has been shown to enhance radiation-induced cytotoxicity in different human carcinoma cell lines (162). In general these pharmaceutical compounds are very attractive though treatment combinations with IR are still in preclinical stage.

The pyrrolo-pyrimidine derivative PKI166 (formerly called CGP75166) is a low molecular weight dual ErbB1/2 tyrosine kinase inhibitor recently developed by Ciba Geigy/Novartis. Proliferative and clonogenic assays performed in our group revealed the radiosensitizing effects of this inhibitor in human cervix and breast carcinoma cell lines when combined with radiation (163). In this thesis, we further analyzed its radiosensitizing potential in another clinically relevant cell line and characterized its mechanism of action.
1.4.3 Vascular Epidermal Growth Factor Receptors as Targets for Radiosensitization

IR does not only target the tumor cells and during the last few years the tumor associated endothelium has emerged as a critical component influenced by radiation for the regulation of tumor growth. Since tumor cells have a high requirement in oxygen and nutrients – due to their uncontrolled growth and proliferation capacities – the maintenance and the new formation of a functional vascular network assuring the supply of these needs is of central importance for tumor survival. The formation of new blood vessels from pre-existing ones – a process named angiogenesis – is now recognized to be essential for both tumor growth and metastasis (164, 165). Blood vessels are essentially composed of endothelial cells that integrate closely with smooth muscle cells, pericytes and the surrounding (extracellular) matrix. Consequently, the tumor associated endothelium and in particular tumor associated endothelial cells are becoming promising and alternative targets for chemo- and radiotherapy (166, 167). Furthermore, targeting the vascular system is an interesting approach since primary endothelial cells of the tumor angiogenic system do not undergo genetic mutations and thus are less prone than genetically unstable tumor cells to develop treatment resistances. Development of inhibitors of angiogenesis essentially focused on the VEGF-signaling (168-172). This is mainly due to the important role of the VEGF as pro-angiogenic factor in pathological situations, neovascularization and enhanced vascular permeability and the advanced mechanistic understanding of the corresponding VEGF-receptors.

Vascular endothelial growth factor (VEGF) receptors and their ligands initiate signaling pathways that play a pivotal role in normal as well as in pathological angiogenesis. Activation of the VEGF-receptors upon ligand binding stimulates a broad spectrum of biological responses in endothelial cells including cell proliferation, growth, migration, survival, differentiation and permeability to macromolecules (173, 174). The VEGF-receptors Flt-1 (Fms-like tyrosine kinase, VEGFR-1) and KDR (kinase insert domain-containing receptor, VEGFR-2) belong to a family of receptor tyrosine kinases which are predominantly located on the surface of endothelial cells (175). Both receptors have seven immunoglobulin-like domains in their extracellular region, a single transmembrane-spanning domain, and an intracellular split tyrosine kinase domain and belong to the same family of receptors as EGFR, PDGFR, c-Kit, c-Fms, Flt-3 and Flt-4 (176, 177). In contrast to VEGFR-1, VEGFR-2 is strongly autophosphorylated upon VEGF-stimulation and VEGF induces signaling processes such as the PI3K/Akt-pathway through direct recruitment of downstream targets to the phosphorylated consensus sites of the VEGFR (178).

Ionizing radiation favors angiogenesis by inducing VEGF production from tumor cells promoting endothelial cell survival in a paracrine fashion (179, 180). The increase in VEGF expression upon irradiation in tumor cells results probably from a hypoxic stress response but also from the IR-induced activation of the ErbB-pathway. This VEGF upregulation can be interpreted as a self-defense mechanism of the tumor that not only protects endothelial cells from radiation damages but also the tumor cells supply of oxygen and nutrients. This was proposed to be a further more indirect mechanism leading to tumor radioresistance after radiation treatment, besides the direct IR-induced tumor radioresistance via ErbB receptors (see above). Thus, blockade of VEGF and its signaling is regarded as an interesting and promising approach for radiosensitization. Moreover, recently the PI3K/Akt signal transduction cascade was identified as crucial element for preventing endothelial cell apoptosis after VEGF activation (181, 182) and as central mediator of endothelial cell survival and angiogenesis (183). Interestingly, IR also activates Akt in endothelial cells in a PI3K-dependent way and the PI3K-inhibitors wortmannin or LY294002 enhance radiation
induced apoptosis and cytotoxicity in these cells (184). Thus, inhibition of the radiation-activated PI3K/Akt-survival pathway might also contribute to an increased antiangiogenic effect eventually leading to radiosensitization after treatment with IR in combination with inhibitors of this survival pathway. However, the upstream entities mediating radiation-induced activation of the PI3K/Akt pathway in endothelial cells are still not known. In this study we were interested to identify which receptor family is involved in this process. Such mechanistical understanding might help to refine antiangiogenic therapies in combination with IR.

Among the different antiangiogenic strategies already in clinical trials (168, 176) none of these has achieved any breakthrough as monotherapy so far. Therefore, now a tendency is raising to combine this class of compounds with classic cytotoxic treatment modalities such as IR. For example, the tumor radiosensitivity could be enhanced by combining IR with the two anti-angiogenic factors angiostatin or endostatin (185, 186). Very promising results were obtained in pre-clinical studies with the anti-VEGF MAb (Bevacizumab) in combination with radiation (125, 179). A pilot phase I study with this antibody and concurrent radiotherapy in patients with advanced head and neck cancer has been recently activated. Further, the anti-VEGFR-2 MAb DC101 has been shown to potentiate IR-induced long term control of human tumor xenografts (187).

During the last few years, different VEGF-receptor tyrosine kinase inhibitors have been developed with very promising results obtained in combination with IR as well (188-191). One of these, SU5416, already entered phase I/II studies as part of a chemoradiotherapy
protocol for treatment of soft tissue sarcoma. We recently gained encouraging data with the phthalazine derivative PTK787/ZK222584 (highly specific for VEGFR-1 and -2) combined with IR on tumor cell control in in vivo mice experiments. Furthermore, cotreatment of primary human umbilical vein endothelial cells (HUVEC) nicely abrogated VEGF-dependent proliferation in an additive way (188). PTK787/ZK222584, denominated in this work by PTK787, not only downregulates VEGF-receptor activity but subsequently targets the PI3K/Akt survival pathway as shown in a zebrafish angiogenesis model system (183). A constitutive active form of Akt rescued blood vessel formation and endothelial survival in zebrafish embryos treated with PTK787. Data from our group indicate that this inhibition is not only on the phosphorylation but also on the Akt protein level in endothelial cells (Riesterer et al., submitted). Regulation at the protein level in endothelial cells might have different implications in regard of cellular toxicity and, ultimately, tumor control compared to a regulation of Akt at the phosphorylation level and further supports the combined treatment approach (254).

**Table 2.** Kinase and autophosphorylation inhibitory profiles of PKI166 and PTK787 (in vitro)

<table>
<thead>
<tr>
<th>IC&lt;sub&gt;50&lt;/sub&gt;, µM</th>
<th>PKI166</th>
<th>PTK787</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Receptor-Type</strong></td>
<td><strong>Kinase</strong></td>
<td><strong>Phosphorylation</strong></td>
</tr>
<tr>
<td>ErbB1</td>
<td>0.001</td>
<td>0.051</td>
</tr>
<tr>
<td>ErbB2</td>
<td>0.011</td>
<td>0.1-1</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>0.962</td>
<td>na</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>&gt; 1</td>
<td>na</td>
</tr>
<tr>
<td>PDGFR</td>
<td>na</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>PKC-α</td>
<td>&gt; 10</td>
<td>na</td>
</tr>
</tbody>
</table>

*Abbreviations:* IC<sub>50</sub>, concentration required to inhibit kinase activity or phosphorylation by 50%; na, not available.
Aim of the study:

The relation of signal transduction cascades, cellular origine and radiosensitization is addressed in this thesis. The relevance of the PI3K/Akt survival pathway in the cellular response to ionizing radiation will be analyzed and discussed in more details. We will lay emphasis especially on its role for radiosensitization since downregulation of this pathway might be a valid strategy to overcome an apoptotic threshold. In parallel we will investigate the mechanism(s) leading to its activation upon radiation stimulation in tumor and endothelial cells. Understanding of such IR-induced processes are of crucial importance to improve combined therapies with radiation.

Combining molecular biology tools and genetically defined cells we used to this end three different and clinically relevant kinase inhibitors recently developed by Novartis (see also above):

a) PKC412 a protein kinase C inhibitor,

b) PKI166 an epithelial growth factor receptor inhibitor and

c) PTK787 a vascular epithelial growth factor receptor inhibitor.

Besides studying their radiosensitzing potential and their mechanism of action, we will also use them to dissect signal transduction cascades induced by ionizing radiation and specifically the PI3K/Akt survival pathway. In particular, we will investigate the interaction of PKC412 with the PI3K/Akt pathway and the importance of PTEN in this context. We will also analyze the radiosensitzing and inhibitory potential of PKI166 in a tumor cell line model and the influence of this inhibitor on the PI3K/Akt cascade. Further, mechanistic studies will be performed using both receptor tyrosine kinase inhibitors PKI166 and PTK787 to elucidate radiation-induced PI3K/Akt-stimulation in endothelial cells and compared it to the radiation-induced Akt-activation profile in tumor cells.
2 MATERIALS AND METHODS

2.1 Cell lines

All cell lines were cultured at 37°C and in 5% CO₂ atmosphere.

All media, except EGM, were supplemented with L-glutamine (1%), penicillin and streptomycin (1%).

EGM was supplemented with 0.4% ECGS/H, 0.1 ng/ml EGF, 1 ng/ml bFGF, 1 μg/ml Hydrocortison, 50 ng/ml Amphotericin B and penicillin and streptomycin (1%).

All media were obtained from Hyclone/Gibco.

Table 3. Cell lines used and their corresponding media.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Description</th>
<th>Basal Medium</th>
<th>Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEFs clonal selected, EIA/ras transformed p53+/+</td>
<td>p53+/+ MEFs were derived from 13.5-day-old mouse embryos and stably transfected with the two oncogenes EIA and T24 H-ras (192)</td>
<td>DMEM</td>
<td>10% FCS, 10% BCS</td>
</tr>
<tr>
<td>MEFs, EIA/ras transformed p53+/+, myrAkt</td>
<td>myrAkt MEFs were constructed during this work</td>
<td>DMEM</td>
<td>10% FCS, 10% BCS</td>
</tr>
<tr>
<td>MEFs, EIA/ras transformed p53+/+, pBabe</td>
<td>pBabe MEFs were constructed during this work</td>
<td>DMEM</td>
<td>10% FCS, 10% BCS</td>
</tr>
<tr>
<td>PTEN+/+ MEFs</td>
<td>PTEN+/+ and PTEN-/- MEFs were kindly provided by H. Wu (193)</td>
<td>DMEM</td>
<td>10% FCS</td>
</tr>
<tr>
<td>PTEN-/- MEFs</td>
<td></td>
<td>DMEM</td>
<td>10% FCS</td>
</tr>
<tr>
<td>A431, human cervix carcinoma, overexpressing erbB1</td>
<td>A431, MDA-MB-468, MDA-MB-231 and LNCaP cells lines were a gift from D. Fabbro (Novartis)</td>
<td>DMEM</td>
<td>10% FCS</td>
</tr>
<tr>
<td>NF9006, mouse mammary carcinoma, overexpressing c-erbB2 (c-neu)</td>
<td></td>
<td>DMEM + Hepes (1%), + non essential amino acids (1%)</td>
<td>10% NBCS</td>
</tr>
<tr>
<td>HUVEC, human umbilical vein endothelial cells</td>
<td></td>
<td>EGM</td>
<td>10% FCS</td>
</tr>
<tr>
<td>φNX-A, human embryonic kidney cell line stably express amphotropic envelope</td>
<td>φNX packaging cell lines are derived from 293T cells stably expressing a temperature sensitive SV40 large T antigen, gag-pol, IRES-CD8 surface marker and were kindly provided by the Nolan laboratory</td>
<td>DMEM</td>
<td>10% FCS</td>
</tr>
<tr>
<td>φNX-E, human embryonic kidney cell line stably express ecotropic envelope</td>
<td></td>
<td>DMEM</td>
<td>10% FCS</td>
</tr>
</tbody>
</table>
2. Materials and Methods

2.2 Chemicals

Table 4. Chemicals

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-inhibitor PKC412 (N-benzoyl-staurosporine, CGP41251)</td>
<td>Novartis Pharma AG</td>
</tr>
<tr>
<td>EGF-receptor-inhibitor PKI166</td>
<td>Novartis Pharma AG</td>
</tr>
<tr>
<td>VEGF-receptor-inhibitor PTK787/ZK222584</td>
<td>Novartis Pharma AG</td>
</tr>
<tr>
<td>Staurosporine</td>
<td>Sigma</td>
</tr>
<tr>
<td>PI3-kinase-inhibitor LY294002</td>
<td>Sigma</td>
</tr>
<tr>
<td>epithelial growth factor (EGF)</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>human vascular epithelial growth factor (VEGF)</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>Caspase-3 colorimetric substrate (Ac-DEVD-pNA)</td>
<td>Biosource International</td>
</tr>
<tr>
<td>AlamarBlue</td>
<td>Sigma</td>
</tr>
<tr>
<td>Typan Blue solution</td>
<td>Sigma</td>
</tr>
<tr>
<td>ECL western blotting detection system</td>
<td>Amersham Pharmacia Biotech</td>
</tr>
<tr>
<td>dATP</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-phospho-Akt (Ser473) antibody</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-phospho-ErbBl (Tyr1086) antibody</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-ErbBl antibody</td>
<td>Oncogene</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
</tr>
<tr>
<td>anti-phospho-VEGFR2 (Tyr951) antibody</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-phospho-VEGFR2 (Tyr996) antibody</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-VEGFR2 antibody</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
</tr>
<tr>
<td>anti-VEGFR2 antibody</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-β-actin, clone AC-15</td>
<td>Sigma</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
</tr>
<tr>
<td>anti-phospho-GSK-3α (Ser21) antibody</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>sheep</td>
<td></td>
</tr>
<tr>
<td>anti-phospho-tyrosine antibody (protein A agarose conjugated), clone 4G10</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
</tr>
<tr>
<td>anti-Akt antibody</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-GSK-3</td>
<td>Upstate Biotechnology</td>
</tr>
<tr>
<td>mouse monoclonal</td>
<td></td>
</tr>
<tr>
<td>anti-cleaved caspase-3 antibody</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>rabbit polyclonal</td>
<td></td>
</tr>
<tr>
<td>anti-mouse IgG secondary antibody, HRP-linked</td>
<td>Amersham Pharmacia Biotech</td>
</tr>
<tr>
<td>anti-rabbit IgG secondary antibody, HRP-linked</td>
<td>Amersham Pharmacia Biotech</td>
</tr>
<tr>
<td>anti-sheep IgG secondary antibody, HRP-linked</td>
<td>Upstate Biotechnology</td>
</tr>
</tbody>
</table>

All other reagents were of the highest purity grade and commercially available.
2.3 Irradiation

Irradiation was carried out at room temperature, using a Pantak Therapax 300kV X-ray unit at 0.7 Gy/min.

2.4 PKC412, PKI166, PTK787, Staurosporine and LY294002 Treatment

PKC412, PKI166, PTK787, Staurosporine and LY294002 were dissolved in DMSO (stock-concentrations were 10 mM for PKC412, PKI166, PTK787 and LY294002 and 1 mM for STP) and stored at -20°C. PKC412 and STP were first ten times diluted in DMSO:H2O (1:1) and further diluted in the corresponding serum-containing media. PKI166, PTK787 and LY294002 were directly diluted in the corresponding media. For combined treatments (IR, serum-, EGF- or VEGF-stimulation), cells were pre-incubated with PKC412 or Staurosporine 4 h, PKI166 or PTK787 1 h and LY294002 30 min prior to the corresponding treatment.

2.5 Proliferation Assay

Cell proliferation was assessed with the colorimetric alamarBlue assay based on the detection of metabolic activity (194, 195). After different time points (24, 48 or 72 hours after treatment) alamarBlue reagent was added in the medium (volume 1/10 of medium), after incubation for four hours. Absorption was measured at 570 and 670 nm using a Dynatech MR5000 spectrophotometer.

2.6 Trypan Blue Viability Assay

Floating and adherent cells were collected at the indicated time points, harvested by centrifugation and resuspended in the corresponding cell medium. The cells were then diluted 1:1 with 0.4% trypan blue solution and scored under the microscope. The results represent the mean of at least two independent experiments with a minimum of 100 cells scored/treatment.

2.7 Cell Fractionation (Cytosolic Extracts)

Cells were harvested by centrifugation and washed with ice-cold PBS. The cell pellet was suspended in 5 volumes of ice-cold buffer A (20 mM Hepes-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 250 mM sucrose, and 0.1 mM PMSF, supplemented with protease inhibitors (5 µg/ml pepstatin A, 10 µg/ml leupeptin, 2 µg/ml aprotinin)). After sitting on ice for 15 min, the cells were disrupted by douncing 15 times with a syringe with a 25 G needle. Cell lysates were centrifuged at 1000xg for 10 min at 4°C and the supernatant was further centrifuged at 100'000xg for 1h. The resulting supernatant (S-100 fraction) was stored at -80°C.

2.8 Protein Determination

Protein concentration of cellular extracts was performed using the Bio-Rad protein assay, which is based on the Bradford method. As standard bovine serum albumin (BSA) was used.

2.9 In vitro Caspase Activity Assay

To determine caspase-3-like activity 75 µg protein (S-100 fraction) were incubated at 37°C with the colorimetric caspase-3 substrate Ac-DEVD-p-nitroanilide (pNA) (100 µM), and 1 mM dATP in a final volume of 120 µl. Control measurements were performed in presence of the caspase-specific inhibitor Ac-DEVD-CHO (20 µM) to correct for unspecific background activity. The cleavage of the caspase substrates was monitored at 405 nm using a
Dynatech MR5000 spectrophotometer.

2.10 MyrAkt-overexpressing Cell Lines

ϕNX-packaging cells were transfected with a total of 8 μg of pBabe(puro) plasmid DNA or its derivative (containing the myristoylated/palmitylated (m/p)-HA-PKBα DNA construct) (196) by calcium phosphate coprecipitation as described (197). The medium containing the retrovirus (supernatant) was harvested 48 h posttransfection, following removal of the precipitate and used to infect the target cells. The E1A/ras transformed MEF target cells were infected at 40% confluence in the presence of polybrene (4 μg/ml; Sigma). Puromycin-selection (2 μg/ml) to enrich a transfected cell population was initiated 30 h after infection and performed over at least 14 days.

2.11 Akt-phosphorylation Measurement and Serum Starvation

PTEN+/+ and PTEN-/- MEFs (1.5x10⁵), NF9006 (2.5x10⁵), A431 (4x10⁵) and HUVE (1x10⁵) cells (2x10⁵) were plated in a 30 mm dish. For Akt-stimulation PTEN+/+ and PTEN-/- MEFs were incubated in serum-free DMEM and after 2 h serum starvation, the medium was replaced by DMEM containing 10% FCS. A431 cells were serum-starved for 24 hours in DMEM with 0.5% FCS. For HUVE cells serum starvation was performed in endothelial cell basal medium supplemented with 0.1% BSA for 12 h. No serum starvation was necessary for Akt-stimulation in NF9006 cells. Drug-treatment and serum or growth factor (EGF, VEGF) stimulation were performed as mentioned. Cells were harvested at different time points by scraping off the cells in 100 μl SDS-sample buffer, heated for 5-10 min at 95°C and stored at -80°C. Samples were then analyzed by western blotting.

2.12 Western Blotting

Samples were analyzed by SDS-PAGE followed by blotting onto PVDF-membranes using a wet system. Membranes were blocked for 1 hour at room temperature in 5 % milk, TBS, 0.1% Tween-20 and then incubated over night at 4°C with the primary antibody. Antibody detection was achieved by ECL-enhanced chemiluminescence using a horseradish peroxidase conjugated secondary antibody (incubation for 1 hour at room temperature). Quantification of the blots was performed with Scion Image computer software.

10%-running gel:

| 30% Acrylamide/0.8% Bis-Acrylamide | 5 ml |
| Running Buffer | |
| (Tris-HCl 1.5 M, pH 8.8, 0.1% SDS) | 3.75 ml |
| 10% APS | 50 μl |
| TEMED | 10 μl |
| H₂O | 6.1 ml |

Stacking gel:

| 30% Acrylamide/0.8% Bis-Acrylamide | 650 μl |
| Stacking Buffer | |
| (Tris-HCl 0.5 M, pH 6.8, 0.1% SDS) | 1.25 ml |
| 10% APS | 25 μl |
| TEMED | 10 μl |
| H₂O | 3.05 ml |
2.13 Membrane Stripping
Membranes were incubated with a stripping-solution (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) for 30 min at 52°C. The membranes were then washed three times with TBS + 0.1% Tween-20, blocked for 1 hour at room temperature with blocking buffer and were then again ready to probe with a different primary antibody.

2.14 Immunofluorescence Labeling and Confocal Laser-scanning Microscopy
HUVEC were treated in 2-well collagen-coated plates (Biocoat, Becton Dickinson) as indicated above, followed by fixation with 3% paraformaldehyde for 15 min and cell permeabilization with 0.5% Triton X-100 in PBS for 2.5 min. The primary rabbit polyclonal anti-VEGFR-2 phosphoTyr996-antibody (Calbiochem) was used at a dilution of 1:50. The secondary Texas Red-X-conjugated affinity-purified goat anti-rabbit IgG (H + L) antibody (Molecular Probes, Eugene OR) was used at a dilution of 1:100. Both antibodies were incubated for 1 h with the cells. Cells were costained with 4,6-diamidino-2-phenylindole (DAPI, 10 µg/ml) for detection of nuclei. The coverslips were mounted on glass slides and embedded in Mowiol (Calbiochem). All steps were performed at RT. A Zeiss Axioplan fluorescence microscope (Zeiss, Germany) equipped with a confocal scanning unit MRC-600 (Bio-Rad, UK) and an argon-krypton laser was used to acquire images, which were subsequently processed using Adobe-Photoshope (System 6.0 San Jose, CA) and reconstructed using simple PC-software.

2.15 Immunoprecipitation
Cells were washed with ice-cold PBS and harvested by scraping off cells in 500 µl of buffer A (20 mM Tris-HCl pH 8.0, 5% Glycerol (m/V), 1% Nonidet P40 (m/V), 2.7 mM KCl, 138 mM NaCl, 1 mM MgCl₂, 1 mM CaCl₂, 50 mM sodium EDTA, 1 mM DTT), supplemented with protease and phosphatase inhibitors (5 mg/ml pepstatin A, 10 mg/ml leupeptin, 2 mg/ml aprotinin, 1 mM Na₃VO₄, 1 mM NaF and 0.1 mM PMSF). After incubation on ice for 30 min the lysate was centrifugated at 15000 x g for 10 min at 4°C. Protein concentration was determined from the supernatant by colorimetric measurement using the Bio-Rad DC protein assay kit II according to the manufacturer’s instructions (Bio Rad Laboratories, Hercules CA). Supernatants (1 mg/ml protein) were incubated with 25 µg Protein A agarose-conjugated anti-phosphoTyr monoclonal antibody (4G10, Upstate Biotechnology, Lake Placid NY) or 2 µg anti-VEGFR-2 polyclonal antibody (Calbiochem) overnight at 4°C and with protein A-conjugated agarose beads (Upstate Biotechnology) at 4°C for 4 hrs. The beads were washed three times in 0.5 ml buffer A, supplemented with all inhibitors, and immunoprecipitates were resuspended in SDS sample buffer (2x) for Western blot analysis.
3 RESULTS

3.1 The PI3K/Akt survival pathway as target for radiosensitizers

3.1.1 The PI3K/Akt-pathway is a target for the antiproliferative and cytotoxic activities of the PKC-inhibitor PKC412

Activation or upregulation of the PI3K/Akt survival pathway often correlates with a cellular stress-resistant phenotype (77, 92). In E1A/ras-transformed MEFs the equilibrium between unphosphorylated and phosphorylated Akt is shifted towards the active form due to a continuous stimulation of PI3K-activity by mutated T24 H-ras (198). We investigated the role of this pathway for the antiproliferative effect of the PKC-inhibitor PKC412. PKC412 is a N-benzoylated staurosporine derivative which is very selective against the conventional α,β,γ-PKC-isoforms (47). To detect an antiproliferative effect of PKC412 in cells with an endogenous high Akt-phosphorylation and -activity level, E1A/ras-transformed MEFs were treated with increasing concentration of PKC412. The metabolic activity was determined 48 hours after treatment with the alamarBlue assay. The alamarBlue assay is a proliferation assay assessing the metabolic activity comparable to the MTT-tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)2,5-diphenylterazolium bromide assay] quantification of cell metabolism.

![Graph](image)

**Figure 3-1** PKC412 decreases proliferative activity in E1A/ras-transformed MEFs. (A) E1A/ras-transformed MEFs carrying a control vector or E1A/ras-transformed MEFs expressing dominant active myrAkt were treated with increasing concentrations of PKC412 (0-1 μM). Proliferative activity was determined 48 h after treatment. (B) The phosphorylation level of Akt was determined in whole cell extracts using an anti-Akt phosphoSer-473 specific antibody. Samples were reanalyzed with an antibody against total Akt to ensure the loading of equal amounts of protein.

The E1A/ras-transformed MEFs were also infected with a high-titer control retrovirus and a retrovirus encoding the dominant and constitutive active myristoylated Akt-construct.
(myrAkt). These two newly produced cell lines enabled us to evaluate the importance of the PI3K/Akt pathway for the antiproliferative effect of PKC412. The proliferative index of the two cell populations as determined by cell counting over 48 hours were similar in absence of treatment. The ElA/ras-transformed myrAkt-MEFs were more resistant to treatment with PKC412 than cells infected with a control retrovirus. After 48 hours the proliferative activity in the control-infected cell population was decreased in a dose dependent way (0-1 μM PKC412) reaching 18% of the activity compared to untreated cells. Interestingly cells expressing the dominant active form of Akt (myrAkt) were less sensitive to treatment with PKC412. High doses of PKC412 (at 500 nM and higher) reduced the proliferative activity only to 50% (Fig. 3-1 A). To control the differential activation level of Akt between both cell lines, we determined the phosphorylation/activity status of Akt in whole cell extracts by western blotting using a Ser-473-site specific anti-phospho-Akt antibody. As expected, a clear increase in phosphorylated Akt was detected in the myrAkt expressing cells in comparison with the control ones (Fig. 3-1 B). The same samples were also analyzed with an anti-Akt-antibody to ensure equal protein loading. A slightly higher amount of total Akt-proteins was present in the cells expressing the dominant active form of Akt over the control ones. However, this difference was less significant than the difference observed in the amount of the active form of Akt in both cell populations.

Further, the cytotoxic effect of PKC412 was compared in both cell populations. The amount of dead cells was quantified using the trypan blue exclusion assay 48 hours after treatment. Treatment with a low dose of PKC412 (200 nM) resulted in minimal cell death in both control and myrAkt-expressing cells (Fig. 3-2). This is similar to the slight antiproliferative effect observed previously in both cell populations at this concentration (Fig. 3-1). In contrary, a high PKC412-concentration (500 nM) induced massive cell death in the control cells (62%). Interestingly, expression of dominant-active Akt partially abrogated the cytotoxic effect of PKC412 (40%, Fig. 3-2), similar to the response observed on the level of proliferation.

Thus these results indicate that the PKC412 antiproliferative and cytotoxic effects are at least in part mediated via inhibition of the PI3K/Akt pathway. In addition, they confirm similar
previous data obtained with Rat1a cells and the corresponding myrAkt-overexpressing cells (52). Additionally, the observed elevated PKC412 antiproliferative and cytotoxic effects in normal MEFs correlates with PKC412-induced activation of the apoptotic machinery (52).

### 3.1.2 Downregulation of the PI3K/Akt-pathway sensitizes for ionizing radiation

We previously reported that PKC-inhibitors sensitize for IR-induced apoptosis in ELA/ras-transformed MEFs. Furthermore, recently several reports have described that inhibition of the PI3K/Akt-survival pathway by LY294002 sensitizes for IR-induced cell death (51, 88, 89). Therefore, we tested the antiproliferative effect of PKC412, IR alone and in combination in ELA/ras-transformed MEFs and cells expressing dominant-active Akt. The partial antiproliferative effect of IR (2 Gy) and PKC412 (200 nM) treatment alone was similar in both cell populations (65% of untreated cells). Combined treatment with PKC412 and irradiation resulted in an at least additive effect in the control cells (20%), however expression of dominant-active Akt considerably abrogated this cooperative effect (40%, Fig. 3-3).

![Figure 3-3 PKC412 sensitzes for treatment with ionizing radiation.](image)

**Figure 3-3** PKC412 sensitzes for treatment with ionizing radiation. ELA/ras-transformed MEFs carrying a control vector and ELA/ras-transformed MEFs expressing dominant active myrAkt were treated with 200 nM PKC412, 2 Gy of IR or in combination and the proliferative activity was determined 48 h after treatment (adapted from (52)).

This last experiment demonstrates that modulation of the PI3K/Akt-pathway influences the cell sensitivity to ionizing radiation. It further indicates that the PKC412 radiosensitizing effect is at least in part mediated via this signaling pathway.

### 3.1.3 PKC412-mediated antiproliferative and cytotoxic effects are influenced by PTEN

A high Akt-phosphorylation and -activity status is found in cells with mutated ras but can also be due to other genetic factors such as the lack of intact PTEN. PTEN is a phosphatase that acts on the D3-position of phosphatidylinositol (3,4,5) triphosphate, a direct product of PI3K-activity and thus PTEN-mutant cells have elevated 3’-phosphorylated phosphatidylinositol levels. LNCaP prostatic cancer cells are characterized by having a PTEN mutation and a high Akt-phosphorylation status (199). Interestingly, previous experiments performed in our laboratory showed a reduced antiproliferative effect of PKC412 as well as an incapability of PKC412 to dephosphorylate and inhibit Akt in these cells (52). However,
one could not exclude that another factor than the lack of functional PTEN was responsible for the attenuated PKC412-effects observed in this cancer cell line.

Therefore, we tested the antiproliferative effect of PKC412 in two genetically identical MEF cell populations which only differ in their PTEN-status, the one being PTEN-positive (PTEN+/+ MEFs) and the other one PTEN-negative (PTEN-/- MEFs). A slightly more pronounced dose-dependent antiproliferative effect of PKC412 was seen in the cell population expressing PTEN in comparison with the PTEN-deficient counterpart upon exposure during 48 hours to 100 nM and 200 nM PKC412 (82 vs 90% and 60 vs 70% respectively; Fig. 3-4).

![Figure 3-4](image)

**Figure 3-4** PKC412 compromises proliferation more efficiently in PTEN-positive than in PTEN-negative MEFs. Both fibroblast cell lines were treated with increasing concentrations of PKC412 (0-2 μM) and the proliferative activity was measured 48 hours after treatment.

Therefore, we tested the antiproliferative effect of PKC412 in two genetically identical MEF cell populations which only differ in their PTEN-status, the one being PTEN-positive (PTEN+/+ MEFs) and the other one PTEN-negative (PTEN-/- MEFs). A slightly more pronounced dose-dependent antiproliferative effect of PKC412 was seen in the cell population expressing PTEN in comparison with the PTEN-deficient counterpart upon exposure during 48 hours to 100 nM and 200 nM PKC412 (82 vs 90% and 60 vs 70% respectively; Fig. 3-4).

![Figure 3-5](image)

**Figure 3-5** The PI3K-inhibitor LY294002 reduces proliferation in both PTEN-positive and -negative MEFs in a similar way. Both fibroblast cell lines were treated with increasing concentrations of LY294002 (0-10 μM) and the proliferative activity was measured 48 hours after treatment.
On the other hand, the PI3K-specific inhibitor LY294002 reduced the proliferative activity of both cell lines in a similar and dose-dependent way independently of their PTEN-status (Fig. 3-5).

To further evaluate the cytotoxic effect of PKC412 and its PTEN-dependence both PTEN-positive and -negative MEF populations were treated with low and high dose of the drug (0.5 and 5 μM, respectively). The amount of dead cells was quantified using the trypan blue exclusion assay. Interestingly, treatment with both PKC412-concentrations resulted in massive cell death in the cell line possessing an intact PTEN (37% and 65% respectively). On the other hand, significant lower amounts of the PTEN-deficient cells were stained trypan blue positive (17% and 25% respectively; Fig. 3-6 A). Less than 5% of dead cells were counted in both untreated cell populations. To analyze if the PTEN-dependent cytotoxic effect of PKC412 might be a particularity of PKC-inhibitors the same experiment was performed with its parent compound staurosporine (STP) – a broad range PKC-inhibitor. Similar results were obtained for this later substance. A dose as low as 10 nM STP already showed a marked difference in cytotoxicity between both PTEN-positive and -negative cell lines (22% vs 12%), while for the higher 50 nM concentration a significant difference was still observed (52% vs 30%; Fig. 3-6 B). Again, in both untreated cell populations less than 5% of the cells were stained trypan blue positive.

This last experiment suggests that the cytotoxicity of PKC-inhibitors in general might be in part dependent on the presence of functional PTEN and also strengthens the PTEN-dependent antiproliferative and cytotoxic effects observed with PKC412. Furthermore, these results demonstrate that the moderate PTEN-dependent antiproliferative effect of PKC412 correlates with its PTEN-dependent cytotoxicity.

**Figure 3-6** PKC412 and STP cytotoxic effects are PTEN-dependent. PTEN-positive and -negative MEFs were treated with increasing concentrations of PKC412 (0-5 μM (A)) or STP (0-50 nM (B)). Cell viability was determined by trypan blue exclusion 48 h after treatment.
3. RESULTS – The PI3K/Akt Survival Pathway as Target for Radiosensitizers

3.1.4 PKC412-mediated inhibition of the PI3K/Akt pathway is PTEN-dependent

Akt-activation is a multistep process and requires phosphorylation of the Akt Ser-473 site. Previous analysis of the inhibitory potential of LY294002 and PKC412 on the PI3K/Akt pathway in LNCaP cells suggested that the two agents do not act in a similar way (52). In addition, downregulation of phosphorylated Akt (Ser-473) in Rat1a cells and MEFs occurred in a different time frame between both substances (within minutes for LY294002 and hours for PKC412, see also (52)). Furthermore, the observed difference in the antiproliferative effect between PKC412 and LY294002 (lightly PTEN-dependent vs PTEN-independent) reinforces the hypothesis of a differential inhibitory mechanism (see above). To determine the role of PTEN in the inhibition of the PI3K/Akt pathway by PKC412 the Akt Ser-473-phosphorylation status was analyzed in both PTEN-positive and -negative MEF populations after treatment with PKC412 and compared to treatment with the specific PI3K-inhibitor LY294002. The mechanism of the PKC412 parental compound STP was also analyzed.

![Figure 3-7](image)

**Figure 3-7** PKC412 and STP downregulate Akt-phosphorylation only in the presence of PTEN. PTEN-positive and -negative MEFs were treated with different concentrations of PKC412 (0.5 and 1 μM), STP (50 and 100 nM) or LY294002 (20 μM). Incubation times in the presence of PKC412 or STP were 4 hours and 30 minutes for LY294002. The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.

It was not necessary to stimulate these cell lines to detect endogenous phosphorylated Akt. Cells were incubated with the different drugs during 30 minutes (LY294002) or 4 hours (PKC412 and STP). Whole cell extracts were probed by western blotting with a Ser-473-site specific anti-phospho-Akt antibody. In both PTEN-positive and -negative cell populations no Akt-phosphorylation was detected after treatment with 20 μM LY294002. In comparison, treatment with 0.5 or 1 μM PKC412 also markedly reduced phosphorylated Akt in the PTEN-expressing cell population. But in contrast, both PKC412-concentrations did not downregulate the Akt-phosphorylation level in the PTEN-deficient parental cell population (Fig. 3-7). In analogy, the phospho-Akt status was also considerably decreased upon preincubation with 50 or 100 nM STP in the PTEN-positive cells, although in a lesser extend than following PKC412-exposure. Similarly, STP-treatment (50 or 100 nM) of the PTEN-negative cells did not result in any detectable reduced Akt-phosphorylation level. The same samples were also
analyzed with an anti-β-actin-antibody to ensure equal protein loading. Thus, these data demonstrate that inhibition of the PI3K/Akt pathway at the level of Akt by PKC412 and STP depends on the presence of intact PTEN, while LY294002 blocks this pathway independently of PTEN.

The PI3K/Akt survival pathway can be stimulated by exogenous factors such as the epithelial growth factor receptor ligand EGF. To investigate if under (EGF-)stimulation, like under unstimulated conditions, PKC412-mediated inhibition of the PI3K/Akt cascade was still PTEN-dependent, a similar experiment as before was performed.

![Figure 3-8](image_url)

**Figure 3-8** Inhibition of EGF-mediated Akt-phosphorylation by PKC412 and STP requires PTEN. Following serum starvation (2 h) PTEN-positive and -negative MEFs were treated with PKC412 (0.5 and 1 μM, 4 h), STP (50 and 100 nM, 4 h) or LY294002 (20 μM, 30 min) and subsequently stimulated with EGF (50 ng/ml) for 30 minutes. The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.

The cells were serum and growth factors starved for 2 hours – to lower the basal phospho-Akt level – and incubated with LY294002 (20 μM, 30 min), PKC412 (0.5 or 1 μM, 4 h) or STP (50 or 100 nM, 4 h) followed by stimulation for 30 minutes with EGF (50 ng/ml) Again, all the three inhibitors downregulated Akt Ser-473-phosphorylation in the PTEN-expressing cell population, while only LY294002 but not PKC412 and STP at all investigated concentrations induced a reduction of the Akt-phosphorylation level in the PTEN-deficient cell population (Fig. 3-8). This confirms that Akt-inhibition mediated by PKC412 and STP, in contrary to LY294002, requires the presence of functional PTEN.

**3.1.5 PKC412 has an antiproliferative effect in ErbB2-overexpressing cells**

Members of the ErbB-receptor family are overexpressed in a large number of human carcinomas and correlate with enhanced radioresistance (121-124, 200). One potential mechanism was recently demonstrated to be mediated through signaling via PI3K-Akt (125, 201, 202). Thus, blocking the PI3K/Akt survival pathway in ErbB-overexpressing cell lines might be a potent strategy to inhibit cell growth in these cell lines and to overcome radioresistance. In this study, we evaluated the antiproliferative and radiosensitizing potential of PKC412 in the mouse mammary carcinoma cell line NF9006 overexpressing ErbB2-receptors.
Cells were treated with increasing PKC412-concentrations (0-1 μM) alone and in combination with irradiation (5 or 10 Gy). The proliferative activity was determined 72 hours after treatment and was markedly decreased after treatment with increasing concentrations of PKC412 and irradiation (Fig. 3-9). PKC412-doses as low as 250 nM reduced the proliferative index to 80% compared to untreated cells, while higher doses (500 nM and higher) led to 45% of untreated cell activity. An antiproliferative cell behaviour was also observed upon radiation-exposure to 5 and 10 Gy alone reaching 55% and 42% of control activity respectively. Combination of radiation with increasing concentrations of PKC412 did not further enhance the antiproliferative capacity of both treatments alone.

Thus, PKC412 – as partial inhibitor of the PI3K/Akt-pathway – showed a clear antiproliferative effect in the ErbB2-overexpressing carcinoma cell line NF9006 but the combination with radiation did not improve this effect.

**3.1.6 The dual ErbB1/2-receptor tyrosine kinase inhibitor PKI166 has an antiproliferative effect in ErbB2-overexpressing cells**

Another strategy to counteract the radioresistant phenotype resulting from ErbB-receptor overexpression in tumor cells is to directly target the ErbB-receptors. Different ErbB-inhibitors have been identified as potent radiosensitizers (recently reviewed in (203-205)). In this study the radiosensitizing potential of the dual ErbB1/2-receptor tyrosine kinase inhibitor PKI166 (Novartis Pharma) was tested *in vitro* against the ErbB2-overexpressing carcinoma cell line NF9006. Therefore, the antiproliferative effect of PKI166 was evaluated alone and in combination with ionizing radiation in these cells. Previous experiments already demonstrated an additive antiproliferative effect when PKI166 was applied in combination with irradiation in two different tumor cell lines overexpressing ErbB1, the human cervix carcinoma cell line A431 and the human breast cancer cell line MDA-MB-468 (163). Here, NF9006 cells were treated with increasing concentrations of PKI166 (0-0.5 μM) alone or followed by irradiation (5 or 10 Gy) and the proliferative activity was measured as metabolic activity 72 hours later.
3. RESULTS – The PI3K/Akt Survival Pathway as Target for Radiosensitizers

(Fig. 3-10). Cell proliferation was considerably decreased after treatment with increasing concentrations of PKI166 reaching 30% of control activity at a concentration of 500 nM.

![Figure 3-10](image)

**Figure 3-10** PKI166- and IR-mediated antiproliferative effects are not additive in NF9006 cells. Cells were treated with increasing concentrations of PKI166 (0-5 μM) and IR-doses (0-10 Gy). The proliferative activity was measured 72 hours after treatment.

Similarly to treatment with PKC412, combination of radiation with increasing concentrations of PKI166 did not further enhance the antiproliferative capacity of both treatments alone. In opposite to the data obtained with the ErbB1-overexpressing cell lines, PKI166 did not show any additive antiproliferative effect in NF9006 cells when combined with radiation.

### 3.1.7 PKI166 inhibits the PI3K/Akt pathway in ErbB2-overexpressing cells

As demonstrated with the PKC-inhibitor PKC412 the activity status of the PI3K/Akt-pathway plays an important role for the radiosensitivity of a cell. In parallel, ErbB-receptor overexpression mediates multiple different cellular responses (principally leading to radioresistance) through activation of the PI3K/Akt-signaling transduction cascade. Thus, blockade of the ErbB2-receptor should result in the inhibition of this survival pathway. The influence of ErbB2-inhibition on the PI3K/Akt-pathway was investigated with the dual ErbB1/2-kinase inhibitor PKI166 and was monitored on the level of Akt phosphorylation. NF9006 cells (overexpressing rat-Neu, which corresponds to ErbB2 in human) were treated for 2 hours with increasing concentrations of PKI166 (0-5 μM) then proteins were extracted and probed against the phospho-Akt-Ser473 antibody by western blotting. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.
3. RESULTS – The PI3K/Akt Survival Pathway as Target for Radiosensitizers

Endogenous high levels of activated/phosphorylated Akt were detected in the untreated sample confirming the downstream activation of the PI3K/Akt-pathway in this ErbB2-overexpressing cell line. The Akt-phosphorylation signal intensity was already considerably reduced following 0.25 μM PKI166 treatment and barely detectable upon incubation with higher drug-concentrations (0.5-5 μM; Fig. 3-11).

Inhibition of Akt-phosphorylation after PKI166-exposure (1 μM) occurred very rapidly and persisted at least for a 6 hours time period (Fig. 3-12). At a concentration of 0.5 μM, PKI166 leads to a considerable reduction in Akt-activation corresponding to the effective dose in the proliferative assay (70% inhibition). This indicates that the antiproliferative effect of PKI166 in ErbB2-signaling dependent cells is, at least in part, mediated via downregulation of the PI3K/Akt-survival pathway. This further suggests that another still not identified pathway might also be responsible for this PKI166-mediated effect.
3. RESULTS – The PI3K/Akt Survival Pathway as Target for Radiosensitizers

3.1.8 PKI166 enhances radiation-mediated activation of the apoptotic machinery in ErbB2-overexpressing cells

Apoptosis is a well-defined and regulated process leading to cellular death and may be activated in diverse stress situations. Combined treatment of PKC412 and ionizing radiation was previously shown to drastically induce apoptotic cell death and to be one possible mechanism responsible for radiosensitization by PKC412 (49, 50). This was demonstrated to be mediated via cytochrome C-release from the mitochondria and subsequent activation of caspase-9 and -3 (51). Further, recent studies identified the PI3K/Akt-pathway as potential mediator for the PKC412 induced apoptosis (52). Here, the impact of PKI166 and radiation alone and in combination on the activation of the apoptotic machinery was investigated in NF9006 cells.

![Graph](image)

**Figure 3-13** Effect of PKI166 and IR on the apoptotic machinery in NF9006 cells. As apoptotic marker, caspase-3-like activity was determined 15 hours after treatment with PKI166 (0.5 μM) or IR (5 Gy) alone and in combination in cytosolic extracts using Ac-DEVD-pNA as a colorimetric substrate. Caspase-3-activity measurements show results from three independent experiments.

To assess apoptosis induction in these cells the effector protease activity (caspase-3-like/DEVDase-activity) was measured in cytosolic S-100 fraction 15 hours after treatment using Ac-DEVD-pNA as colorimetric caspase-3-substrate (Fig. 3-13). A significant increase in caspase-3 activity was observed after both PKI166 (0.5 μM) and IR (5 Gy) treatment alone in comparison to untreated cells (2 and 3 times respectively). However, combined treatment of PKI166 with IR induced caspase-3 to a much higher level than in the control sample (about 10 times) and has an at least additive effect compared to both treatments alone.

We still do not understand how PKI166 sensitizes to IR and why this inhibitor does not enhance the antiproliferative effect of radiation in NF9006 cells. Interestingly, similar results were observed in MDA-MB-231 cells. In this latter cell line – expressing normal level of ErbB1/2-receptors – PKI166 did not improve the antiproliferative effect of IR. This might be due to the weak dependence on the ErbB-receptor signaling of this cell population. However, PKI166-pretreatment of both ErbB1-overexpressing cell lines A431 and MDA-MB-468 clearly enhanced the antiproliferative and cytotoxic effects of radiation in these cell populations (see appendix Fig. A-B).

Together with the data obtained at the Akt-phosphorylation level, these results suggest that inhibiting the PI3K/Akt-survival pathway with PKI166 in ErbB2-overexpressing cells
sensitizes these cells to radiation-induced apoptosis. However, apoptosis-induction is probably not the principal process leading to the reduced metabolic activity observed in the proliferation assay in these cells, since combined treatment of PKI166 (0.5 μM) and IR (5 Gy) did not further decrease the antiproliferative effect mediated by the drug alone.

3.2 Ionizing radiation induces the PI3K/Akt survival pathway in tumor and endothelial cells

Ionizing radiation induced activation of receptor tyrosine kinases (RTK) and their related downstream signaling entities is a highly investigated area to understand the cellular stress response to IR (129, 206). In the following, mechanistic studies were performed to evaluate the influence of radiation on the PI3K/Akt-survival pathway in tumor and endothelial cells.

3.2.1 Ionizing radiation activates Akt in tumor cells

The PI3K/Akt pathway is activated by different growth-promoting signals and is also under influence of EGF. EGF-binding to the ErbB1-receptor is leading to activation of the ErbB1-receptor tyrosine kinase thereby activating the PI3K/Akt-signaling transduction pathway. The ErbB1-overexpressing A431 cell line was used as a tumor model system to determine PI3K/Akt pathway-activation in tumor cells upon radiation-exposure. Cells were seeded and cultured in the corresponding media containing 10% FCS. The next day the cells were serum starved for 24 hours by replacing the culture-medium by medium containing 0.5% FCS. Under serum starvation the endogenous Akt-phosphorylation level is kept at the minimum due to absence of an activating stimulus. The Akt phosphorylation status in A431 cells was investigated after irradiation with a dose of 5 Gy at different time intervals using the Ser-473 site-specific antiphospho-Akt antibody (Fig. 3-14).

**Figure 3-14** Ionizing radiation induces Akt-phosphorylation in A431 cells. Cells were stimulated with 5 Gy irradiation for different times (0-120 min) or EGF (50 ng/ml, 30 min). The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.
A clear increase in the phosphorylation level was observed at 10, 20 and 30 min after irradiation. At 60 and 120 min the signal intensity was drastically reduced but still above the basal level distinguished in untreated extracts. As positive control sample treatment with EGF (50 ng/ml) for 30 min strongly induced Akt-phosphorylation. This confirms that the PI3K/Akt survival pathway is activated downstream of the ErbB1 receptor upon EGF-ligand stimulation.

Thus, these results indicate that the PI3K/Akt-survival pathway is upregulated after radiation-exposure in A431 carcinoma cells, probably as a stress response. These results complete recent data demonstrating IR-induced activation of PI3K in this same cell population and IR-induced Akt-phosphorylation in MDA-MB-231 carcinoma cells (131).

3.2.2 Ionizing radiation activates Akt in human endothelial cells

Given the importance of the tumor vascular compartment in tumor growth control, the activation-status of the PI3K/Akt pathway upon IR-exposure was investigated in endothelial cells. Human umbilical vascular endothelial cells (HUVEC) were selected as an endothelial cell model system. To determine PI3K/Akt pathway-activation in endothelial cells the Akt phosphorylation status was quantified in HUVE cells after treatment with low doses of IR using the Ser-473 site-specific antiphospho-Akt antibody. The cells were serum-starved for 12 h and the Akt phosphorylation status was then analyzed by western blotting of whole cellular lysates following irradiation with 2 Gy (Fig. 3-15).

A clear increase in the phosphorylation level was observed at 20, 30, 60 and 120 min after irradiation. This is in agreement with recent analysis reported during the course of our experiments (184). A dose-dependent effect on Akt phosphorylation was investigated after treatment with increasing doses of IR (0-10 Gy). Interestingly irradiation with doses as high as 10 Gy did not further increase the Ser-473-Akt phosphorylation level (Fig. 3-16).
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

Figure 3-16 Ionizing radiation induces Akt-phosphorylation in human endothelial cells (dose-titration). Following 12 h serum-deprivation, HUVE cells were stimulated for 30 min with increasing doses of radiation (0-10 Gy). The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.

In analogy to the radiation-mediated Akt-activation observed in tumor cells, these results indicate that treatment with ionizing radiation stimulates the PI3K/Akt survival pathway in endothelial cells as well. This suggests that radiation induces this survival pathway in cells of different origin.

3.2.3 Epithelial and vascular endothelial growth factor activate Akt with distinct time courses in human endothelial cells

The PI3K/Akt pathway is activated in proliferating cells via growth factor receptor stimulation, and interestingly IR-induced Akt phosphorylation has been demonstrated in epithelial tumor cells to be mediated through activation of ErbB-receptors (131). Thus, IR might also induce Akt phosphorylation in HUVE cells via upstream receptor tyrosine kinases. To investigate the mechanism of IR-dependent Akt activation in greater detail, we analyzed at first the effect of Akt phosphorylation in endothelial cells in response to the two intrinsic ligands of the ErbB- and VEGF-receptor systems. HUVE cells were serum deprived for 12 h before treatment with the two growth factors EGF (50 ng/ml) and VEGF (20 ng/ml) respectively, followed by analysis of the Ser-473-Akt phosphorylation status by western blotting of whole cellular protein extracts. Akt phosphorylation could be observed 5 and 10 min after EGF-stimulation (Fig. 3-17).
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

Figure 3-17 EGF induces Akt-phosphorylation in human endothelial cells. HUVE cells were stimulated with 50 ng/ml EGF for different times (0-360 min). The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.

However, growth factor-specific upregulation of the Akt phosphorylation status was only detected as early as 10 min following stimulation with VEGF and high phosphorylation levels remained visible for a prolonged time period at 20 and 30 min after treatment (Fig. 3-18).

Figure 3-18 VEGF induces Akt-phosphorylation in human endothelial cells. HUVE cells were stimulated with 20 ng/ml VEGF for different times (0-120 min). The phosphorylation level of Akt was determined in whole cell extracts by western blotting using anti-Akt phosphoSer-473 specific antibody. Samples were reprobed with an antibody against β-actin to ensure the loading of equal amounts of protein.

The delayed kinetics of Akt phosphorylation in response to VEGF mirrored Akt stimulation following irradiation. Further, these results show that the PI3K/Akt pathway can be activated via both growth factors (EGF or VEGF) in endothelial cells.
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

3.2.4 VEGF- and ErbB-receptor specific activation of Akt

Clinically relevant inhibitors of the VEGF-receptor system (the phtalazine derivative PTK787/ZK222584) and the ErbB1/2-receptors (the pyrrolopyrimidine derivative PKI166) were used to dissect whether activation of specific receptor tyrosine kinases is required for IR-induced Akt phosphorylation. Both the ErbB1/2-inhibitor PKI166 and the VEGFR-inhibitor PTK787 inhibit the kinase activity of their respective receptors in the submicromolar range with an overlapping inhibitory effect at higher doses levels (207) and Table 2 in the introduction part). To determine the required concentration for specific receptor inhibition, growth factor-dependent Akt activation was first tested in HUVE cells and the ErbB1-overexpressing human tumor cell line A431. Both cell lines were serum-starved for 12 h (HUVEC) or 24 h (A431) and stimulated with EGF (50 ng/ml) with or without pretreatment with PKI166 (500 nM) or PTK787 (100 nM) for 1 h. HUVE cells were treated with EGF for 5 min and A431 cells for 30 min. EGF-induced Akt phosphorylation was completely abrogated in A431 and HUVE cells, respectively, when cells were pretreated with PKI166 (Fig. 3-19).

On the other hand cellular pretreatment with PTK787 did not affect EGF-induced Akt phosphorylation in neither cell line (Fig. 3-20).
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

Further, Akt phosphorylation was also tested in HUVEC cells in response to VEGF. The cells were treated similarly as mentioned above, instead of growth factor stimulation which was performed with VEGF (20 ng/ml, 15 min). While the ErbB-inhibitor PKI166 did not downregulate VEGF-dependent Akt phosphorylation, the phosphorylation status was strongly decreased on pretreatment with the VEGF-receptor inhibitor PTK787 (Fig. 3-21). These results confirm that EGF- and VEGF-induced Akt activation is mediated through their corresponding receptor tyrosine kinases. They further demonstrate the growth factor receptor-specific inhibitory potential of the two receptor tyrosine kinase inhibitors and validate the use of both inhibitors to dissect the potential link between receptor tyrosine kinases and IR-induced PI3K/Akt stimulation in tumor and endothelial cells.

Figure 3-20 ErbB-receptor dependent activation of Akt is not inhibited by the VEGF-receptor inhibitor PTK787. Serum starved A431 (A) and HUVE cells (B) were preincubated for 1 h in the absence or presence of 100 nM PTK787 followed by stimulation with EGF (50 ng/ml) for 30 and 5 min respectively. The Akt-phosphorylation level was quantified as mentioned above. In all experiments samples were reanalyzed with an antibody against β-actin to ensure the loading of equal amounts of protein.
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

3.2.5 Receptor-dependent Akt stimulation by ionizing radiation in tumor and endothelial cells

Using these pharmacological inhibitors we tested receptor tyrosine kinase dependent Akt phosphorylation following irradiation in endothelial cells and in the tumor cell line A431. A431 cells were serum starved, pretreated with PKI166 (500 nM, 1 h) or PTK787 (100 nM, 1 h) and irradiated with 5 Gy of ionizing radiation for 30 min. The ErbB-receptor inhibitor completely abrogated IR-induced Akt phosphorylation while in opposite the VEGF-receptor inhibitor did not affect Akt phosphorylation in these cells (Fig. 3-22).
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

**Figure 3-22** ErbB-receptor-dependent Akt stimulation by ionizing radiation in tumor cells. A431 cells were serum-deprived for 24 h followed by preincubation in the absence or presence of 0.5 μM PKI166 (A) or 100 nM PTK787 (B) and irradiated with 5 Gy of IR. The phosphorylation level of Akt was determined in whole cell extracts using an anti-Akt phosphoSer-473 site specific antibody 30 min following irradiation. Samples were reanalyzed with an antibody against β-actin to ensure the loading of equal amounts of protein. All experiments were repeated at least twice.

**Figure 3-23** VEGF-receptor-dependent Akt stimulation by ionizing radiation in endothelial cells. HUVEC cells were serum-deprived for 12 h followed by preincubation in the absence or presence of 0.5 μM PKI166 (A) or 100 nM PTK787 (B) and irradiated with 5 Gy of IR for 30 min. The phosphorylation level of Akt was determined in whole cell extracts using an anti-Akt phosphoSer-473 site specific antibody. Samples were reanalyzed with an antibody against β-actin to ensure the loading of equal amounts of protein. All experiments were repeated at least twice.
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

Analogous experiments were performed in the endothelial cell line. Interestingly, PKI166 did not decrease the IR-induced Akt phosphorylation status in HUVE cells while pretreatment with PTK787 downregulated IR-dependent Akt phosphorylation in these cells (Fig. 3-23). These data strongly suggest a differential receptor tyrosine kinase dependent stimulation of the PI3K/Akt pathway by IR in tumor and endothelial cells respectively.

3.2.6 Activation of the VEGF- and ErbB-receptor by ionizing radiation

Similar to growth factor stimulation, irradiation results in defined phosphorylation of Tyr-residues in the cytoplasmic domain of RTKs which serve as docking sites for signaling entities of further downstream signal transduction pathways such as the PI3K/Akt-pathway. To demonstrate a direct activation of the specific receptor tyrosine kinases in response to irradiation, the activity status of the ErbB-receptor and the VEGF-receptor was analyzed in A431 cells and in HUVE cells, respectively.

First, the influence of ionizing radiation on ErbB-receptor activation was evaluated in the A431 carcinoma cell line. Cells were serum starved during 24 hours and then irradiated with a radiation dose of 5 Gy for different time intervals (0-20 min). Detection of ErbB-receptor phosphorylation was performed with a Tyr-1086 site-specific antiphospho-ErbB1 antibody in whole cellular extracts. An increase of the phosphorylation level was already observed at 1 and 2 min following irradiation (Fig. 3-24).

![Figure 3-24 Ionizing radiation activates the ErbB1-receptor in tumor cells. Following 24 h serum-deprivation A431 were irradiated with 5 Gy and analyzed at the indicated time points (0-20 min). The phosphorylation level of ErbB1-receptors was determined in whole cell extracts using an anti-ErbB1-receptor phosphoTyr-1086 site specific antibody. Samples were reanalyzed with an antibody against ErbB1-receptor to ensure the loading of equal amounts of protein.](image)

A minimal shift of a slower migrating band could be observed (columns 2 and 3), representing most probably a hyperphosphorylated form of the receptor. The radiation-induced ErbB-receptor phosphorylation identified at a single discrete tyrosine residue in this experiment confirms and completes previous results obtained in the same cell line by Schmidt-Ullrich and colleagues (129).
To further determine activation of VEGF-receptors in response to irradiation, the receptor phosphorylation status was analyzed in endothelial cells. HUVE cells were serum-starved for 12 h and stimulated with a 2 Gy radiation dose with or without pretreatment with PTK787 (100 nM) for 1 h. VEGF-receptor phosphorylation was detected with a Tyr-951 site specific antiphospho-VEGFR-2 antibody in whole cellular extracts. Interestingly an increase of the phosphorylation level was observed at 5 min following radiation exposure (Fig. 3-25).

![Figure 3-25](image)

**Figure 3-25** Ionizing radiation activates VEGF-receptor 2 in endothelial cells. Following 12 h serum-deprivation HUVE cells were preincubated in absence or presence of PTK787 (100 nM, 1 h) and irradiated with 2 Gy for 5 min. The phosphorylation level of VEGF-receptors was determined in whole cell extracts using an anti-VEGFR-2 phosphoTyr-951 site specific antibody. Samples were reanalyzed using anti-VEGFR-2 or anti-β-actin antibodies to ensure the loading of equal amounts of protein.

Treatment with PTK787 completely abrogated radiation-induced VEGF-receptor phosphorylation in these cells. The slightly shifted and slower migrating band observed upon irradiation alone in comparison to control or combined treated samples supports a hyperphosphorylated and activated status of the receptor after stimulation with radiation. To confirm these data immunohistochemical analysis of endothelial cells were performed by confocal laser-scanning microscopy. HUVE cells were serum-starved for 3 hours and irradiated for 5 min with 2 Gy in the presence or absence of PTK787 (500 nM, 1 h preincubation). Immunofluorescent labeling was performed with the Tyr-996 site specific antiphospho-VEGFR-2 polyclonal antibody. In parallel the nuclei were stained with DAPI to facilitate cellular localization. In comparison to control cells, irradiated cells displayed a markedly enhanced, almost continuous rim staining at the site of the plasma cell membrane. On the other hand pretreatment of cells with PTK787 prior to irradiation resulted in a less pronounced rim staining with only a few dotted structures, similar to the staining of control cells (Fig. 3-26).
3. RESULTS – Ionizing Radiation Induces the PI3K/Akt Survival Pathway in Tumor and Endothelial Cells

Figure 3-26 Ionizing radiation activates the VEGF-receptor in human endothelial cells. HUVE cells were serum-deprived for 3 h in the absence or presence of 500 nM PTK787 and then irradiated with 2 Gy. The cells were fixed 5 min after treatment and immunofluorescence labeling was performed with the rabbit polyclonal anti-VEGFR-2-phosphoTyr-996 site specific antibody. Nuclei were stained with DAPI. The cells were subsequently examined by confocal laser-scanning microscopy. The experiments were repeated at least three times and representative images are displayed.

Together with the data showing RTK inhibitor-dependent control of IR-induced Akt phosphorylation, these results demonstrate specific VEGF-receptor-mediated activation of the PI3K/Akt pathway in endothelial cells and ErbB-receptor-dependent activation of this survival pathway in the A431 tumor cells.
4 DISCUSSION

4.1 The PTEN/PI3K/Akt survival pathway as target of PKC412

Introduction of constitutively active genes into cells represents a powerful tool to evaluate the importance of a specific protein or pathway for a given biological endpoint. In this study a retroviral system was adapted as strategy for the gene transfer. With this method we demonstrated that the antiproliferative and cytotoxic effects of the N-benzoylated staurosporine derivative and protein kinase C inhibitor PKC412 are mediated, at least in part, via downregulation of the PI3K/Akt survival pathway. Oncogene-transformed mouse fibroblasts were successfully infected with a control virus carrying an empty vector or with a virus expressing a dominant-active form of Akt. The antiproliferative and cytotoxic effects of PKC412 in cells transformed with the control vector was rescued in these cells when expressing constitutive active forms of Akt. The antiproliferative effect of PKC412 in these cells might results from its cell killing capacity essentially mediated via apoptosis induction (52). Furthermore, our results also indicates that PKC412 inhibits the PI3K/Akt pathway upstream of Akt, eventhough the exact mechanism is still unclear.

Several recent studies have pointed out the important role of the tumor suppressor PTEN as negative regulator of the PI3K/Akt signaling cascade principally by reversing the enzymatic reaction catalyzed by PI3K (97-100). Experiments in two isogenic cell lines differing only in their PTEN-status (PTEN+/+ vs PTEN/- MEFs) suggest the presence of PTEN as being a critical component for the PKC412-mediated inhibition of the PI3K/Akt pathway. PKC412 clearly decreased Akt-phosphorylation in the PTEN positive cell population but failed at dephosphorylating Akt in the PTEN negative counterpart. Furthermore, staurosporine showed an identical PTEN-dependent regulation of Akt-phosphorylation than its derived compound PKC412. However, LY294002, the specific PI3K-inhibitor, completely blocked Akt-phosphorylation in both cell lines independently of the PTEN-background, most probably due to direct inhibition of 3'-phosphoinositide generation. Results on cell killing efficacy revealed that PKC412 and staurosporine depend on an intact PTEN for their cytotoxic effect. The PTEN-dependence of PKC412 is weaker in proliferative assays, nevertheless its antiproliferative effect is slightly more pronounced in PTEN positive cells than in the PTEN negative ones. LY294002, however, displayed an identical antiproliferative effect in both cell populations. This alleviated PTEN-dependence of PKC412 on the proliferative level may result from the different molecular entities targeted by this “not so specific” protein kinase C inhibitor, thereby influencing PTEN-independent signaling pathways linked to cell proliferation. These results indicate that PKC412 and staurosporine do not downregulate the Akt-phosphorylation level at the Ser-473 residue by the same mechanism than LY294002 and that they do not directly inhibit PI3K at least at the concentrations used for these studies.

Only limited studies investigated the effect of STP or PKC412 on the PI3K/Akt-pathway and mostly elevated concentrations of STP were applied (27, 29, 59, 208, 209). PKB/Akt is structurally related to protein kinase A and C. However, based on the IC50-concentrations required for STP and PKC412 to inhibit purified Akt-kinase in vitro (IC50>10 µM) it is unlikely that Akt is directly inhibited by PKC412 in vivo as reported previously (210). A direct inhibition of the upstream phosphoinositide-dependent kinase-1 (PDK-1) by staurosporine has recently been demonstrated in vitro and in vivo but interestingly inhibition of PDK-1 only affected Thr-308-Akt-phosphorylation but not Ser-473 and further indicate that Ser-473 is phosphorylated by a so far not-identified kinase (208). PKC412 also directly inhibits PDK-1 although only at high concentrations (IC50=1.72 µM) (208). Different PKC-isoforms interact in vitro with the PI3K/PDK1/2/Akt-lipid-mediated multienzyme-complex.
4 Discussion – The PTEN/PI3K/Akt Survival Pathway as Target of PKC412

(58) and might affect the phosphorylation status and activity of Akt. Interestingly, the specific PKCζ isoform can also directly be co-immunoprecipitated with Akt from cell extracts though this isoform acts as a negative regulator of Akt (94). PKCζ was also proposed to function as an adaptor, associating with a staurosporine-insensitive PDK2 enzyme that catalyzes the phosphorylation of Ser-473 of Akt (211). Furthermore, the affinity of PKC412 towards PKCζ is in the micromolar range (IC50=10 μM) and this low affinity further supports that this isoform is not the responsible target for the observed effect of PKC412. In a recent study it has been shown that PTEN modulates phosphorylation of Akt on Ser-473 but not on Thr-308 (212). Hence one might suspect PKC412 to be involved in the regulation of the interaction between different molecular entities such as PI3K, PKCζ, PTEN, PDK2 and Akt eventually leading to Akt Ser-473 dephosphorylation. Thus, protein kinase C and its inhibitors modulate in a so far unknown way this multi-enzyme complex and we cannot rule out that the relevant target of PKC412 is not a protein kinase C-isoform. This will require a detailed analysis to investigate which factors influence the response to this class of PKC-inhibitors and which protein is the ultimate target of PKC412.

Our results demonstrate that PKC412 and staurosporine downregulate the PI3K/Akt-survival pathway only in presence of intact PTEN and this downregulation contributes to the PTEN-dependent cytotoxic effect of both inhibitors. Since induction of apoptosis is one possible mechanism responsible for staurosporine and PKC412 mediated cytotoxicity (51, 52), one might postulate that this process is PTEN-dependent as well. This would be in agreement with recent findings demonstrating that exogenous PTEN augments staurosporine induced apoptosis in PTEN-deficient cells by downregulating the PI3K/Akt-pathway (213). Lower concentrations of staurosporine are required to achieve a similar cytotoxic effect than with its derivative PKC412 (factor of about 100 times). The multiple additional targets of staurosporine however renders it to toxic for use in the clinic. The narrow therapeutic window achieved with staurosporine might be resulting from a not so selective discrimination of this drug between healthy and malignant cells. PKC412 is less effective but also less toxic than staurosporine and leads consequently to milder side effects in patients. It is therefore tested for its anticancer activity in vitro and in vivo and is currently under clinical investigations. It will be important to investigate its activity profile on the molecular level in healthy and malignant cells to understand its enhanced effect in the malignant environment. In addition to Akt-inhibition, other growth promoting anti-apoptotic pathways signaling mechanism might be affected by PKC412 to cooperate to its full effect. Identification of these additional signaling pathways will increase our view of the apoptotic network and its crosstalks with the PI3K/Akt-pathway.

One recently identified new target of PKC412 is the Flt3-receptor. Flt3 is mutated and constitutively active in approximately 30% of all patients with AML (acute myelogenous leukemia). Mutations in Flt3 have not been seen in nonhematopoietic cancers, perhaps because expression of Flt3 is limited to only few tissues outside of the blood and immune system. PKC412 was shown to be selectively toxic to leukemic cells expressing mutant Flt3 and therefore inhibition of Flt3 represents a promising approach to the treatment of AML. This example illustrate that tyrosine kinase inhibitors such as the protein kinase C inhibitor PKC412 might target other molecular entities than the ones initially anticipated and this might influence their clinical application.

Only recently constitutive activation of Akt was demonstrated to promote cellular survival and resistance to combined treatment modalities in non-small cell lung cancer cells (88). Constitutive and inducible Akt activity promotes resistance to chemotherapy, trastuzumab (ErbB2-antibody), or tamoxifen in breast cancer cells (214). Likewise, during the last few years several studies revealed the importance of PTEN in modulating response to chemo- and
radiotherapy. Loss of PTEN correlates with therapy resistance and restoration of PTEN leads to treatment (re-)sensitization in cells of different tumor origin (95, 102-104, 215-220). Thus, the PI3K/Akt survival pathway and the status of the different components within are critical factors in the response of malignant cells to combined therapies and particularly radiotherapy.

We previously reported that the clinically relevant PKC-inhibitor PKC412 and its parent compound staurosporine sensitize tumor cells to irradiation in vitro and in vivo and demonstrated that the combined treatment of IR with PKC412 results in the activation of the apoptotic machinery or cell cycle arrest depending on the cellular genetic background (see introduction Fig. 1-3 and (49, 51)). We further demonstrated that the radiosensitizing effect of PKC412 is also mediated at least in part via the PI3K/Akt-pathway suggesting that treatment of cells with PKC412 decreases an Akt-regulated stress-response threshold (52). Here, we confirm that PKC412 definitively downregulates this pathway and identify a component (PTEN) upstream of Akt as critical mediator of this effect.

Major pathways that determine the resistance of tumor cells to ionizing radiation and are thus important for radiosensitization are poorly defined. Our studies with the PKC-inhibitor PKC412 show that the PI3K/Akt-pathway is important for radiosensitization. Mechanistic analysis on PKI166 from this work and from previous experiments indicate a strong correlation between inhibition of the PI3K/Akt-pathway by this ErbB1/2-inhibitor and its radiosensitizing potential. They also indicate that the genetic background of the targeted cell population is a critical factor for the functioning capacity of a drug (i.e. the presence of PTEN for PKC412). Further, experiments with the specific PI3K-inhibitor LY294002 performed in our lab show that inhibition of the PI3K/Akt-pathway by the specific PI3K-inhibitor LY294002 also sensitizes tumor cells (EIA/ras MEFs) to ionizing radiation in vitro (221). This is a further indication that this pathway is important for radiosensitization.

In the meantime other groups published similar results verifying these observations (89, 125, 202, 222). McKenna and colleagues recently demonstrated the radiosensitization potential of LY294002 in vivo as well with nude mice bearing tumor cell xenografts (with activating mutations in the Ras oncprotein) (90). However, this was a kind of “proof of principle” that inhibition of the Ras to PI3K/Akt pathway could be clinically useful, since concerns exist about the side effects and toxicity of this drug in humans. The group of Brognard tested LY294002 in multiple NSCLC (non small cell lung cancer) cell lines (88). They demonstrated that especially cell lines with a high level of phosphorylated Akt were sensitized to ionizing radiation and chemotherapy by LY294002. In these cell lines apoptosis was markedly increased and clonogenic survival was inhibited after combined treatment. LY294002 radiosensitized also cells bearing mutant ras oncopgenes, but the survival of cells with wild-type ras was not affected (89). More important, this study also shows that additional well known pathways investigated were less crucial for radioresistance and radiosensitization. The Raf-MEK-MAPK-pathway-inhibitor PK98059, the Ras-MEKkinase-p38-pathway-inhibitor SB203580 and the p70S6K-inhibitor rapamycin failed to sensitize bladder carcinoma cells (with mutant ras oncogene) to ionizing radiation. Hence, inhibition of the mammalian target of rapamycin (mTOR) a downstream target of Akt with rapamycin was shown to sensitize U87 xenografts to fractionated radiation therapy (223). And interestingly, the radiosensitizing effect of rapamycin in this glioblastoma multiforme derived cell line could be observed in vivo, but not in vitro. This might be due to the strong antiangiogenic effect mediated by this drug (224). These observations demonstrate a very special and important role of the PI3K/Akt-pathway in the field of radiation treatment.

Clinical studies go even further and claim that the PI3K/Akt-pathway-status might become a prognostic factor for the response to radiation therapy. Only recently it could be shown that
the phosphorylation status of Akt, evaluated by immunohistochemical staining of tumor cells from head-and-neck-cancer patients, is related to treatment outcome. A lower level of phospho-Akt correlated with a better local tumor control. In the same study in vitro experiments with a radioresistant squamous head-and-neck cancer cell line (SQ-20B) revealed once more that inhibition of elements in the PI3K/Akt-pathway (ras, EGFR or PI3K) lead to radiosensitization (125).

4.2 ErbB-Receptors and Radiosensitization

Experiments performed with the dual ErbB1/B2-receptor inhibitor PKI166 have demonstrated that this tyrosine kinase inhibitor sensitizes ErbB2-overexpressing cells to radiation-induced apoptosis in mouse mammary carcinoma NF9006 cells. PKI166 in combination with irradiation had a synergistic (or at least additive) effect on the activation of the apoptotic machinery as measured in a caspase-3 activity assay. However, cotreatment with PKI166 did not result in any clear enhancement of the radiation-induced antiproliferative effect in these cells. This might be explained by a small percentage of cells undergoing apoptosis and that the principal mechanism leading to inhibition of proliferation in this cell population upon drug and radiation exposure might be mediated via cell cycle arrest and that this effect is not cumulative (IR + PKI166). This further suggests that the antiproliferative effect of PKI166 in these cells is mediated in part via downregulation of the PI3K/Akt-pathway but also via other (most probable MAPK-related cascade) signaling cascades downstream of the ErbB-receptors. However, the relative contribution of the PI3K/Akt-pathway for the PKI166-mediated antiproliferative effect was determined as part of our experiments. Importantly, PKI166 does neither enhance the antiproliferative effect of radiation in NF9006 cells nor in MDA-MB-231 cells. This latter cell line is probably not highly dependent on the ErbB-signaling, since it does express normal level of ErbB-receptors. In contrary, the ErbB1/PI3K/Akt-pathway is dominant in the two ErbB1-overexpressing cell lines A431 and MDA-MB-468, therefore inhibiting this pathway with PKI166 might lead to the enhancement of the IR-mediated antiproliferative effect and cytotoxicity observed in these cells. NF9006 cells overexpress ErbB2-receptors, we therefore expected a similar phenotypical pattern by this dual ErbB1/2-receptor inhibitor combined with IR in this cell population than in the ErbB1-overexpressing cell lines. However, unknown genotypical differences between these cell populations might be responsible for these phenotypical discrepancies.

It is noteworthy that lag of a radiosensitizing effect by PKI166 in vitro does not imply that this kinase inhibitor has no radiosensitizing potential in vivo. Several recent studies identified the tumor microenvironment – and particularly tumor associated endothelial cells – as an important target of PKI166 (144, 225).

ErbB-receptor dysregulation has been implicated in therapeutic resistance to diverse agents across many cancer types. Direct associations have been reported between ErbB-receptor overexpression and radioresistance. Numerous studies show that ErbB blockade in combination with ionizing radiation leads to enhanced cytotoxicity in multiple tumor cell types (head and neck, non small cell lung carcinoma, ovarian, colon, breast). However, it is still not well understood how inhibition of ErbB signaling sensitizes for IR on the cellular and tumor level.

Several mechanisms might contribute to ErbB-inhibition related radiosensitization. First, ErbB-receptor overactivation occurs frequently in many cancer cells and this often correlates with survival and growth advantages. Second, IR induces the production of growth promoting factors such as EGF and TGFα, leading to an enhanced autocrine stimulation of ErbB-receptors (130, 226, 227). Thus, ErbB-inhibitors might suppress this survival and growth
Discussion — ErbB-Receptors and Radiosensitization

Dysregulation due to receptor-overactivity or upregulated ligand production thereby potentiating the cytotoxic effect of irradiation. Furthermore, importantly, radiation itself was shown to directly stimulate ErbB-receptors leading to the phosphorylation and activation of the tyrosine kinase domain (129, 228). The exact mechanism inducing the kinase phosphorylation is still the focus of intense research in this field. This IR-induced receptor-mediated signaling might be part of a self-defense mechanism of the irradiated cell and results paradoxically in cell-growth-stimulation. Results published during the course of our experiments as well as data from our own experiments (see below) indicate that this growth-stimulating signal is mediated via the PI3K/Akt-pathway downstream of ErbB-receptors (131). Thus, blockade of this radiation-induced process by using ErbB-receptors inhibitors represents one supplementary rationale to hinder the resulting pro-survival signal and thereby cooperate with IR to commit its anti-proliferative and cytotoxic effects. Schmidt-Ullrich and colleagues suggested that ErbB-inhibitors might directly act in an antiproliferative way thereby controlling accelerated tumor repopulation during repetitive irradiation and decrease the capacity for DNA-damage repair in excessively proliferating cells. However, these inhibitors also radiosensitize tumor cells presumably through downregulation of overactivated survival cascades. Since the PI3K/Akt-survival pathway is also activated by ligand-mediated ErbB-receptor stimulation or in ErbB-receptor-overexpressing cells, radiation resistance and sensitization might be conferred via this pathway (125, 163, 229, 230).

We demonstrate by comparing transformed MEFs with or without constitutive active Akt that the observed PKC412-mediated antiproliferative and cytotoxic effects could be explained in part by the downregulation of the PI3K/Akt-pathway. Activation of Akt was detected in serum deprived, but EGF-stimulated, PTEN positive and negative MEFs suggesting a link in these cell lines between ErbB-receptors, upstream, and Akt, downstream, of PI3K/PTEN. PKC412 inhibited EGF-mediated phosphorylation of Akt in the PTEN positive cell population confirming the ErbB/PI3K/Akt signaling cascade as target for inhibition by this PKC-inhibitor. Furthermore, treatment of NF9006 cells with increasing doses of PKC412 or PKI166 indicates the antiproliferative potential of both inhibitors in cells characterized by a high endogenous ErbB-receptor signaling. Thus, in analogy to PKC412, the antiproliferative effect mediated by the ErbB1/2-inhibitor might result from the downregulation of the PI3K/Akt-pathway. Since this ErbB2-overexpressing cell population revealed a strong Akt-activity level, an intact signaling from the receptor via the downstream PI3K/Akt cascade was suspected in this cell line. As expected, PKI166 blocked the signaling from the receptor and inhibited the phosphorylation of Akt in these cells. Downregulation of Akt-phosphorylation could be already observed in a concentration range with a minimal effect on the proliferative level (50 - 250 nM). However, the strict requirement of this pathway for PKI166-mediated growth arrest is not proved by these experiments and it is not clear yet which other pathways are required. But keeping in mind the results achieved with the PKC-inhibitor showing that the PI3K/Akt-pathway is important for radiosensitization it would be of no surprise if PKI166 also sensitizes for IR by the inhibition of an upstream target of this cascade. Together with previous data showing downregulation of Akt-phosphorylation by PKI166 in the ErbB1-overexpressing A431 cell line (163), we demonstrate here that this dual ErbB1/2 inhibitor is also effective in ErbB2-overexpressing cells and leads to the downregulation of the PI3K/Akt survival pathway in this cell line as well. However, although inhibition of this pathway favors radiosensitization of A431 cells in vitro, no radiosensitization could be observed in the ErbB2-overexpressing cell line tested in this study. Nevertheless, recent data suggest the PI3K/Akt-pathway to be the major pathway for Trastuzumab (an antibody against ErbB2) mediated radiosensitization of breast cancer cells in vitro (201). Consequently, the ErbB/PI3K/Akt signaling cascade represents a promising target for inhibition with a possible
implication in the radiosensitization process. Thus, the identification of a specific signal transduction pathway that plays an important role in radiosensitization is very important and would therefore reveal more targets that could be addressed with new molecular defined compounds. This might support a combined treatment modality with IR (Fig. 4-1).

A link was recently demonstrated between the PTEN-status in tumor cells and ErbB-receptor inhibition efficacy. In ErbB-receptor overexpressing MDA-MB-468 tumor cells loss of PTEN was shown to counteract the antitumor action of ErbB-receptor inhibitors (ZD1839, C225) (231). The authors demonstrated that re-expression of PTEN in these PTEN-null cells restores the receptor dependence of PI3K- and Akt-activation. Additionally, resistance to ZD1839 (Gefitinib) in PTEN-null ErbB-overexpressing tumor cells could be overcome by introducing wildtype PTEN (217). Both studies underline the critical role of the PTEN/PI3K/Akt pathway in the antitumor effects of ErbB-inhibitors and suggest that patients with ErbB-overexpressing tumors and concomitant amplification of the PI3K/Akt cascade might benefit from the combined blockade of the ErbB- and PI3K/Akt-signaling. This further illustrates the importance not only of the pharmacogenetic profiling of each particular compound but also to gain insight into the cellular genetic background of a tumor to optimize drug- or treatment-efficacy.

ErbB-receptors are overexpressed in many human solid carcinomas and this correlates with a
chboro- and radioresistant phenotype. Inhibitors of the ErbB-receptors are of highest clinical priority as novel molecular drugs targeting cancer cells with overexpressed ErbB-receptors. In contrast to ErbB2-overexpression, which is generally due to gene amplification and predicts response to Trastuzumab therapy, the overexpression is only one of the several mechanisms by which ErbB-receptor deregulation occurs and does not always associate with activity, and data regarding the correlation between receptor overexpression and response to ErbB-receptor targeting agents are conflicting (232). Furthermore, ErbB-inhibition combined with IR, as already showed in numerous studies, leads to enhanced cytotoxicity in multiple tumor cell lines and improved tumor growth control in different xenograft models (138, 151, 152). However, the relative contribution of ErbB1 and ErbB2 as well as their respective activity-status for the sensitization to IR on the cellular and tumor level still remains unclear (233-235). As example, recent data obtained in several breast tumor cells indicate that ZD1839 (ErbB1-inhibitor) action requires an intact ErbB1 and is effective in cells expressing high levels of ErbB2, independent of ErbB1 expression (235). Thus, this lack of correlation between ErbB-overexpression or -activity and drug efficacy underline the importance for a better understanding of the ErbB-receptor biology as well as a more precise characterization of the mechanism(s) of action of these novel molecular compounds targeting this class of receptors.

ZD1839 is the ErbB-receptor kinase inhibitor most advanced in clinical trials and it was approved in may 2003 by the US Food and Drug Administration (FDA) for the treatment of patients with advanced NSCLC previously treated (unsuccessfully) with, or intolerant of, chemotherapy. It is also in advanced stage of development (phase II clinical trials) in metastatic breast cancer (MBC). However, the failure of any therapeutic benefit in adding ZD1839 to standard conventional polychemotherapy in advanced NSCLC was reported and serious adverse effects, mainly in studies conducted in Japan, are of concern. The somewhat disappointing initial efficacy results of ZD1839 in the treatment of patients with advanced breast cancer underscore the need for a better understanding of the ErbB-receptor biology. Additionally, there are several possible explanations for the failure to demonstrate a benefit. First, inadequate dosing or scheduling and therefore inadequate delivery of the drug to the tumor might result in suboptimum target modulation. Second, the study populations have usually advanced stage diseases (with malignant cells having accumulated diverse and heterogeneous mutations), which are fairly refractory to conventional treatments. Third, there might be an important dilution of the benefit in a small cohort of patients with tumors sensitive to ErbB-receptor inhibition by a larger cohort of patients with insensitive tumors. Therefore, patients might need to be selected for clinical trials based on the presence of markers thought to predict for sensitivity of the tumor to ErbB-inhibition. Additionally, further evaluation of ErbB-inhibiting drugs in different settings is needed, such as less heavily pre-treated patients and probably in combination with other drugs or therapies, like radiotherapy. Since large molecular heterogeneous patient populations will most likely not benefit from molecular targeted compounds as monotherapy, combining such novel pharmacologic substances with broad-range cytotoxic therapy, like radiotherapy, is most likely to be successful in clinical protocols.

The concern about ZD1839 does not have to hide the recent success of Glivec (the first tyrosine kinase inhibitor approved by the FDA) for the treatment of chronic myelogenous leukemia (CML) and Trastuzumab for the treatment of metastatic breast cancer (MBC). Glivec was discovered in an initial screening for protein kinase C inhibitors, however it showed specific inhibition of the Bcr-Abl oncoprotein, a unique feature of CML. Interestingly and in contrary to ZD1839, both Glivec and Trastuzumab were assessed in patients with cancers known to express the relevant target and in whom the target was likely to be contributing to the malignant phenotype. By contrast, ErbB-inhibitors are likely to have...
antitumor activity in only a fraction of patients with a particular tumor histology due to lack of target expression or activation, or lack of relevance of the ErbB-signaling pathway to tumor growth. These examples illustrate the potential and successes recently achieved with novel molecular targeted drugs in the treatment of cancer. This further indicates that efforts have to be made, not only in the identification of the mechanism of action and pharmacogenetic of a drug, but also in the characterization of the genetic profile of each patient to optimize therapies.

4.3 Differential Receptor Induction by Ionizing Radiation

During the course of radiotherapy ionizing radiation will not only affect tumor cells but also tumor-associated endothelial cells in the microenvironment. Understanding of the impact of radiation on both cell compartments and on the signaling cascades it influences is of central importance to improve radiotherapy.

The relevance of the Akt protein kinase for cellular survival has been investigated in different cell types and in response to many stress factors (53, 54, 236, 237). Radiation-induced Akt activation in endothelial cells has recently been demonstrated to contribute to an increase in radioresistance both in vitro and in vivo, however, the upstream molecular entities leading to an activated PI3K/Akt-pathway have not been addressed so far in endothelial cells (184). In this work, we have compared RTK-dependence of radiation-induced PI3K/Akt-activation in tumor cells with the activation pattern in endothelial cells using clinically relevant inhibitors of the ErbB1/2-class receptors and the VEGF-receptor. Both growth factor ligands EGF and VEGF stimulated Akt-phosphorylation through their corresponding growth factor receptors, which could be abrogated by the specific receptor tyrosine kinase inhibitors PKI166 and PTK787, respectively, indicating an intact growth factor-induced signaling network from the RTKs to Akt in endothelial cells. Interestingly, IR-dose and -time dependent activation of Akt in these cells closely followed the VEGF- but not the EGF-mediated activation-pattern of this survival pathway. Radiation-induced Akt-phosphorylation was strongly downregulated by the VEGFR-specific RTK-inhibitor in the endothelial cells while the ErbB-receptor-inhibitor did not decrease IR-induced Akt-stimulation in these cells. An opposite receptor-dependence for radiation-induced Akt-phosphorylation was observed in the tumor cell line A431. Specific VEGF-independent, but VEGF receptor-mediated Akt-activation was further corroborated through the direct activation of the PTK787-sensitive VEGFR-2 by IR (Fig. 4-2).

Both enhanced cell proliferation and DNA repair as well as reduced apoptosis are part of a cytoprotective response and are induced in response to growth factor ligand- and IR-activated RTKs. IR-induced RTK-activation has been intensively investigated for ErbB-receptor tyrosine kinases in tumor cells, however the exact mechanism for their activation is still unclear (205, 238). The generation of reactive oxygen and nitrogen species may shift the steady-state tyrosine phosphorylation status of RTKs to its phosphorylated active form due to the deactivation of critical cysteine residues in the catalytic center of corresponding protein phosphatases (239-244). On the other hand reactive oxygen species also affect lipid bilayer composition and rigidity which might ultimately lead to a proximity effect and transphosphorylation of plasma membrane integrated RTKs (245). Whether inhibition of PTEN protein phosphatase activity plays a role in IR-mediated RTK activation is still not known. This might be a further possibility, since oxidative stress – i.e. generation of reactive oxygen species – has been recently shown to tightly regulate this phosphatase (246). Thus, IR might modulate PTEN in a similar way as oxidative stress. Interestingly, while ligand-independent, IR-induced RTK-auto-/transphosphorylation is induced to a lower level in
comparison to ligand-mediated RTK-activation, downstream Akt-phosphorylation is induced to a similar extent by IR and the corresponding growth factor (130, 247, 248). We also observed a lower level of IR-induced VEGF-receptor activation in endothelial cells, but again the level of Akt phosphorylation was enhanced to a similar extent by IR and VEGF. Activation of multiple ErbB-receptor isoforms in parallel might explain that low level of RTK-phosphorylation on the individual isoform level is still sufficient to induce strong downstream signaling in a cumulative way or to overcome a signaling threshold. This might also apply to the low level of receptor phosphorylation of the different VEGFR-isoforms which could be determined by western blotting in response to IR. More important, IR-induced site-specific VEGFR-2 phosphorylation could be detected by confocal microscopy on the individual cell level and could be abrogated by pretreatment with the RTK-specific inhibitor PTK787.

![Scheme of the differential PI3K/Akt-pathway activation in tumor and endothelial cells by ionizing radiation](image)

**Figure 4-2** Scheme of the differential PI3K/Akt-pathway activation in tumor and endothelial cells by ionizing radiation. Ionizing radiation dependent PI3K/Akt-pathway activation is mediated by ErbB-receptors in tumor cells (A431) and VEGF-receptors in endothelial cells (HUVEC), subsequently promoting cell survival. Ligand-specific activation is represented by dotted arrows. The IR-specific activation is represented by solid arrows.

Ligand-dependent activation of RTKs are mainly determined by the affinity of the ligand to the corresponding receptor-isoform. In depth analysis by Schmidt-Ullrich and colleagues on the level of ErbB-receptors revealed that IR-induced RTK-activation does not discriminate between the different ErbB-receptor-isoforms and their different ligand-affinities. It is rather the expression level of the isoforms that predetermines IR-mediated RTK isoform-activation (130). These studies have mainly been performed in tumor cells in which ErbB-mediated
signaling dominate proliferation and survival processes not least because of a high expression level of these RTKs. We used primary, not established proliferating endothelial cells for these studies which most probably resemble in vivo endothelial cell structures in regard of RTK expression. Thus, the differential RTK-dependence of IR-induced Akt phosphorylation might be due to a shifted RTK-expression profile towards elevated VEGF-receptors in endothelial cells in comparison to ErbB-receptor overexpression in ErbB-receptor-dominated tumor cells or due to a change in the relative importance of a specific pathway. However, we cannot exclude a distinct activation of VEGF-receptors by IR which might be linked to inter- and intramolecular crosstalks between the different VEGF-receptor isoforms or the release of trapped signaling agonists such as VEGF or PI GF (249). A detailed quantification and analysis of PI3K-recruitment to the different VEGF-receptor isoforms will further clarify the mechanism of RTK-dependence for PI3K/Akt-pathway activation in response to irradiation.

As already mentioned above, ionizing radiation is a very powerful therapy against cancer because of its cytotoxicity mainly induced by DNA-damage. But IR also induces stress responses promoting cell survival and repopulation eventually leading to treatment resistance. Activation of the ErbB-receptors followed by downstream activation of the PI3K/Akt-pathway upon IR-exposure was proposed to be a central step favoring tumor cell survival and proliferation. Moreover, this mechanism was shown to stimulate ErbB-receptor mediated VEGF expression from tumor cells indirectly promoting an angiogenic response or at least an anti-apoptotic stimulus, which further contributes to enhanced radioresistance (138).

However, irradiation targets both tumor and endothelial cells and interestingly the radiosensitivity of the tumor microvasculature and microvascular damage strongly contributes to the tumor response to radiation (250). Thus, targeting an intrinsic treatment threshold in endothelial cells of the tumor vasculature sensitizes the tumor for ionizing radiation. Besides the cytotoxic effect of IR on the endothelial cell level, a direct cytoprotective stress response is induced by the activation of the PI3K/Akt-pathway further promoting endothelial cell survival. Consequently, PI3K-inhibitors such as LY294002 or wortmannin increase IR-induced cytotoxicity in endothelial cells and also enhance radiation-induced tumor growth delay in animal tumor models. Unfortunately, these inhibitors are overly toxic for clinical studies.

Other preclinical studies demonstrated that specific inhibitors of angiogenesis such as neutralizing anti-human VEGF$_{165}$-antibodies and anti-VEGFR-2 monoclonal antibodies enhance radiation-induced tumor growth control. Further, many studies are performed in vitro and in vivo using small molecular pharmacological inhibitors of the VEGFR-2 tyrosine kinase, such as PTK787, in combination with IR and demonstrate a strongly potentiated effect of this combined treatment modality compared to each treatment modality alone (132, 179, 185, 188). A combined treatment modality using anti-VEGF-antibodies in combination with IR does induce an enhanced antiangiogenic effect by decreasing the growth and survival promoting effects mediated by the growth factor ligand VEGF. On the other hand the combined use of a specific clinically relevant VEGF-receptor tyrosine kinase inhibitor will additionally abrogate direct IR-induced and receptor-mediated signaling in the endothelial tumor compartment further contributing to a cooperative anti-tumoral effect of IR (Fig. 4-3).

An ideal radiotherapy protocol would be radiation combined with both ErbB- and VEGF-receptor inhibitors, thus targeting the IR-induced receptor-mediated activation of PI3K/Akt-signaling in both the tumor and endothelial cell populations. A recently developed dual ErbB/VEGF-kinase receptor inhibitor is currently investigated in pre-clinical studies. Initial in vivo experiments in mice tumor xenografts (NF9006 cells) performed in our lab revealed a very rapid and complete tumor regression and a relatively long time-period to relapse. It will be interesting to compare these results with the data resulting from a similar experiment.
currently performed in our group with the VEGF-receptor inhibitor PTK787. Additionally, the use of an Akt-inhibitor might be very promising, since this might block the IR-induced signaling in both compartments as well, however in a receptor-independent way.

Recently Deguelin, a natural plant product, was shown to induce tumor cell specific apoptosis in transformed pre-malignant and malignant human bronchial epithelial cells (251, 252). Mechanistically Deguelin inhibits PI3K activity and reduces phospho-Akt levels, with minimal inhibitory effect on the MAPK-pathway. Interestingly, and not reported so far for ErbB-receptor inhibitors like Trastuzumab, C225 or ZD1839, Deguelin effectively inhibits Akt not only in malignant but also pre-malignant cells. Like Deguelin, other inhibitors of the PI3K/Akt-survival pathway should now also be tested for their activity in cancer model systems (88). Also the combination of the VEGF-receptor inhibitor PTK787 plus Deguelin (or an ErbB-inhibitor or a PKC-inhibitor) with ionizing radiation could be an attractive concept for a maximal local tumor control effect. Interestingly, in a recent report the combination of PTK787 and PKI166 sensitized for standard chemotherapeutic treatment (with gemcitabine) in a metastatic human pancreatic cancer model (253). With such novel “dual” radiosensitization one targets the Akt pathway simultaneously in tumor cells and in tumor associated endothelial cells.
4.4 General Discussion

During the course of radiotherapy, radiation will affect cells of different biological compartments including the tumor and the tumor associated endothelium. IR-induced tumor cellular responses have been the main focus of research in this field for decades. Only in the last few years the intercellular communication network between the tumor and its microenvironment (and particularly tumor associated endothelial cells) as well as the direct influence of IR on tumor associated endothelial cells have gained attention. A very large spectrum of responses are induced by radiation on the individual cellular level, the principal ones are detrimental to the cell (e.g. DNA-damage, programmed cell death) and are counterbalanced by more beneficial ones (e.g. DNA-repair, pro growth- and survival-signaling). Our knowledge still comprises a small part of this vast signaling system and only now we are beginning to understand how the different responses induced by IR interact on the molecular level. Likewise, only a few of the genetic factors important and characteristic for a radioresistant or radiosensitive cell phenotype are known. While classical radiobiology was focused primarily at increasing damages produced by IR at the DNA-site to enhance cell killing, nowadays interest is raising at modulating cellular stress responses independent of DNA-damage to enhance the effect mediated by IR-induced DNA-damage, thereby overcoming treatment resistance. Thus, the central part of this work consisted in understanding the role and the mechanism of regulation of a major signaling cascade (i.e. the PTEN/PI3K/Akt survival pathway) for radiosensitization. For this, we used protein-kinases inhibitors that are already well advanced in preclinical or clinical evaluation as monotherapy (i.e. PKC412, PKI166, PTK787) and that might potentially work as radiosensitizers. Such small molecular targeted compounds are important not just for the treatment of diseases, but also as reagents to understand more about the physiological role of different protein kinases and signaling cascades they are involved in.

The major critical pathways determining the resistance of tumor cells to ionizing radiation and thus important for radiosensitization are poorly defined. In this thesis, we investigated the importance of the PI3K/Akt-survival pathway for the radiosensitization potential of the PKC-inhibitor PKC412 with a genetic system and characterized the molecular mechanism by which PKC412 influences the PI3K/Akt cascade. We demonstrated the antiproliferative potential of the ErbB-receptor tyrosine kinase inhibitor PKI166 and its enhanced cytotoxicity in combination with IR in ErbB-overexpressing cells. The antiproliferative and cytotoxic effects induced by this ErbB-inhibitor correlated well with PKI166-dependent downregulation of the ErbB-mediated signaling to PI3K/Akt.

The genetic background of a cell is critical for its sensitivity or resistance to radiation. Tumor cells are usually bearing mutations rendering them particularly resistant to stress stimuli and favoring cell survival. Therefore, tumor cells might become especially dependent on a particular mutated pathway for their survival and proliferation. Mutations in elements at different levels are found in the ErbB/PI3K/Akt cascade (e.g. overexpression of ErbB, PI3K, Akt or activating Ras mutations or PTEN deficiency) leading to enhanced signaling and subsequently responsible for a higher intrinsic radioresistance. Blocking such important – and in tumor often overactivated – signaling pathways represent a promising strategy to sensitize cells to ionizing radiation.

Since upregulation of the PI3K/Akt-pathway can have different origins, different compounds are not expected to modulate it in a similar way. This is nicely illustrated by the finding that PKC412 and LY294002 (the PI3K-inhibitor) have a different mode of action on this pathway. PKC412 in contrary to LY294002 requires intact PTEN to inhibit the PI3K/Akt-pathway and exerts its antiproliferative and cytotoxic effects. Importantly, these results suggest that
PKC412 is not a direct inhibitor of an element in this signal transduction cascade but probably signals upstream of PTEN by a so far unresolved mechanism. Further, these findings might have important implications for clinical setting and advise against the use of PKC412 for treatment of PTEN-mutated tumors. (PTEN is mutated in as few as about 10% of leukemia patients with Flt3-mutations.) Given the encouraging preclinical data observed with this drug on tumor growth control in animals grafted with Flt3-mutated tumors, the first clinical results for the treatment of this category of leukemia are tinged with high expectations. Interestingly, PTEN-mutations are a rather late event in tumor progression. Thus, there might be increased chances of success for PKC412-based therapies, if cancer is diagnosed early. As previously described for Deguelin, PKC412 could also be exploited for its efficacy in pre-malignant tumor cells with wild type PTEN-status (pre-cancer in a high risk patient population) or in tumor associated normal cells (e.g. endothelial cells). Whether PI3K/Akt is selectively upregulated in both types of cells remains to be determined and also whether a therapeutic index (e.g. in situ cancer vs. normal tissue) is still seen with such a proposed treatment. However, downregulation of activated Akt does not automatically imply a decrease in tumor cell proliferation due to PI3K/Akt-survival pathway inhibition. Downstream mutations or activation of alternative survival pathways may occur. Though such survival salvage mechanisms are less likely to occur in non-transformed endothelial cells.

ErbB-receptor overactivity is frequently seen in tumor cells. This might result from overexpression of the receptor or activating mutations in its intracellular kinase domain. Small molecular tyrosine kinase inhibitors will preferentially inhibit cells expressing receptors with a constitutive kinase activity. Since such tumor cells probably even lack an intact ligand-binding domain, they might not respond to ligand binding inhibitors. Thus, inhibitors targeting the extracellular ligand binding domain (e.g. monoclonal antibodies) will principally affect receptor-overexpressing cells.

Altogether, these findings illustrate the importance to gain as much information as possible at the individual level about the cellular genetic background, intracellular signaling pathways and essential elements within to improve treatment efficacy.

Ionizing radiation also directly induces the PI3K/Akt-survival pathway, probably as a stress response. This phenomenon seems to be ubiquitous in cells, since it is observed in tumor as well as in endothelial cells. In tumor cells this signal is mediated via activation of receptors of the ErbB-family often already overexpressed in this category of cells. Interestingly, endothelial cells – which possess intact ErbB to PI3K/Akt signaling – respond to the IR-induced stress stimulus by VEGF-receptor mediated activation of the PI3K/Akt cascade. This cell-differential receptor-mediated stimulation of the PI3K/Akt signaling most probably reflects the distinct receptor expression profile in each particular cell population (i.e. high ErbB-receptor expression in tumor cells versus elevated VEGF-receptor expression in endothelial cells). The direct activation of the ErbB/PI3K/Akt pathway by radiation is a central step for tumor cell survival and proliferation favoring repopulation during the course of the (fractionated) radiotherapy protocol. Further, this mechanism induces the production of VEGF-ligands from tumor cells, thereby promoting endothelial cell survival and possibly angiogenesis and is interpreted as an additional tumor (indirect) protective response contributing to enhanced radioresistance. Blockade of these IR-induced protective responses represents an additional rationale to combine inhibitors of the ErbB/PI3K/Akt signaling with radiation, subsequently potentiating IR-cytotoxicity. Moreover, since radiation directly promotes endothelial cell survival by a similar but not identical mechanism as in tumor cells (i.e. activation of VEGFR/PI3K/Akt signaling), targeting this cell compartment is expected to further improve tumor radiosensitivity. Importantly, in combination with radiation the use of a VEGF-receptor inhibitor (i.e. PTK787) will be preferred to an anti-ligand antibody (i.e. anti-
VEGF), since such a receptor inhibitor will additionally abrogate the direct IR-induced and receptor-mediated signaling in endothelial cells.

The selection of a cocktail of single target molecular compounds for a specific patient is difficult and too expensive in clinical practice. Therefore, a compound with either a multilevel activity – to intercept potential alternative salvage survival pathways – or a key target activity within a tumor modulating a specific survival signaling pathway is of major interest. Attractive is certainly a multi-compartment treatment concept to target the same survival pathway simultaneously in tumor and (tumor associated) endothelial cells. Further, patient selection based on genetic and/or proteomic screenings needs to be generalized in order to individualize and improve molecular targeted chemo-radiotherapy.

This work focused to get a better comprehension of the mechanisms by which ionizing radiation and small clinically relevant molecular compounds alone or in combination modulate an important signaling cascade for radiosensitization: the PI3K/Akt-survival pathway.
5 OUTLOOK

In the future, it will be of central importance to determine the pharmacogenetic profile of novel molecular defined anticancer agents to optimize their efficacy. This is not only essential for their use as monotherapy, but probably eventhough more determinant for combined therapies, i.e. with ionizing radiation and thus for their radiosensitizing potential. Consequently, characterization of the pharmaco- as well as radiogenetic profiles of such compounds will undoubtedly lead to their fine-tuned and improved use.

In this regard, the results presented here raise a number of questions of interest. In this work we demonstrate a PTEN-dependent cell killing mediated by PKC412. Further, it has been shown that exogenous PTEN augments staurosporine induced apoptosis in PTEN-deficient cells by downregulating the PI3K/Akt-pathway (213). Thus, it would be interesting to investigate if apoptosis is a relevant process for the observed PKC412-mediated cell killing effect by comparing apoptosis induction following PKC412-treatment between PTEN+/+ and PTEN-/- MEFs.

Treatment of NF9006 cells with increasing doses of PKC412 or PKI166 indicates the antiproliferative potential of both inhibitors in cells characterized by a high endogenous ErbB-receptor signaling. The antiproliferative effect mediated by PKI166 correlates with the downregulation of the PI3K/Akt pathway. In analogy to PKI166, the elucidation of the influence of PKC412 on the PI3K/Akt-cascade in NF9006 cells would also be important. Additionally, the effect of both PKI166 and PKC412 on the MAPK-proliferative pathway in this cell population might contribute to a more detailed understanding of the antiproliferative mechanism mediated by both inhibitors. This might be achieved by expressing constitutive active members of this pathway (i.e. MEK, ERK1/2) in this cell population and comparing the antiproliferative capacity of PKI166 and PKC412 respectively in both normal and transformed cells. Analysis of PKI166 and PKC412 on cell cycle arrest in NF9006 cells might further help to characterize the mechanism of action of both compounds.

PKC412 was shown to be selectively toxic to leukemic cells expressing mutant Flt3 and therefore inhibition of Flt3 represents a promising approach to the treatment of AML (48). In this context, it would be important to test the relevance of the presence of an intact PTEN for the inhibitory effect of PKC412 in (leukemia) cells expressing Flt3. This should be performed at best by comparing two isogenic cell lines, the one expressing normal wildtype PTEN and the other one with defective PTEN.

Another important question concerns the PKI166-radiosensitization potential in ErbB2-overexpressing cells. Although cotreatment of NF9006 cells with PKI166 and ionizing radiation did not result in any enhancement of the radiation-induced antiproliferative effect in these cells, it might be worth to test PKI166 alone and in combination with IR in other ErbB2-overexpressing cell lines. In this way we should gain insight if this phenomenon is peculiar to NF9006 cells or if it applies more generally to ErbB2-overexpressing cells.

Regarding the effect of PKI166 and PTK787 in vivo, it would be important to compare their effect with the newly developed dual ErbB/VEGF-kinase receptor inhibitor AEE788. Furthermore, we might test these different inhibitors with fractionated irradiation against allograft and spontaneously growing murine mammary tumor models. This will not only allow a comparison on the efficacy between each compound, but also the impact and importance of the vascular system for the effect of each inhibitor will be better characterized - since allograft and spontaneously growing tumors diverge essentially in their vascular system. The elucidation of the exact mechanism leading to IR-mediated receptor-phosphorylation (ErbB-receptors, VEGF-receptors) is highly actuel and of central importance. To test if membrane-trapped growth factors released within minutes following radiation play a role in
this process, we might preincubate cells with antibodies targeting the appropriate growth factors — i.e. EGF and VEGF in tumor and endothelial cells respectively — and analyze the activity/phosphorylation of Akt as molecular readout. To test for the importance of the presence of the receptor extracellular domain, cell lines expressing receptors lacking their extracellular domain represent interesting systems for investigation.

Further, it would be also important to analyze the relevance of PTEN in the IR-mediated receptor phosphorylation process (i.e. first, quantification of receptor-phosphorylation after IR in presence and absence of PTEN; second, immunoprecipitation of receptors before and after IR and comparison of co-immunoprecipitated PTEN amounts).

Finally, a detailed quantification and analysis of PI3K-recruitment to the different VEGF-receptor isoforms will further clarify the mechanism of RTK-dependence for PI3K/Akt-pathway activation in response to irradiation. This might be performed for example by immunoprecipitating specific VEGFR-isoforms following irradiation and subsequent detection of co-immunoprecipitated PI3K.

In conclusion, multilevel PI3K/Akt pathway modulation and single key target modulation within the PI3K/Akt pathway represent one promising approach for current translational research in radiation oncology. This might be complemented with a “dual radiosensitization-approach” by targeting (common) pathways in different tumor compartments (e.g. tumor and endothelial cells). In addition, the pharmaco- and radiogenetic profiles of each compound should be carefully characterized and compared with the individual genetic tumor profile to optimize therapies. Finally, this might be complemented with radioproteomic profiling, which will further help to identify the posttranslational modifications critical in relation to ionizing radiation. These investigations can lead to the development of novel potential radiosensitizers/protectors.

In the future, efforts have to be intensified in the multidisciplinary field of radiation oncology to improve and strengthen interactions between medicine, physics and biology. Only with a harmonized multidisciplinary teamwork successes in chemo-radiotherapy will be achieved.
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APPENDIX

A)

Antiproliferative effect of PKI166 and IR alone and in combination against different human p53-mutated carcinoma cell lines.

Figure A Inhibition of the proliferative activity after PKI166 and IR-treatment in three different cell lines. MDA-MB-468, MDA-MB-231 and A431 cells were treated with 5 μM and 5 Gy or 10 Gy respectively. Proliferative activity was measured 24, 48, 72 and 96 h after treatment. (Absence of error bars is due to minimal standard deviations.)
B) Clonogenic survival assay with increasing concentrations of PKI166 and IR against the two p53-mutated tumor cell lines A431 and MDA-MB-468.

**Figure B** Clonogenic assay showing the radiosensitizing effect of PKI166 in two EGFR-overexpressing cell lines. MDA-MB-468 and A431 cells were treated with increasing concentrations of PKI166 (0-5 μM) and IR (0, 2, 5, 10 Gy) depending on the radiosensitivity of cell lines in the proliferation assay. Cells were allowed to grow for 6-9 days. Clonogenic survival fraction was calculated as described in (49). (Absence of error bars is due to minimal standard deviations.)
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