Pharmacologic Inhibitor of DNA-PK, M3814, Potentiates Radiotherapy and Regresses Human Tumors in Mouse Models

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ABSTRACT

Physical and chemical DNA-damaging agents are used widely in the treatment of cancer. Double-strand break (DSB) lesions in DNA are the most deleterious form of damage and, if left unrepaired, can effectively kill cancer cells. DNA-dependent protein kinase (DNA-PK) is a critical component of nonhomologous end joining (NHEJ), one of the two major pathways for DSB repair. Although DNA-PK has been considered an attractive target for cancer therapy, the development of pharmacologic DNA-PK inhibitors for clinical use has been lagging. Here, we report the discovery and characterization of a potent, selective, and orally bioavailable DNA-PK inhibitor, M3814 (pepsoertib), and provide in vivo proof of principle for DNA-PK inhibition as a novel approach to combination radiotherapy. M3814 potently inhibits DNA-PK catalytic activity and sensitizes multiple cancer cell lines to ionizing radiation (IR) and DSB-inducing agents. Inhibition of DNA-PK autophosphorylation in cancer cells or xenograft tumors led to an increased antitumor activity of IR and led to complete tumor regression at nontoxic doses. Our results strongly support DNA-PK inhibition as a novel approach for the combination radiotherapy of cancer. M3814 is currently under investigation in combination with radiotherapy in clinical trials.

Introduction

To ensure the accurate maintenance and transfer of genetic information to progeny, mammalian cells have evolved sophisticated mechanisms to sense DNA damage, coordinate its repair, and prevent potential tumorigenic effects; this is collectively known as the DNA damage response (DDR). Defects in the DDR contribute to genomic instability and represent one of the key hallmarks of cancer (1). DNA can be damaged by multiple endogenous and exogenous factors. Many established therapeutic modalities, such as radiotherapy and chemotherapy that attack cancer cell DNA are in clinical use but provide limited benefit to patients with cancer. This is due, at least in part, to the competence of tumor cells to deal with DNA damage (2).

Diverse types of lesions can be generated in DNA, ranging from base modifications to strand breaks, leading to large deletions or genomic rearrangements. Of those, double-strand breaks (DSBs) are considered the most harmful and can have lethal consequences for the cells and organism if left unrepaired (3). DSB repair is accomplished through two major pathways, homologous recombination-guided repair (HR) and nonhomologous end joining (NHEJ; refs. 3, 4). HR requires an intact DNA strand as a template for break repair and is restricted to the S and G2 phases of the cell cycle. Therefore, HR is considered less error prone than NHEJ. Conversely, NHEJ repairs DSBs in the absence of a template and leads to alterations in the repaired DNA. However, NHEJ is functional in all phases of the cell cycle and is believed to participate in the repair of over 80% of DSBs induced by ionizing radiation (IR) in cancer cells (5).

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase and a key driver of NHEJ repair, working in co-ordination with five additional factors, Ku70, Ku80, XRCC4, ligase IV, and Artemis (6). A heterodimer consisting of Ku70 and Ku80 binds specifically to DSBs, recruits and activates the catalytic subunit DNA-PKc, which in turn recruits the XRCC4/ligase IV heterodimer responsible for resealing the break. Trimming of the DSB ends may require Artemis and other DNA polymerases specialized in repair-mediated DNA polymerization. The activation of DNA-PK through autophosphorylation is essential for proper execution of the repair process (7, 8).

DNA-PK-knockout mice are viable, suggesting that pharmacologic inhibition will not affect essential functions in mammalian organisms and may be tolerated for the duration of standard cancer therapy regimens (9, 10). Several lines of experimental evidence suggest that inhibition of DNA-PK activity can effectively sensitize cancer cells to exogenous DSB DNA damage, such as IR and certain types of DSB-inducing chemotherapies (11–17). These conclusions are derived from experiments using molecular biology approaches (RNAi) to suppress DNA-PK expression or early chemical inhibitors as laboratory tools in cultured cancer cells. However, most of these tool compounds lacked the specificity and pharmacologic properties needed to establish a proof of principle for selective DNA-PK inhibition as a therapeutic approach in relevant vivo models.
Here, we describe M3814, a novel potent and selective pharmacologic DNA-PK inhibitor. We show that M3814 effectively suppresses DSB repair in cancer cells in a DNA-PK-dependent manner and strongly potentiates the antitumor effect of IR and DSB-inducing chemotherapy in vitro and in vivo. Clinically relevant 6-week studies using fractionated radiation and M3814 in two human xenograft models demonstrated complete and durable tumor regression, providing a strong rationale for clinical testing.

Materials and Methods

Cell lines and reagents

M3814 (MSC2490484A) was synthesized in the Department of Medicinal Chemistry at Merck KGaA, Darmstadt, Germany (18). Cell lines were obtained commercially (ATCC, ECACC, JCRB, RIKEN) and cultured in medium recommended or previously tested for these cell lines (A549, BxPC-3, FaDu, HT-29; Dulbecco’s Modified Eagle Medium (DMEM)/10% fetal calf serum (FCS)/10% CO2; Calu-6: DMEM/10% fetal bovine serum (FBS)/non-essential amino acids (NEAA)/10% CO2; Capan-1: DMEM/15% FBS/10% CO2; DU-145: MEM alpha/10% FBS/10% CO2, KB-4: DMEM/Hams F12 1/1/10% FCS/2 mM/mM/L glutamine/5% CO2; B-16F1, 11008). Immuno affinity chromatography.

PK assays

DNA-PK enzymatic assays were performed at concentrations of adenosine triphosphate (ATP) near the K_m (10 μM/L) or at 1 mM/L for time-resolved fluorescence energy transfer (TRET). DNA-PK purified from HeLa nuclear extracts was preincubated with M3814 at different concentrations (4.0E-15–3.0E-5 M) of vehicle for 15 minutes at 22°C. The reaction was started by addition of purified c-myc-tagged p53 (23-034; Eurofins) and ATP, and then incubated for approximately 30 minutes at 22°C. Reactions were subsequently stopped and antibodies were added (anti-phospho-p53(Ser15)-Eu (61P08KAY; Cisbio); anti-conv (61MYC Conc; Ciscbio)), concentrated for 2 hours, plates were analyzed in an EnVision (PerkinElmer) microplate reader (excitation at 540 nm, emission: 665 and 615 nm). Data were normalized to a dimethyl sulfate (DMSO) control, and the half maximal inhibitory concentration (IC50) values were determined by nonlinear regression analysis.

Protein and lipid kinase profiling was performed at Merck Millipore. Recombinantly produced protein and lipid kinases were used in enzyme activity assays. Protein kinase reactions were initiated with Mg-ATP and stopped after 40 minutes by addition of phosphoric acid. The transfer of radiolabeled γ-phosphate to peptide substrates was quantified by scintillation counting of peptide substrates immobilized on filter membranes. A nonradioactive assay [homogeneous time resolved fluorescence (HTRF)] was used for lipid kinases. The “percent of effect” activity was determined compared with vehicle-treated controls corrected for background activity. M3814 was tested at 1 μM/L (some cases 10 μM/L) or serially diluted for IC50 determination.

Western blot analysis

Exponentially growing HCT-116 and FaDu cancer cell lines were seeded in 12-well plates. The next day, the medium was removed, and cells were incubated with fresh medium containing a serial dilution of M3814 (concentration range: 1.5E-09–2.5E-05 M) and 10 μM/L bleomycin (for detection of phosphorylated DNA-PK) or 3 mM/L hydroxyurea (for detection of phosphorylated checkpoint kinase [CHK1]) for 6 hours. Cells were washed and lysed using HGN buffer. After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to nitrocellulose membranes and incubated with primary antibodies (1:2,000 anti-total DNA-PK, ab70250, Abcam; 1:1,000 anti-pSer2056 DNA-PK, ab18192, Abcam; 1:1,000 anti-pSer345 CHK1,2348, Cell Signaling Technology; 1:200 anti-total CHK1,sc8408, Santa Cruz Biotechnology) overnight at 4°C. Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using Lumi-Light PLUS (Roche). Luminescence was recorded using a VersaDoc luminescent imager (Bio-Rad). pDNA-PK and pCHK1 values were normalized to total protein, and the bleomycin/hydroxyurea and DMSO controls were set to 0 and 100, respectively. Concentration–response curves were fitted using a nonlinear regression method to determine IC50 values. The experiment was repeated at least twice.

Immunofluorescence studies

A375 cells were seeded on eight-well glass chamber slides precoated with fibronectin. After 48 hours, the medium was removed and fresh medium without serum was added. The next day, the medium was removed and fresh medium with and without 1 μM/L M3814 was added 30 minutes prior to IR (2.4 Gy); the cells were further incubated for 0.5, 2, 4, 6, 8, or 24 hours. Cells were fixed with methanol and stained with primary antibodies against phosphohistone H2AX (1:8,000 dilution, γH2AX Ser139; clone 20E3, Cell Signaling Technology, 9718) overnight at 4°C, and subsequently with Alexa Fluor-conjugated secondary antibodies (1:2,000 dilution, anti-rabbit IgG; Invitrogen A, 11008). Immunofluorescence was determined at 40× magnification, γH2AX foci were counted in 50 to 80 nuclei, and the numbers of cells with more than 10 γH2AX foci per nucleus were counted and expressed as a percentage of total nuclei.
Table 1. Cellular profiling of M3814 in cancer cell lines.

<table>
<thead>
<tr>
<th>Cancer cell line</th>
<th>Origin</th>
<th>Blomycin-induced pDNA-PK, IC50 (M)</th>
<th>Clonogenicity ± irradiation, IC50 (M)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0 Gy</td>
</tr>
<tr>
<td>M059J</td>
<td>Brain</td>
<td>n.d.</td>
<td>4 × 10⁻⁵</td>
</tr>
<tr>
<td>M059K</td>
<td>Brain</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>HCT-116</td>
<td>Colon</td>
<td>1 × 10⁻⁷</td>
<td>&gt;1 × 10⁻⁵</td>
</tr>
<tr>
<td>HT29</td>
<td>Colon</td>
<td>1 × 10⁻⁷</td>
<td>2 × 10⁻⁷</td>
</tr>
<tr>
<td>FaDu</td>
<td>Head and neck</td>
<td>6 × 10⁻⁸</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>A549</td>
<td>Lung</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>Calu-6</td>
<td>Lung</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>EBC-1</td>
<td>Lung</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>NCI-H460</td>
<td>Lung</td>
<td>1 × 10⁻⁶</td>
<td>3 × 10⁻⁵</td>
</tr>
<tr>
<td>BxPC-3</td>
<td>Pancreas</td>
<td>2 × 10⁻⁷</td>
<td>1 × 10⁻⁵</td>
</tr>
<tr>
<td>KP-4</td>
<td>Pancreas</td>
<td>4 × 10⁻⁷</td>
<td>3 × 10⁻⁴</td>
</tr>
<tr>
<td>MiaPaCa</td>
<td>Pancreas</td>
<td>5 × 10⁻⁷</td>
<td>&gt;5 × 10⁻⁵</td>
</tr>
<tr>
<td>DU145</td>
<td>Prostate</td>
<td>5 × 10⁻⁷</td>
<td>2 × 10⁻⁵</td>
</tr>
</tbody>
</table>

Abbreviation: DNA-PK, DNA-dependent protein kinase; Gy, Gray unit; IC50, half maximal inhibitory concentration.

IHC

FaDu xenograft tumors were fixed in 4% buffered formaldehyde and embedded in paraffin. Sections (3 μm) were deparaffinized and heated to 96 to 100°C for epitope retrieval. Sections were incubated with primary antibodies (10 μg/mL anti-murine CD31, clone SZ31, DIA-310, Dianova; 1:50 anti-human yH2AX, clone 20E3, 9718, NEB), and subsequently with Alexa Fluor-conjugated secondary antibodies (1:250 anti-rat IgG AF488; 1:250 anti-rabbit IgG AF594; A-11037, Invitrogen). Immunofluorescence was imaged at 40× magnification and the yH2AX area under nuclei (AF594 positive)/area nucleus (DAPI positive) was quantified. Data from vehicle- and drug-treated animals were compared by one-way analysis of variance (Kruskal–Wallis test) followed by Dunn multiple comparison test (two-sided, GraphPad Prism). Data are presented as the mean ± standard error of the mean (SEM).

Colony formation assay

Cell survival was tested in response to IR at doses of 2.4 and 4.8 Gy using a Faxitron RX-650 irradiation device (Tucson). Exponentially growing cancer cells (for cell lines, see Table 1; Fig. 1D) were seeded on six-well plates. Twenty-four hours later, cells were treated with a serial dilution of M3814 (typical concentration range: 2 × 10⁻⁵–5 × 10⁻⁷ M) for 1 hour prior to radiation. After an additional 24 hours in the presence of M3814, the medium was substituted with fresh culture medium without compound. Cells were incubated for several days to weeks until visible colonies could be detected. Colonies were stained with neutral red or crystal violet and quantified using a Gelcount Scanner (Oxford Optronix). Inhibition under each condition (single concentration and IR dose) was determined in singlicate in at least three independent experiments. Raw data were normalized by setting the cell number under DMSO treatment without IR to 100%. Concentration–response curves were fitted using a nonlinear regression method to determine IC50 values.

ELISA assays

Autophosphorylation of DNA-PK on serine 2056 was assessed by ELISA with a pDNA-PK antibody (0.125 μg/mL, EM9912), a DNA-PK antibody (0.5 μg/mL, WH0005591M2) for capture, and a biotinylated DNA-PK antibody (0.15 μg/mL, Abcam, ab79444) for detection. The PathScan Phospho-Chk2 (Thr68) Kit (Cell Signaling Technology) was used according to the manufacturer’s instructions to determine CHK2 phosphorylation on threonine 68. Exponentially growing cancer cells were treated with a serial dilution of M3814 (3E-10–3E-05 M) and 10 μmol/L bleomycin for 6 hours. Cell lysates prepared in HNGT buffer were incubated with a solid phase-bound capture antibody and further processed with a detection antibody and reagents. Phospho-DNA-PK and total DNA-PK chemiluminescence was quantified with a Mithras LB940 reader (Berthold). The absorbance signal for phospho-CHK2 was measured at 450 nm using a Sunrise reader (Tecan). Phospho-DNA-PK values were normalized to total DNA-PK values; the bleomycin and DMSO controls were set to 0 and 100%, respectively. For the PathScan Phospho-Chk2 (Thr68) Kit (Cell Signaling Technology), background-corrected values were analyzed by setting the mean value of bleomycin-treated controls to 0% and transforming the absorbance values measured for the compound-treated samples to percentages. IC50 values were calculated by nonlinear regression. Each experiment was repeated at least three times.

Cancer cell line profiling in combination with IR or other drugs

Radiosensitization of 92 cancer cell lines and resting peripheral blood mononuclear cells (PBMCs) by M3814 was performed at Oncolead. Cell viability was determined with 3 Gy IR (Cobalt-60 source), M3814 (5 μmol/L–5 nmol/L), and a combination of 3 Gy IR and M3814 (5 μmol/L–5 nmol/L). Treated cells were incubated for 120 hours, fixed, stained with sulforhodamine B, and quantified colorimetrically (18). EC50 (half-maximal effective concentrations) values were calculated from the concentration response data. Because 50% inhibition of viability was not observed in a substantial proportion of cell lines, EC50 data were displayed and analyzed.

Drug combination profiling of M3814 with 72 antitumor agents was performed in 34 cancer cell lines at Oncolead. Cell viability was determined with 72 drugs in concentration response, M3814 at a fixed concentration of 0.3 μmol/L, and a combination of both. After 120-hour incubation, cells were fixed, stained with sulforhodamine B, and quantified colorimetrically (19). Bliss independence was used to calculate synergy (20). In brief, the Bliss independence method compares the observed effect size Emeasured of a drug combination with the calculated effect, with Ecalc assuming complete independence of the drug effects (Ecalc = EDrug1 + EDrug2 – EDrug1EDrug2). Calculated
M3814 in combination with IR inhibits DNA-dependent protein kinase (DNA-PK) activity and reduces cancer cell survival in a DNA-PK-dependent manner. 

A, Chemical structure. 

B, Concentration-dependent inhibition of DNA-PK autophosphorylation (pDNA-PK) by M3814 in bleomycin-treated cancer cell lines, HCT-116 and FaDu, as shown by Western blotting. Total (t)DNA-PK was used as a loading control. Representative images are shown.

C, M3814 (1E-6 M) in combination with ionizing radiation (IR) (2.4 Gy) suppressed double-stranded break repair, as assessed by the number of γH2AX foci in cells. A high basal level of γH2AX foci was noted in A375 cells; therefore, the percentage of cells with >10 γH2AX foci was calculated and the time course of foci dynamics is plotted (bottom). Representative immunofluorescent images of vehicle-treated A375 cells without IR (top image) and 30 minute after IR (2.4 Gy; bottom image) are shown. D, M3814 (4E-7 M, 2E-6 M) in combination with IR (2.4 Gy, 4.8 Gy) reduced colony formation in FaDu and HCT-116 cancer cells in a clonogenic cell survival assay. Visible colonies were stained with neutral red. Representative images of at least three assays are shown. E, M3814 in combination with IR reduced colony formation of glioblastoma cell lines in a DNA-PK-dependent manner. M3814 combined with IR had no effect on colony survival versus vehicle in the DNA-PK-deficient glioblastoma cell line, MO59J (top). With increasing concentrations of M3814 combined with IR, the surviving fraction of colonies was reduced versus vehicle in the DNA-PK-proficient glioblastoma cell line, MO59K (bottom). Other abbreviations: DMSO, dimethyl sulfoxide.
Bliss values represent the mean values from all concentration combinations of a specific drug combination (synergy > 0.1; 0.1 ≤ additivity ≤ 0.5; antagonism — 0.1).

In vivo efficacy and pharmacokinetic/pharmacodynamic studies

In vivo efficacy data were generated in squamous cell head and neck FaDu (ATCC, HTB-47™) and non-small cell lung cancer (ATCC, HTB-177™) NCI-H660 human xenograft models in mice. The study designs and animal usage were approved by local animal welfare authorities (Regierungspräsidium Darmstadt, Germany, protocol registration numbers DA4/Anz.397 and DA4/Anz.398). Seven- to nine-week-old female NMRI (nu/nu) mice were used (Charles River Laboratories). Mice received subcutaneous injections in the right thigh with 2.5 million FaDu or NCI-H660 cells. When tumor xenografts reached a mean volume of 50 to 115 mm³, mice (n = 10 per treatment arm, randomized from 15 mice per arm to obtain a similar mean and median within the treatment groups) received IR (2 Gy, Y-RAD320 irradiation cabinet, Precision X-ray Inc.; settings: 10 mA, 250 kV, 58 cm focus skin distance collimator, 2 mm Al filter). Mice were irradiated in groups of 10 and were anesthetized during the irradiation process. IR was administered locally by positioning the tumor-bearing part of the leg in the beam path while shielding other body parts of the mice with lead. M3814 was formulated in vehicle (0.5% Methocel, 0.25% Tween20, 300 mM citrate buffer, pH 2.5) and administered orally at different doses, or in combination with IR for 5 days/week for 1 or 6 weeks. Mice were irradiated 10 minutes after oral administration of M3814. Tumor length (L) and width (W) were measured with calipers and tumor volumes were calculated using the formula V = L × W²/2.

Pharmacodynamic (PD) data were generated in FaDu human xenograft models in mice. M3814 was administered orally once (5, 25, or 100 mg/kg) in combination with 10 Gy IR. Animals were sacrificed at different time points (0.16, 1.5, 3, 8, or 24 hours) and tumors were removed, frozen in liquid nitrogen, lysed with HGNT buffer, and homogenized using a Precellys-24 homogenizer. Cell lysates were transferred to capture antibody-coated (mouse anti-DNA-PK) microtiter plates (Mesoscale; L15XB-6) and detected with either total DNA-PK (concentration range: 1.0E-09 mol/L) or phospho-DNA-PK antibodies (mesoscale, R32AD-1) at a high ATP concentration close to Kᵦ (10 mol/L). At a high ATP concentration (1 mol/L), the potency was reduced more than 30-fold, suggesting that M3814 competes with ATP for binding to DNA-PK.

M3814 exhibited a high degree of selectivity when tested using a broad panel of serine/threonine, tyrosine, and lipid kinases (Table 2). Only eight of 284 recombinantly expressed protein/lipid kinases, including mutant kinases, were inhibited by at least 50% at 1 µmol/L. M3814. All represented wild-type isoforms or mutant versions of lipid kinases of the phosphoinositide 3-kinase (PI3K) family (Supplementary Table S1). PI3K kinases, ATM, ATR, mammalian target of rapamycin (mTOR), and DNA-PK, are members of the PI3K-related kinase family, which is characterized by high similarity in the kinase domain (21). Despite their high similarity, PI3K lipid kinase isoforms were affected with strongly reduced potency (>100-fold split). Other family members, ATR, ATM, and mTOR, were even less sensitive to M3814 (Table 2).

M3814 selectively inhibits DNA-PK activity and DSB repair in human cancer cell lines

M3814 inhibited DNA-PK autophosphorylation on Ser2056, a marker for DNA-PK activity in bleomycin-treated cancer cells, determined by Western blot analysis (Fig. 1B) or ELISA (Table 1). In bleomycin-treated HCT-116 cells, M3814 did not inhibit phosphorylation of the ATM kinase substrate CHK2 (Table 2). In contrast, a moderate and concentration-dependent increase in CHK2 phosphorylation was observed, suggesting that DNA-PK blockade may trigger a compensatory upregulation of DSB repair (22). Hydroxyurea-induced CHK1 phosphorylation is induced by activation of ATR. M3814 did not block CHK1 phosphorylation, suggesting that ATR is not inhibited in cells. Phosphorylation of protein kinase B (AKT) on serine 473 in

### Results

M3814 is a potent and selective inhibitor of DNA-PK activity

M3814 (MSC2490484A) is the product of a drug discovery screening and optimization program performed at Merck KGaA, Darmstadt, Germany. The structure of M3814 is shown in Fig. 1A and its synthesis described (18). Key pharmacologic properties of M3814 are summarized in Table 2. M3814 inhibits DNA-PK activity with 0.6 µmol/L I₅₀ at an ATP concentration close to Kᵦ (10 mol/L). At a high ATP concentration (1 mol/L), the potency was reduced more than 30-fold, suggesting that M3814 competes with ATP for binding to DNA-PK.

M3814 showed selectivity against a broad panel of serine/threonine, tyrosine, and lipid kinases (Table 2). Only eight of 284 recombinantly expressed protein/lipid kinases, including mutant kinases, were inhibited by at least 50% at 1 µmol/L. M3814. All represented wild-type isoforms or mutant versions of lipid kinases of the phosphoinositide 3-kinase (PI3K) family. (Supplementary Table S1). PI3K kinases, ATM, ATR, mammalian target of rapamycin (mTOR), and DNA-PK, are members of the PI3K-related kinase family, which is characterized by high similarity in the kinase domain (21). Despite their high similarity, PI3K lipid kinase isoforms were affected with strongly reduced potency (>100-fold split). Other family members, ATR, ATM, and mTOR, were even less sensitive to M3814 (Table 2).

### Table 2. M3814 potency and selectivity.

<table>
<thead>
<tr>
<th>Assay</th>
<th>I₅₀ (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-PK, IC₅₀ [10 µmol/L ATP]</td>
<td>0.6</td>
</tr>
<tr>
<td>DNA-PK, IC₅₀ [1,000 µmol/L ATP]</td>
<td>20</td>
</tr>
<tr>
<td>Protein kinases panel profiling</td>
<td>276/284 not inhibited ≥50% at 1 µmol/L</td>
</tr>
<tr>
<td>PK kinases family</td>
<td></td>
</tr>
<tr>
<td>ATM</td>
<td>10,000</td>
</tr>
<tr>
<td>ATR</td>
<td>2,800</td>
</tr>
<tr>
<td>hP3Kalpha</td>
<td>330</td>
</tr>
<tr>
<td>hP3Kbeta</td>
<td>250</td>
</tr>
<tr>
<td>hP3Kgamma</td>
<td>&gt;1,000</td>
</tr>
<tr>
<td>hP3Kdelta</td>
<td>95</td>
</tr>
<tr>
<td>mTOR</td>
<td>&gt;10,000</td>
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<tr>
<td>HU-induced pS345-CHK1</td>
<td>&gt;25,000</td>
</tr>
<tr>
<td>Bleomycin-induced pT68-CHK2</td>
<td>&gt;5,000</td>
</tr>
<tr>
<td>Constitutive pS473-AKT in PC3</td>
<td>3,800</td>
</tr>
</tbody>
</table>

**Abbreviations:** ATM, ataxia-telangiectasia mutated; ATP, adenosine triphosphate; ATR, ATM and rad3-related; CHK, checkpoint kinase; DNA-PK, DNA-dependent protein kinase; HU, hydroxyurea; IC₅₀, half maximal inhibitory concentration; mTOR, mammalian target of rapamycin; PI3K, phosphoinositide 3-kinase; PIKK, phosphatidylinositol 3-kinase-related kinase.
PC3 prostate cancer cells is driven by inactivation of phosphatase and tensin homolog (PTEN) and generation of phosphatidylinositol-3,4,5-triphosphate (PIP3) by PI3K activity (23). M3814 only moderately inhibited the phosphorylation of AKT, which is consistent with its weak inhibitory activity on the PI3K isoforms.

The reduced level of DNA-PK autophosphorylation by M3814 suggests that DNA-PK-dependent DSB repair by NHEJ is inhibited in cancer cells. To monitor the DSB level and repair kinetics in cancer cells, γH2AX-foci were counted at different time points after IR (Fig. 1C). Thirty minutes after IR, the number of cells with more than 10 foci increased and subsequently returned to baseline at 24 hours. Upon addition of M3814 (1 μmol/L), the reduced number of foci over the recovery period was significantly inhibited compared with the DMSO-treated control, indicating that DNA-PK inhibition effectively suppressed DSB repair.

M3814 sensitizes cancer cells to IR

M3814 reduced colony formation in combination with IR in a concentration-dependent manner but showed no or limited activity in the absence of IR (Fig. 1D; Table 1). Overall, the antitumor potency correlated well with the inhibition of DNA-PK autophosphorylation, indicating that DNA-PK inhibition sensitizes cells to IR. The ability of M3814 to inhibit DNA-PK autophosphorylation and reduce colony formation was observed in all cancer cell lines tested, regardless of tumor origin. However, level of inhibition varied depending on the cancer cell line (Table 1). The antitumor effect of M3814 was due to suppressing DNA-PK catalytic activity; in the glioblastoma cell line, MO59J, which lacks DNA-PK activity (24), increasing concentrations of M3814 did not further enhance sensitization to IR (Table 1; Fig. 1E). However, the DNA-PK-proficient cell line, MO59K, isolated from the same tumor specimen, was less sensitive to IR alone but was sensitized by M3814 in a concentration-dependent manner, suggesting that pharmacologic inhibition of DNA-PK is the key mechanism of radiosensitization. Enhancement factors at 10% colony survival (EF10) for M3814 reached values between 2.5 and 6 in the 0.11 to 1 μmol/L concentration range.

Suppression of colony formation is a widely used test to assess radiosensitization. However, its application is limited because many cancer cell lines do not form countable colonies (25). To assess M3814 activity in combination with IR in a large panel of cancer cell lines, we used a sulforhodamine B-based cell growth/viability assay. A random collection of 92 cancer cell lines, representing multiple tumor types, was used in the assay. M3814 inhibited cell growth with a mean EC50 of 2.1 μmol/L (Fig. 2A; Supplementary Table S2). The effect on growth/viability in the absence of IR was more pronounced than in the colony formation assay, probably due to extended exposure to the compound. Nevertheless, the potency of inhibition by M3814 alone was regarded as rather moderate. In combination with 3 Gy IR, synergistic growth inhibition was observed in all cell lines, with a mean 12.4-fold half
maximal effective concentration (EC\(_{50}\)) shift in sensitization (mean \(EC_{50(\text{M3814+IR})} = 0.17 \mu\text{mol/L}\); Supplementary Table S2; Supplementary Fig. S1). Taken together, our data suggest that by inhibiting DNA-PK activity and DSB repair, M3814 sensitizes cancer cells to IR through impaired colony outgrowth or proliferation/viability.

**M3814 synergistically enhances the activity of DSB-inducing agents**

To investigate the combination potential of M3814 with other anticancer agents, 35 randomly selected cancer cell lines were profiled with 72 drugs, representing diverse antitumor mechanisms of action. The inhibitory effect of each agent was measured in the presence or absence of 300 nmol/L M3814 and Bliss synergy was calculated (20). Bliss scores were classified as synergistic (>0.1), additive (-0.1 ≤ X.0 ≤ 0.1), or antagonistic (≤-0.1; Fig. 2B; Supplementary Fig. S2). No significant combination effect was observed with the majority of tested drugs in most cancer cell lines, indicating that M3814 does not broadly synergize with anticancer agents. Notably, M3814 synergy across different cell lines was observed with the radiomimetic drug, bleomycin, and the topoisomerase inhibitors, doxorubicin and etoposide. These agents induce DSBs via radical generation or topoisomerase 2 inhibition, indicating that DNA-PK is important for repair of these lesions. Although sporadic synergism or antagonism was observed in a few individual cell lines, implying that the genetic make-up of the cancer cell lines may contribute to the combination effect, the overall ability of M3814 to synergistically enhance the activity of other drugs was clearly defined by their mechanism of action. Only three of 72 drugs showed significant synergism in >34 cancer cell lines; those drug combinations are known to induce DSBs. These results clearly indicated that M3814 activity is derived from its molecular mechanism of action.

**M3814 inhibits radiation-induced DNA-PK autophosphorylation and DSB repair in tumor xenograft models**

Next, we asked whether M3814 could inhibit its target and the repair of IR-induced DSBs in tumor tissues in vivo. We performed Pharmacokinetic (PK)/PD analyses in tumor xenograft studies. Initial results indicated that detection of DNA-PK autophosphorylation at low IR doses of 1 to 5 Gy is technically difficult and would not allow accurate quantification, while a higher IR dose (10 Gy) resulted in a detectable increase of ψSer2056 in DNA-PK, which returned to baseline levels over 24 hours (Fig. 3A). Following oral administration of a single dose of 25 mg/kg M3814, the plasma concentration of the parent compound increased to approximately 3 μmol/L 1.5-hours postdose, followed by elimination over the next 24 hours. IR-induced Ser2056 phosphorylation of DNA-PK was suppressed at 1.5 hours to below baseline levels and remained low 3 hours after IR. The signal increased at 8 hours, when the plasma concentrations of M3814 were lower, and returned to baseline levels at 24 hours. Autophosphorylation of DNA-PK was reduced in a concentration-dependent manner at 1.5 hours (Fig. 3B).

To assess the effect of DNA-PK inhibition on DSB repair, we developed an immunofluorescence-based assay for quantification of γH2AX foci in tumor tissue (Fig. 3C, see Materials and Methods), which was used to measure γH2AX levels in FaDu tumors. A single dose of IR (10 Gy) was given in the presence or absence of M3814 (200 mg/kg) and γH2AX signals were quantified in xenograft tumors obtained at different intervals post-IR. M3814 administration changed the kinetics of the γH2AX signal compared with vehicle-treated tumors, indicating suppression of DSB repair (Fig. 3C and D). Taken together, these results indicate that M3814 administration inhibited IR-induced DNA-PK activation, leading to increased DSB levels, both consistent with the exposure dynamics of M3814 in mouse plasma.

**Preincubation of human blood with M3814 attenuates DNA-PK phosphorylation**

PD data obtained from human tumor biopsies helps to confirm that the drug reaches its molecular target in the target tissue. However, the possibility to sample tumor tissue in early clinical trials is rather low, making it difficult to build a robust PK/PD model. Analyses of PD biomarkers from surrogate tissues, such as blood, offer the possibility for regular sampling in parallel to routine PK analyses. For this purpose, we developed a PD biomarker assay to detect DNA-PK autophosphorylation in peripheral blood cells. DNA-PK phosphorylation was induced by incubation of PBMCs from healthy human donors with bleomycin for 4 hours (Fig. 3E). Preincubation of human blood with different concentrations of M3814 ranging from 1 nmol/L to 30 μmol/L attenuated DNA-PK phosphorylation in a concentration-dependent manner. The potency of inhibition was within the same range observed for cancer cell lines. These data demonstrated that PD analyses from surrogate tissue are feasible and may be useful for clinical exploration of M3814.

**M3814 strongly potentiates IR efficacy in xenograft models of human cancer**

M3814 demonstrated good oral bioavailability and PK in mice (Supplementary Table S5; Supplementary Fig. S3); thus, further studies were performed to assess antitumor activity and evaluate safety in mouse xenograft models. Subcutaneously established FaDu or NCI-H460 tumors were treated with a 5-day fractionated radiation regimen (2 Gy IR fraction per tumor/mouse per day), with or without M3814 administered by oral gavage 10 minutes before IR. In both tumor models, tumor growth was moderately but significantly retarded with IR alone (Fig. 4A and B). However, co-administration of M3814 led to enhanced tumor growth inhibition in both tumor models. Tumor growth inhibition was dependent on the dose of M3814 administered (Fig. 4A). Additional human xenograft models of different origin were investigated (A549, BxPC3, Capan-1, and HCT-116) using the same IR regimen. Again, significant tumor growth inhibition was seen when M3814 was combined with IR for 1 week (Supplementary Fig. S4). NHEJ repair is independent of the cell-cycle phase, fast, and contributes to the repair of most DSB lesions during the first hours after radiation (26). Indeed, the results of scheduling experiments of M3814 in relation to IR support this hypothesis (Supplementary Fig. S5). When M3814 was administered 10 minutes prior to IR, the combination benefit was clearly superior than when administered 3 or 6 hours after IR. Because M3814 is rapidly cleared in mice, administration of an additional, consecutive dose of M3814 (first dose 10 minutes before IR, second dose 3 hours later) also improved antitumor activity. However, administration of M3814 for additional days after IR did not further improve antitumor efficacy indicating that DSBs have already been repaired.

In clinical practice, fractionated radiation regimens are commonly used for the treatment of multiple solid tumor types. The duration of radiation therapy with curative intent usually extends several weeks of therapy and applies fractionated doses of 1.8 to 2.0 Gy, for a total dose of approximately 50 to 70 Gy, depending on the tumor type and location (27). Therefore, we applied a 6-week radiotherapy regimen (5 days on, 2 days off) with 2 Gy fractions to subcutaneously implanted mouse xenograft tumors and investigated the effect of different doses of M3814 together with IR (Fig. 4C and D; Supplementary Fig. S6). In
Figure 3.
M3814 inhibits radiation-induced DNA-dependent protein kinase (DNA-PK) activation leading to increased double-stranded break (DSB) levels in tumor tissue from mice xenograft models; assessment of DNA-PK phosphorylation and inhibition by M3814 in human blood. A, Time course of DNA-PK phosphorylation in the FaDu tumor xenograft model in response to ionizing radiation (IR; 10 Gy) alone (black line) or the combination of M3814 (25 mg/kg) with IR (10 Gy, red line). Data from a representative pharmacokinetic (PK)/pharmacodynamic (PD) study are shown. The ratio of phosphorylated to total DNA-PK was determined in five tumors per treatment group and time points, and the mean ± standard error of the mean are presented. The mean plasma concentration of M3814 over time following oral administration is shown in green. B, The ratio of phosphorylated to total DNA-PK was determined in FaDu xenograft tumors at baseline (no IR), after 10 Gy IR alone, and following treatment with IR (10 Gy) combined with M3814 (5, 25, 100 mg/kg). Tumors were processed 1.5 hours after treatment (five tumors per group). C, Impaired DSB repair over time in FaDu tumors following treatment with vehicle (left image) or 200 mg/kg M3814 (right image) and IR (10 Gy). Representative immunofluorescence images detecting γH2AX in tumor sections are shown (Scale bar = 2 μm). D, Quantification of time-dependent resolution of γH2AX staining from the experiment described under C. E, DNA-PK phosphorylation was induced by incubating whole blood from healthy donors with bleomycin for 4 hours (left). Preincubation of whole blood with M3814 attenuated bleomycin-induced DNA-PK phosphorylation in a concentration-dependent manner, confirming target modulation by M3814 in human blood samples. Representative data from a single donor are shown (blood from six different donors was tested). Other abbreviations: DMSO, dimethyl sulfoxide; RT, radiotherapy.
the FaDu head and neck cancer model, IR led to a partial regression of tumors during the treatment phase (Days 1–42); however, tumor growth progressed upon cessation of IR (Fig. 4C). With increasing doses of M3814 administered orally 10 minutes before each IR fraction, the tumor response was significantly enhanced, with increased tumor growth retardation at 5 and 10 mg/kg and complete regression of all tumors observed at 25 and 50 mg/kg doses. Of note, the strong response to combination therapy extended beyond treatment throughout the observation period (Days 43–106). Because the FaDu model is sensitive to IR alone (28), we assessed the impact of combination therapy in the relatively radio-resistant non–small cell lung cancer model, NCI-H460 (Fig. 4D). Indeed, this model was significantly less responsive to IR alone compared with FaDu tumors. During the treatment period, H460 xenografts continued to grow following treatment with IR alone. However, co-administration of M3814 and IR significantly increased the antitumor response during the treatment and observation period. In five of 10 animals treated with 25 mg/kg M3814 plus IR, tumors progressed during the observation period.
In general, the combination of M3814 plus IR was well-tolerated (Supplementary Fig. S6). During the treatment period, animals in all treatment groups showed a moderate loss of body weight (less than 10%), likely due to the daily (5 days on, 2 days off) treatment procedure, including anesthesia, oral gavage, and IR over 6 weeks. However, the body weight loss was fully reversible, and mice recovered during the observation period. In the FaDu efficacy study, grade 1 acute dermatitis was observed in only three of 10 mice in the combination group receiving 50 mg/kg M3814 after 42 days of treatment; however, this was fully reversible after 14 days. No signs of dermatitis were observed in the other groups (29).

Applying clinically relevant IR treatment regimens to human tumor models in mice confirmed the remarkable antitumor activity of M3814 in combination with IR, which warrants clinical exploration.

**Discussion**

M3814 is a potent inhibitor of DNA-PK catalytic activity with remarkable selectivity against most of the 284 protein kinases we tested, including the closest members of its own kinase family. Cell-based experiments indicated that the in vitro selectivity is retained in cancer cells, albeit with reduced potency due to high ATP concentrations and possibly other factors, such as the nuclear localization of the target. Neither the ATR-CHK1 nor ATM-CHK2 pathways were significantly inhibited by M3814 in cancer cells at concentrations that effectively suppressed the DNA-PK pathway. Residual inhibitory activity against three closely related lipid kinases, PI3Kα/β/δ, has been noted. However, phosphorylation of AKT in PTEN-mutated prostate carcinoma cells, which can be potently blocked by PI3K inhibitors, was only marginally inhibited.

M3814 inhibited DSB repair and sensitized cancer cells to IR. Treatment of cancer cells with M3814 attenuated foci resolution, indicating inhibition of DSB repair. Radio-sensitization was observed in most tested cancer cell lines, regardless of tissue origin, indicating inhibition of DSB repair. Radio-sensitization was only marginally inhibited.

Inhibitors of prostate carcinoma cells, which can be potently blocked by PI3K inhibitors, was only marginally inhibited.

M3814 inhibited DSB repair and sensitized cancer cells to IR. Treatment of cancer cells with M3814 attenuated foci resolution, indicating inhibition of DSB repair. Radio-sensitization was observed in most tested cancer cell lines, regardless of tissue origin, and confirmed using two different assay formats. The level of synergy varied moderately between cancer cell lines, suggesting that other factors have a subtle influence on sensitivity to the combination treatment. However, as expected, there was a good correlation between sensitivity to IR alone and to combination treatment with M3814. These suggest that the DNA-PK inhibitor enhances existing sensitivity to radiation rather than engaging a different antitumor mechanism. Strikingly, the glioblastoma line MO59J, which is devoid of DNA-PK activity (29), could not be radiosensitized by M3814, whereas the DNA-PK-proficient cell line, MO59K, which originates from the same tumor specimen, was sensitive to M3814. These data indicate that inhibition of DNA-PK kinase activity is the key mechanism underlying Radiosensitization by M3814. Taken together, our results suggest that inhibition of the canonical NHEJ pathway is responsible for the strong antitumor effects of M3814 when combined with IR.

Profiling M3814 in combination with 72 diverse established and developmental antitumor agents revealed potential for combination with the chemotherapeutics bleomycin, etoposide, and doxorubicin. These synergistic combinations were anticipated on the basis of their mechanisms of action, which involve the generation of DSBs through inhibition of the topoisomerase enzymatic cycle leading to the generation of Top2:DNA complexes. DNA-modifying agents (alkylators, antimetabolites, and topoisomerase 1 inhibitors) did not synergize with M3814, because their primary mechanism of repair does not require the canonical NHEJ. Similarly, multiple other mechanisms represented by the large panel of tested drugs do not provide combination benefit, further indicating that M3814 acts exclusively by inhibiting NHEJ.

Exposure-dependent inhibition of DNA-PK autophosphorylation, and the subsequent delay in DSB repair kinetics observed in xenograft tissues in vivo, indicated that M3814 possesses all the necessary properties to evaluate the therapeutic potential of DNA-PK inhibition in xenograft models. To this aim, we selected two xenograft tumor models with different sensitivity to IR and the most relevant clinically established regimen for radiation therapy; 6-week fractionated radiation dosing. Pilot 1-week fractionated radiation studies showed that the combination of M3814 and IR is beneficial over IR alone. However, the 6-week animal trials, which modeled a clinically relevant fractionated radiation regimen with curative intent, demonstrated strong and durable antitumor activity of the M3814/IR combination. Local tumor radiation with M3814 exposures predicted to be achievable in man caused durable tumor regression in multiple animals in both models. In general, M3814 was well-tolerated with moderate but completely reversible body weight loss. In one xenograft study grade 1 radiation-induced dermatitis was observed in three out of ten animals receiving the highest dose of M3814 (50 mg/kg) in combination; however, the symptoms were fully reversible. Lower M3814 doses, which also produced complete regressions, did not lead to a skin reaction. However, the limited toxicological data presented here do not sufficiently address the impact of M3814 on other normal tissues in the radiation field, especially toxic effects that manifest themselves significantly later post-radiation treatment. Further combination studies with M3814 and dedicated toxicologic assessments will help to better evaluate radiation-related toxicities.

Data on the discovery and characterization of a novel DNA-PK inhibitor, M3814 presented in this manuscript offer a basis to explore its activity in combination with radiotherapy and other DSB-inducing therapies in the clinic. Furthermore, the data provide preclinical proof of concept for selective pharmacologic inhibitors of DNA-PK as combination partners of clinically established regimens for radiation therapy. Currently ongoing clinical investigations with M3814 in both monotherapy and radiotherapy combination settings should answer many outstanding questions regarding their therapeutic potential (ClinicalTrials.gov IDs: NCT02316197, NCT02516813).

**Disclosure of Potential Conflicts of Interest**

F.T. Zenke, C. Sirrenberg, T. Grombacher, T. Fuchs and A. Blaukat are employees of Merck KGaA, Darmstadt, Germany; and have stock or ownership interests (including patents) in Merck KGaA, Darmstadt, Germany. A. Zimmermann, H. Dahmen, U. Pehl, C. Wilm and C. Amendt are employees of Merck KGaA, Darmstadt, Germany. V. Kirkin was an employee of Merck KGaA, Darmstadt, Germany at the time of study conduct. L.T. Vassilev is an employee at EMD Seronova, Billerica, MA, United States; a business of Merck KGaA, Darmstadt, Germany.

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References


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