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Repair of DNA Double-Strand Breaks in Human Cells

Susanne Nyström

Göteborg 2005

Institute of Laboratory Medicine
Department of Clinical Chemistry and Transfusion Medicine
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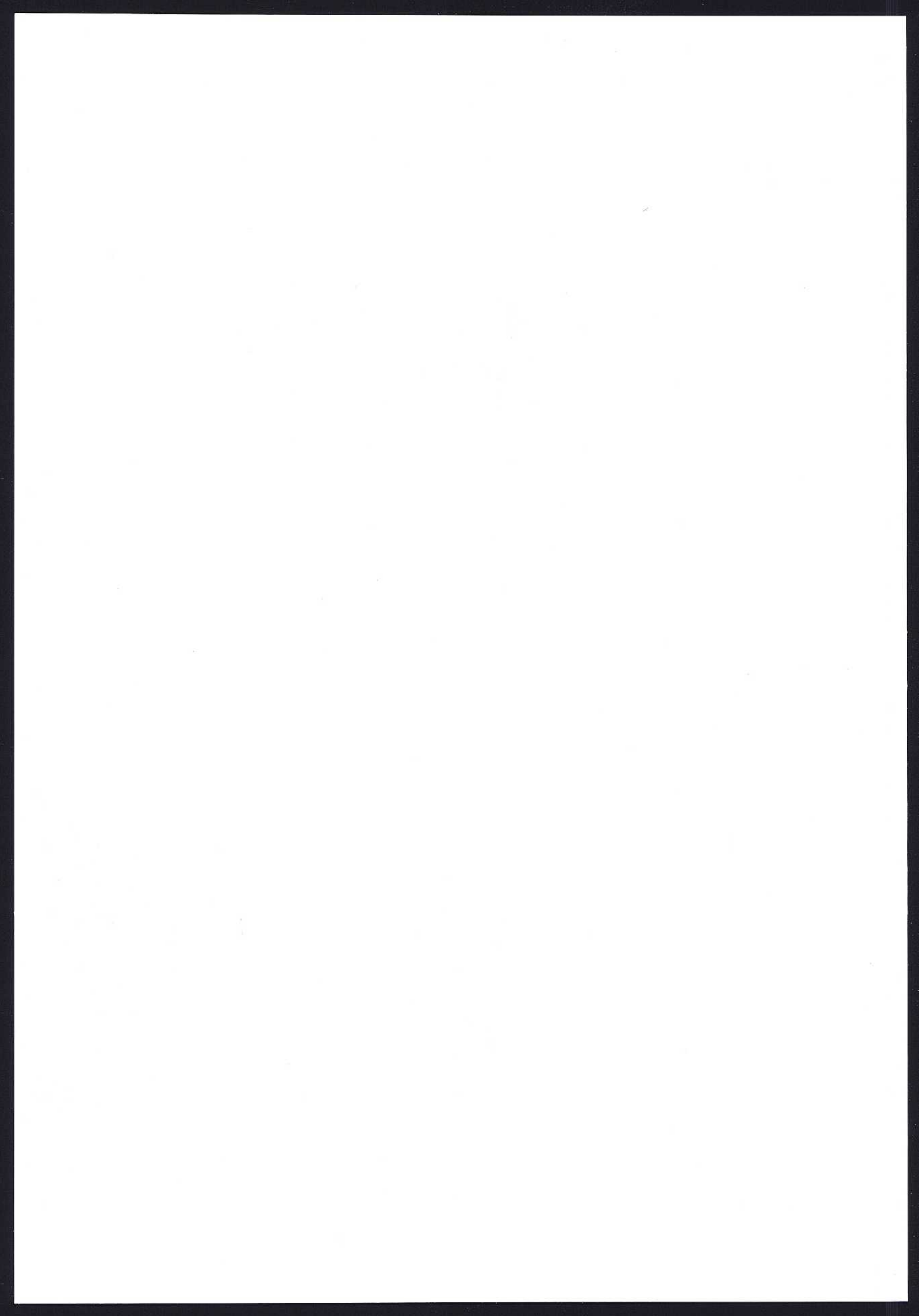

Repair of DNA Double-Strand Breaks in Human Cells

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Göteborgs Universitet kommer att offentligen försvaras i föreläsningssal Ivan Ivarsson, Medicinaregatan 3, fredagen den 9:e september 2005, kl. 9.00

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DNA-dependent Protein Kinase Catalytic Subunit: Structural Requirements for Kinase Activation by DNA ends.
J. Biol. Chem. 2002 January 25; 277 (4):3020-3029

II Mårtensson S , Nygren J , Osheroff N, Hammarsten O.
Activation of the DNA-dependent protein kinase by drug and radiation-induced DNA strand-breaks.
Radiat Res. 2003 Sep; 160(3):291-301

III Elmroth K, Nygren J, Mårtensson S, Ismail IH, Hammarsten O.
Cleavage of cellular DNA by calicheamicin γ1.
DNA Repair (Amst). 2003 Apr 2;2(4):363-74

IV Ismail HI, Mårtensson S, Moshinsky D, Rice A, Tang C, Howlett A, McMahon G, Hammarsten O.
SU11752 inhibits the DNA-dependent protein kinase and DNA double-strand break repair resulting in ionising radiation sensitisation.
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Göteborg 2005

Dissertation Abstract

Repair of DNA Double-Strand Breaks in Human Cells

Susanne Nyström

Institute of Laboratory Medicine, Department of Clinical Chemistry and Transfusion Medicine, Sahlgrenska University Hospital, Göteborg University, Göteborg, Sweden

DNA is continuously subjected to degradation. Therefore, our cells need to constantly repair its DNA to prevent mutations and in the long run cancer. In mammalian cells, when DNA is broken right off, a double-strand break (DSB) is produced, and the ends are ligated by a process called non-homologous end-joining (NHEJ). NHEJ involves a protein kinase, DNA-PK that binds and becomes activated by the DNA ends. DNA-PK is composed of the DNA-binding subunit Ku and the catalytic subunit DNA-PKcs. The overall aim of the present investigation was to take a closer look at the repair of DSBs in human cells and the involvement of the DNA-PK in this process.

Using purified enzymes and kinase assays, we studied the activation of DNA-PK by different forms of broken DNA ends. DNA-PK was activated by DNA-ends containing a wide variety of modifications. This indicated that DSBs that remain undetected by DNA-PK are rare. The only exception was DSBs generated by the cancer drug etoposide. In this case the DSB is covered by topoisomerase II and can therefore not be recognized by DNA-PK. We also studied DNA-PK activation by DNA strand breaks generated by agents used in cancer treatment. DSBs produced by bleomycin, calicheamicin γ1, and different kinds of radiation activated DNA-PK to maximal levels.

We also compared the cleavage of cellular DNA and purified plasmid DNA by calicheamicin γ1 (CLM), a drug used in cancer treatment. Our findings show that the ratio of DNA DSBs to single-strand breaks (SSB) in cellular DNA was 1:3, close to the 1:2 ratio observed when CLM cleaved purified plasmid DNA. CLM-induced DSBs were repaired slowly but completely and resulted in a normal and strong DSB-response in cells. The high DSB/SSB ratio, specificity for DNA and the even damage distribution makes CLM a superior drug for studies of the DSB-response.

Finally, we identified and characterized a DNA-PK-inhibitor, SU11752. SU11752 was a potent and selective inhibitor of DNA-PK. Results showed that SU11752 inhibited DNA-PK by ATP competition and sensitized cells 5-fold to ionizing radiation (IR) by inhibition of DSB repair. SU11752 defines a new class of drugs that serves as a starting point for development of specific DNA-PK inhibitors. Loss of DNA-PK results in increased sensitivity to IR due to inefficient repair of DNA DSBs. Over-expression of DNA-PK in tumor cells conversely results in resistance to IR, because DSBs are repaired quickly. It is therefore possible that inhibition of DNA-PK will enhance the preferential killing of tumor cells by radiotherapy.

Keywords: *DNA-PK; DNA double-strand break, DSB; DNA single-strand break, SSB; DNA damage; ionizing radiation, IR; DNA repair; calicheamicin γ1; bleomycin; etoposide; topoisomerase II; radiotherapy; radiosensitizer; ATM*

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Susanne Nyström

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Department of Clinical Chemistry and Transfusion Medicine
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Abstract

Our DNA is continuously subjected to degradation. Therefore, our cells constantly need to repair their DNA to prevent mutations and, in the long run, cancer. In mammalian cells, when DNA is broken off, a double-strand break (DSB) is produced, and the ends are ligated by a process called non-homologous end-joining (NHEJ). NHEJ involves a protein kinase, DNA-PK, which binds and is activated by the DNA ends. DNA-PK is composed of the DNA-binding subunit Ku and the catalytic subunit DNA-PKcs. The overall aim of the present investigation was to take a closer look at the repair of DSBs in human cells and the involvement of the DNA-PK in this process.

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We also studied DNA-PK activation by DNA strand breaks generated by agents used in cancer treatment. DSBs produced by bleomycin, calicheamicin $\gamma 1$, and different kinds of radiation activated DNA-PK to maximum levels.

We also compared the cleavage of cellular DNA and purified plasmid DNA by calicheamicin $\gamma 1$ (CLM), a drug used in cancer treatment. Our findings show that the ratio of DNA DSBs to single-strand breaks (SSB) in cellular DNA was 1:3, close to the 1:2 ratio observed when CLM cleaved purified plasmid DNA. CLM-induced DSBs were repaired slowly but completely and resulted in a normal, strong DSB response in cells. The high DSB/SSB ratio, specificity for DNA and the even damage distribution all make CLM a superior drug for studies of the DSB response.

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Abbreviations

53BP1	protein 53-binding protein-1
AP-site	apurinic/apyrimidinic site
AT	Ataxia-telangiectasia
ATLD	Ataxia-telangiectasia like disorder
ATM	Ataxia-telangiectasia Mutated
ATP	adenosine triphosphate
ATR	ATM and Rad3-related
BER	base excision repair
BRCA1/2	breast cancer susceptibility protein 1/2
Chk2	checkpoint kinase 2
CLM	calicheamicin
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
dsDNA	double stranded DNA
EMSA	electromobility shift assay
ERCC1	excision repair cross complementation group 1
FAR	fraction of activity released
Gy	gray
H2AX	histone 2 variant AX
HJ	Holliday junction
HR	homologous recombination
IR	ionizing radiation
Kbp	kilo-base pair
kDa	kilo Dalton
LET	linear energy transfer
Lig4	ligase 4
Mbp	mega-base pair
MDC1	mediator of DNA damage checkpoint 1
MMR	mismatch repair
Mre11	meiotic recombination 11
MRN	Mre11/RAD50/Nbs1 complex
MRX	Mre11/RAD50/Xrs2 complex
mTOR/FRAP	mammalian target of rapamycin/FKB12-rapamycin binding
NBS	Nijmegen breakage syndrome
Nbs1	Nijmegen breakage syndrome protein 1
NER	nucleotide excision repair
NHEJ	non-homologous end-joining
P53	protein 53
PBS	phosphate-buffered saline
PCC	premature chromosome condensation
PI3K	phosphatidylinositol-3 kinase
PIKK	phosphatidylinositol-3 kinase-like kinase

PNK	polynucleotide kinase
PVDF	polyvinylidene difluoride
Rag1/2	recombination activating gene 1/2
RecA	recombination protein A
RNA	ribonucleic acid
ROS	reactive oxygen species
RPA	replication protein A
RS	recombination signal
SCID	severe combined immunodeficiency
SDS	sodium dodecyl sulphate
SDSA	synthesis-dependent strand annealing
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMG1	supressor of morphogenesis in genitalia-1
SSA	single-strand annealing
SSB	single strand break
ssDNA	single stranded DNA
T	thymidine
TAE	tris-acetate-EDTA
TARDIS	trapped in agarose DNA immunostaining
TdT	terminal deoxytransferase
TE	tris-EDTA
Topo I/II	topoisomerase I/II
TSB	total strand breaks
UV	ultraviolet
WRN	Werner protein
XPF	Xeroderma Pigmentosum Complementation group F
XRCC3/4	X-ray cross-complementary gene 3/4
γH2AX	phosphorylated histone 2 variant AX

This thesis is based on the following publications, referred to by their Roman numerals in the text:

I **DNA-dependent Protein Kinase Catalytic Subunit: Structural Requirements for Kinase Activation by DNA ends.**
S. Mårtensson, O. Hammarsten
J. Biol. Chem. 2002 January 25; 277 (4):3020-3029

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DNA Repair (Amst). 2003 Apr 2;2(4):363-74

IV **SU11752 inhibits the DNA-dependent protein kinase and DNA double-strand break repair resulting in ionising radiation sensitisation.**
Ismail Hassan Ismail, Susanne Mårtensson, Deborah Moshinsky, Audie Rice, Cho Tang, Anthony Howlett, Gerald McMahon and Ola Hammarsten.
Oncogene 2004 Jan 29;23(4):873-82

Äntligen!

DNA

By 1944 it had become clear that the DNA in our cells contained the genetic code [1], and in 1953 James Watson and Francis Crick suggested a model for the molecular structure of DNA [2]. Since then, many discoveries have been made about this double helical molecule, although many others are still waiting to be revealed.

DNA stands for DeoxyriboNucleic Acid. The building blocks of DNA are the nucleotides, composed of a phosphate group, a sugar moiety (deoxyribose), and a base attached to each sugar. The nucleotides are linked together into a strand, one after another, with the bases placed in a specific order. The bases from two strands pair together in a certain manner, with adenine binding to thymidine and cytosine to guanine, thus creating a DNA ladder, with the bases as the steps with the sides consisting of the sugar moieties, i.e. the DNA backbone. The strands are thus complementary and one can be used as a template for the other.

The DNA is packed extremely tightly in the cells. The isolated human DNA molecule is 2 meters long, and every cell carries a copy of the DNA with it. Since the human cell in general is approximately 0.01 mm (or 10 μm) in diameter, the DNA has to be very tightly packed in order to fit into the cell. The DNA is threaded around proteins called histones, which greatly reduces its length. The histones and the DNA are then coiled together in a spiral (helix) to form chromosomes, fitting into the nucleus of the cell.

DNA damage

The DNA in our cells is continuously subjected to alteration and degradation. More than 20,000 nucleotides are damaged in every cell every day. Damage can result, for instance, in loss of bases (abasic sites, AP-sites), cross linking between the two DNA strands, mismatches, single-strand breaks (SSBs) where one of the DNA strands is broken off, and double-strand breaks (DSBs), where both DNA strands are broken off. If damage accumulates in the DNA, cellular response signals can trigger apoptosis (programmed cell death), but they can also result in mutations, which enhance the risk of cancer. Many of this damage is spontaneous, arising as consequences of normal metabolism and errors that arise during replication, recombination and repair [3]. For instance, mispairing of bases during DNA synthesis results in mismatches, which can, in turn, lead to mutations. Alterations in or elimination of bases also occurs simply because of the inherent instability of the chemical bonds present in the nucleotides [3].

Two major forms of spontaneous damage occurs in DNA; deamination of bases and loss of bases [4]. Three of the bases present in DNA: adenine, cytosine and guanine, have protruding amino groups. If these amino groups are lost, the base is deaminated, which may result in mutations [3]. Deamination can occur if the base is attacked by reactive oxygen species (ROS) produced during cellular metabolism.

When cytosine is deaminated, for example, it is converted into uracil, a base not normally found in DNA, but in RNA. Uracil pairs with adenine, and thus the deamination of cytosine can lead to a transition mutation if present during DNA synthesis. The cell has a specific set of DNA repair enzymes that remove uracil and replace it with cytosine. The enzyme Uracil-glycosylase cleaves the bond that links uracil to the DNA backbone, leaving an AP-site [4]. The AP nuclease cleaves the backbone next to the AP-site, the sugar moiety in the DNA backbone is removed, and DNA polymerase and ligase fill the gap in the DNA strand with the correct base.

Damage can also arise from exogenous agents such as ionizing radiation (IR), UV radiation, and chemical agents, including drugs used for cancer treatment, and other chemicals present in our environment.

The DNA double-strand break, DSB

The DSB is one of the most toxic types of damage that arise in DNA. If they are not repaired correctly, one single DSB, or at most ten [5], can potentially lead either to chromosomal fragmentation and cell death or to chromosomal translocations and cancer. Because DNA is continuously subjected to degradation, our cells constantly need to repair their DNA to prevent mutations and, in the long, run cancer. The extreme toxicity of the DSB is used in cancer treatment, where drugs that specifically introduce DSBs are used frequently.

The definition of a DSB is two SSBs located less than 12 base pairs apart in an organism living at 37°C.

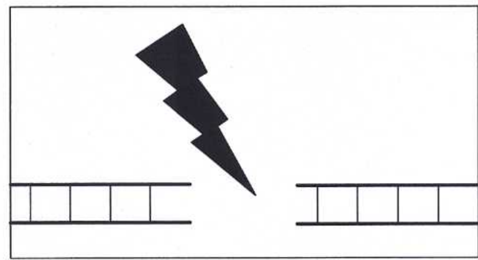

Figure 1. *Both strands of the DNA helix are broken, and a DSB is formed.*

A DSB does not have to be the result of an accident. A DSB can also occur naturally during normal cell metabolism to initiate recombination between homologous chromosomes, and when replication forks stall and collapse, a process that is thought to occur frequently during the S-phase [6]. DSBs are also introduced during V(D)J recombination, which is a site-specific recombination process that

generates the molecular diversity of the immune system. These pathways are very strictly regulated, with DSBs occurring as intermediates, and many of the enzymes involved in DSB repair are also used in these programmed processes [7]. Since improperly repaired DSBs may lead to chromosomal aberrations and cancer, the repair of DSBs is absolutely essential to the maintenance of genomic integrity [8, 9].

DNA double-strand break signaling

The eukaryotic cell cycle is into between four phases: G1, S, G2 and M. The cell cycle also has checkpoints that are strictly regulated, and at which progression through the cell cycle can be arrested to ensure that the genome remains intact.

DNA damage activates intricate signaling responses that can trigger cell cycle checkpoint arrest, apoptosis [10, 11] or repair pathways. These signaling systems are also in close cooperation with the replication and transcription mechanisms, to prevent broken or rearranged chromosomes from being passed on to daughter cells [7]. The basic principle of recognizing and signaling DNA damage is that a protein recognizes the damage and is then activated and transports the signal to other proteins involved in the signaling cascade.

Ataxia Telangiectasia Mutated (ATM) is a key protein kinase involved in signaling the DSB response [10, 12, 13]. ATM is a serine/threonine kinase and a member of a subgroup of the phosphatidylinositol-3-kinase (PI3K) family, known as the PI3K-like protein kinase family (PIKK). PI3K-like protein kinases are involved in many cellular processes, including cell cycle progression, growth and signal transduction. The mammalian protein kinases of this subgroup are ATM, ATR (ATM- and RAD3-related), SMG1 (suppressor of morphogenesis in genitalia-1), mTOR/FRAP (mammalian target of rapamycin/FKB12-rapamycin binding) and DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase) [10], and they all share homology in the kinase domain. ATM and DNA-PKcs respond primarily to DSBs [10], ATR and SMG1 respond to DSBs as well as UV-light-induced damage, and mTOR/FRAP does not respond to DNA damage, but nutrient levels, mitogenic stimuli, and is also involved in cellular growth control.

ATM responds rapidly to DSBs with increasing kinase activation, and signals by phosphorylating numerous other proteins involved in the DSB response process [10]. In undamaged cells, ATM exists as an inactive dimer or multimer [14]. When the DNA is damaged, each ATM molecule phosphorylates another. The molecules are released from each other and become active monomers within minutes after DSBs are introduced in the genome [14]. Once activated, ATM phosphorylates and activates several downstream targets, including Chk2, p53, Nbs1, BRCA1 and also itself, subsequently leading to cell cycle arrest [15]. The histone H2AX is one of the first proteins to be phosphorylated in an ATM-dependent manner shortly after damage [16, 17]. DNA-PKcs is also required for optimal phosphorylation of H2AX [18]. H2AX is phosphorylated (γH2AX) over a 2 mega-base pair area flanking the DSB [16], and probably serves as amplification of the DSB signal. This leads to recruitment of several additional proteins at the break site, for instance 53BP1, MDC1, BRCA1 and the MRN complex [19]. The roles of H2AX and the proteins that accumulate at the site of the DSB are unknown [11]. Cells lacking H2AX

display radiosensitivity and DSB repair defects, demonstrating its importance in DSB signaling and repair [20].

In response to DSBs, the MRN complex (composed of Mre11, RAD50, and Nbs1) appears to be a sensor protein [13]. Accumulating evidence puts MRN upstream of ATM, and indicates that MRN senses DNA breaks directly, binds to DNA, unwinds the ends, recruits ATM via Nbs1, and dissociates the ATM dimer [13]. The MRN complex may also be involved in processing the ends of a DSB before repair.

DNA-PKcs is involved in repairing DSBs, and has only recently been implicated in signaling of DSBs. Results reported on different cell lines and mouse strains are, however, inconclusive [21]. DNA-PKcs have been reported to phosphorylate the tumor suppressor protein p53 in response to DSBs, which activates apoptosis [22]. On the other hand, studies with DNA-PKcs-deficient cell lines have shown that the p53-mediated response to DNA damage is normal in the absence of DNA-PKcs activity [23]. So DNA-PKcs do not seem to be primarily involved in initiating cell cycle arrest in response to DSBs [24], or perhaps other proteins have overlapping functions. However, DNA-PKcs seems to play an important role in signaling apoptosis, since IR-induced apoptosis is suppressed in the absence of DNA-PKcs [25]. Another suggested function of DNA-PK is as a molecular scaffold, to target other repair proteins to damaged DNA [26, 27].

ATM deficiency results in ataxia-telangiectasia (AT), a disease characterized by defective cell cycle checkpoints, elevated cancer susceptibility, radiosensitivity, genomic instability, and variable immunodeficiency [28]. AT cells are able to repair most of the DSBs present in the DNA, however, a residual number of DSBs remain unrepaired. The basis underlying this defect is unknown [29, 30].

Mre11 and Nbs1 (both part of the MRN complex) deficiencies lead to an ATM-deficient phenotype, with radiosensitivity and defects in cell cycle checkpoints [31]. Mutations in the human Mre11 gene are responsible for an Ataxia Telangiectasia (AT)-like disorder (ATLD) which clinically resembles AT [32]. Deficiency or mutation in Nbs1 leads to Nijmegen breakage syndrome (NBS), with microencephaly, chromosomal instability, radiosensitivity and cancer susceptibility [28].

Measurements of DNA double-strand breaks

The FAR assay

FAR stands for Fraction of Activity Released, a method based on measurements of the fraction of DNA that is small enough (about 10 Mbp) to enter a gel. In this assay, the cells are first harvested after treatment, molded into an agarose plug, and lysed. The plugs are then subjected to agarose gel electrophoresis. If the DNA contains two DSBs sufficiently close, it will enter the gel, whereas larger fragments remain in the plug. The relative amount of DNA entering the gel is proportional to the number of DSBs. The migrating DNA is then compared with the DNA in the

plug (figure 2). This can be done by staining the DNA with, for example, ethidium bromide, and scanning the gel with an image analyzer. The FAR assay, as compared with for instance neutral filter elution, has the advantage of avoiding shearing forces that may damage the DNA by molding the cells into agarose.

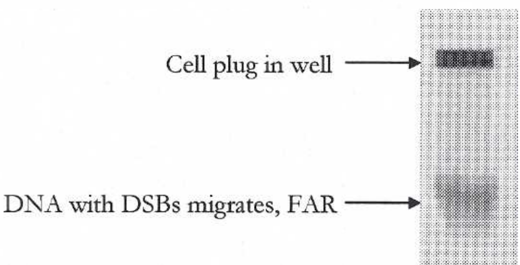

Figure 2*. The FAR value obtained from comparing the DNA in the lane with the non-migrating DNA in the plug (left) is compared with the FAR value obtained in untreated cells (right).*

Using constant field gel electrophoresis, only DNA fragments less than 20 kbp in size can be separated, since larger fragments migrate at the same velocity [33]. With pulsed field gel electrophoresis, larger DNA fragments can be separated than with standard constant field gel electrophoresis. By alternating the direction of the electrical fields at regular intervals, the DNA is forced to constantly reorient in new directions, resulting in better size separation [34]. When examining the total amount of DNA released from the plug, pulsed field gel electrophoresis gives the same results as constant field gel electrophoresis [35, 36].

A modified version of constant field gel electrophoresis, graded field gel electrophoresis was presented [37] in 1995, allowing separation of DNA fragments according to molecular weight at X-ray doses less than 100 Gy. Observations that large fragments migrate only at low voltages, whereas small fragments can also migrate at higher voltages [38] lead to development of the method of stepwise increase of the field strength [37]. Larger fragments are trapped in the agarose, while smaller migrate. The more the field strength is increased, the smaller the fragments that are trapped. Thus, a stepwise increase of the field strength results in separation of the DNA fragments released from the plug. For DSB-inducing agents that induce damage randomly, both constant field gel electrophoresis and pulsed field electrophoresis can be used.

A recent report shows that the FAR assay overestimates the number of X-ray-induced DSBs, since other types of DNA damage are converted to strand breaks during high-temperature lysis [39]. In accordance with this report, to avoid heat-labile sites being turned in to DSBs, lysis should be performed at temperatures below 20°C.

Neutral elution

Neutral elution is a technique based on the rate at which DNA DSBs elute through a membrane filter under non-denaturing conditions [40]. Cells are collected on filters in columns and lysed under conditions that preserve the double-stranded nature of the DNA. The DNA in the cells is then eluted slowly, and the fraction of DNA eluted from the filter can be used to estimate the amount of DSBs. If more DSBs are introduced, smaller DNA fragments are created, and a larger amount of DNA will be eluted, whereas the chromosomal DNA is retained on the filter and cannot pass through it. If the DNA is labeled before lysis and elution, the amount of DNA that is eluted, compared to the DNA that is retained on the filter, can be estimated. The principle behind this method is not quit clear, and calibration is needed in order to measure an exact number of DSBs [41]. The amount of total strand breaks (TSBs) (SSBs+DSBs) can also be measured with this method, using alkaline conditions that separate the DNA strands, thus allowing for detection of SSBs and DSBs, instead of neutral conditions [42].

Premature chromosome condensation (PCC)

Premature chromosome condensation (PCC) is a method that can be used to examine chromosome breaks, that may result from DNA damage [43]. Many investigators have found a linear dose-response relationship for the induction of chromosome breaks immediately after exposure to radiation [44, 45]. After introduction of DNA damage, chromosome condensation is induced, and the resulting chromosome fragments are counted.

Chromosome analysis can only be done in the mitotic stage of the cell cycle when chromosomes are condensed, which makes it very difficult to obtain enough mitotic chromosomes for analysis [46]. With PCC, chromosome analysis can be preformed at any stage of the cell cycle, greatly increasing the number of cells that can be analyzed [47]. Cells are treated with a mitosis-promoting factor to promote premature condensation. The chromosomes then condense, the cells are collected using centrifugation, and lysed. The cells are subsequently fixed, mounted on microscopic slides and air dried. The chromosomes can then be colored, and the number of chromosome fragments counted.

Histone 2AX phosphorylation (γH2AX)

H2AX is a histone 2 variant that is phosphorylated in the proximity of DSBs by the phosphatidylinositol-3-kinase (PI3K)-like kinases ATM, ATR and DNA-PK [48]. Within minutes of the induction of DSBs, foci of phosphorylated H2AX (γH2AX) are formed over a 2 Mbp area flanking the DSB, probably serving as an amplification of the DSB signal [16]. The number of γH2AX increases until a plateau is reached after 10-30 minutes of IR damage, and about 2,000 H2AX molecules are phosphorylated for each DSB [49]. A primary fluorophore-linked antibody against phosphorylated H2AX can be visualized with a fluorescence

microscope, and the number of foci correlates with the number of DSBs. It is possible to detect one single γH2AX focus within the nucleus, probably reflecting one single DSB, making this the most sensitive method for detecting DSBs [49, 50].

Neutral sucrose gradient centrifugation

Neutral sucrose gradient sedimentation [51] is a method in which DNA fragments are separated by centrifugation through a sucrose density gradient according to their molecular mass. A centrifuge tube containing a sucrose gradient solution with the higher concentration (greater density) at the bottom is prepared. Cells are carefully lysed on top of the sucrose gradient in a thin layer, since large DNA fragments are easily broken during pipetting, owing to shearing forces [52, 53]. The sucrose gradients are then spun in an ultracentrifuge and the DNA from the cells sediment in the sucrose gradient. If all the molecules are of the same shape (e.g. all are linear double-stranded DNA), the larger DNA fragments will migrate further into the gradient. Fractions are then collected, and can be counted in a liquid scintillation counter if the DNA has been prelabeled. The sucrose gradient can be calibrated with DNA fragments of a known size so that the molecular weight of the sample can be determined. If both the size standards and the DNA are radio labeled, they can be detected in very low concentrations, and the molecular weight of the DNA fragment of interest can be measured.

The Comet assay

The comet assay was first described in 1984 [54], and is a gel electrophoresis method used for visualizing and measuring DNA strand breaks in individual cells. The comet assay is simple and sensitive, and allows measurements of DNA damage in individual cells, which makes it suitable for patient material.

The proposed mechanism is that DNA is organized in large supercoils, which can be relaxed by strand breaks in the DNA, and thus stretched by the electrophoresis [54]. With different protocols, it is possible to measure both SSBs and DSBs, as well as damage to bases and DNA inter-strand crosslinks [55, 56]. Specific DNA sequences can also be labeled, with antibodies or fluorescence *in situ* hybridization [57].

In the comet assay, a single-cell suspension is mixed with agarose and mounted on a microscopic slide. After the agarose gel has solidified, the cells are immersed in lysis solution to lyse the cell membranes and remove lipids and proteins. When DSBs are to be measured, neutral lysis solution is used. To measure the total amount of strand breaks (TSBs) (SSBs+DSBs), alkaline lysis solution is used. After lysis, the cells are put in unwinding solution to unwind the DNA strands and allow DNA migration during agarose gel electrophoresis. The unwinding solution is neutral for measurement of DSBs, and alkaline for measurement of TSBs. The cells are subsequently subjected to electrophoresis, the DNA stained with a fluorescent dye, and viewed with fluorescence microscopy. If the DNA contains strand breaks, it can migrate out of the cell nucleus during gel

electrophoresis. The DNA in the cell will then resemble a comet, with the undamaged DNA in the head (nucleus), and the DNA with strand breaks in the tail. The relative fluorescence intensity of the head and tail can then be compared (normally expressed as percentage of DNA in tail), to assess the extent of DNA damage. To measure SSBs, two experiments must be performed; one measuring the TSBs and one measuring the DSBs, and the amount of DSBs is then subtracted from the total amount of strand breaks.

DNA unwinding assay

DNA strand breaks can be analyzed by using the DNA unwinding assay [58]. Alkaline solution is gently added to the cell suspension prepared for strand break analysis. The unwinding procedure is performed on ice, using weak alkaline solution to obtain partial DNA strand separation, in darkness, for 30 min, and the more strand breaks the DNA contains, the more DNA will unwind, and the more DNA will become single stranded [41]. After neutralization, the DNA is fragmented and single and double stranded DNA is separated on hydroxyl apatite columns, using different buffer strength. Single stranded DNA binds more tightly to hydroxyl apatite columns than do double stranded DNA, and with increasing buffer strength, ssDNA will elute more slowly than dsDNA. If the DNA is labeled, the activity of ^{14}C in each sample can be measured using, for example, liquid scintillation. Alternately, a dye can be used that fluoresce differently when bound to single stranded or double stranded DNA.

Repair of DNA damage

Our cells have several repair systems to detect and repair different kinds of DNA damage, with specific enzymes capable of recognizing certain forms of DNA damage.

Excision repair is used to repair a wide range of alterations to DNA [4]. There are three types of excision repair: base excision repair (BER), nucleotide excision repair (NER) and mismatch repair (MMR). In all of them, the damaged base is recognized and removed, and the gap is filled using the undamaged complementary DNA strand as a template.

BER repairs abasic sites (AP sites) and removes damaged and modified bases such as uracil. BER also removes pyrimidine dimers as well as bases damaged by IR [4]. The damaged base is recognized by specific DNA glycosylases and removed. An AP site is then formed and recognized by AP endonuclease, which cleaves the DNA strand. The abasic deoxyribose is removed by a phosphodiesterase, the gap is filled by DNA polymerase and ligated by DNA ligase.

NER functions similarly to BER, although this system removes longer parts of DNA. Nucleases cleave the DNA strand on both sides of the damage, a helicase unwinds the strands, and the oligonucleotide with the damaged bases is removed

[4]. The gap is filled by DNA polymerase and ligated by DNA ligase. There is also an alternative form of NER, called transcription-coupled repair, which specifically repairs damage in genes that are being transcribed. If DNA damage is present in a gene that is being transcribed, it blocks transcription. Transcription-coupled repair is similar to NER, however the proteins involved in the recognition recognize the stalled polymerase instead of the actual DNA damage.

MMR recognizes bases that are mismatched during DNA replication [4]. Enzymes involved in this repair system recognize and remove the mismatched bases. These enzymes are also able to distinguish the parental strand from the newly synthesized DNA strand, since the parental DNA strand is methylated. The parental strand is thus left untouched, whereas the newly synthesized strand with the mismatch is repaired. In *E.coli*, the MutS protein recognizes the mismatched base, and forms a complex with the MutH and MutL proteins. MutH cleaves the DNA strand with the mismatched base, and MutL and MutS excise the DNA between the strand break and the mismatched base, together with an exonuclease and a helicase. DNA polymerase and DNA ligase then fill in and ligate the gap.

Mammalian cells have homologues to these proteins, but the mechanism differs from the one in *E. coli*. The strand-specificity of the MMR proteins is not determined by DNA methylation. Instead the parental and new DNA strand appear to be distinguished by SSBs present in the newly replicated DNA, or the association of the MutS and MutL homologues with the replication machinery. The MutL and MutS homologues bind to the mismatched base and direct the excision of the DNA between an SSB and the mismatched DNA base.

Repair of DNA double-strand breaks

The two major pathways involved in the repair of DSBs are non-homologous end-joining (NHEJ) and homologous recombination (HR). These two mechanisms differ primarily in that HR requires a homologous DNA template, while NHEJ puts any two ends together (figure 3). NHEJ is dominant in the G0 and G1 phases of the cell cycle when a sister chromatid is not available, and HR is dominant during DNA replication when appropriate homologous templates are available [6]. In general, inactivating mutations in genes involved in NHEJ lead to greater radiation hypersensitivity than mutations in HR genes, suggesting that NHEJ is the dominant pathway for removing IR-induced DSBs [6]. HR is the main pathway involved in yeast, whereas NHEJ is suggested to be the dominant DSB repair pathway in mammalian cells [59]. This could be attributable to the fact that only a small part of the mammalian genome codes for genes and regulatory elements, and that the cells are able to cope with small sequence changes [6], and that most parts of the yeast genome contain coding DNA, making HR dominant to ensure genomic stability [60]. Genomic instability in animals with defects in both pathways states that they are both important in higher eukaryotes [61]. It is not entirely clear why the cell chooses one or the other pathway to repair a DSB and there has been a great deal of speculations regarding this question. It is likely that the balance of NHEJ and HR depends on the type and location of the lesion, among other factors [6], and also on where in the cell cycle the DSB is introduced. The human protein RAD52, involved

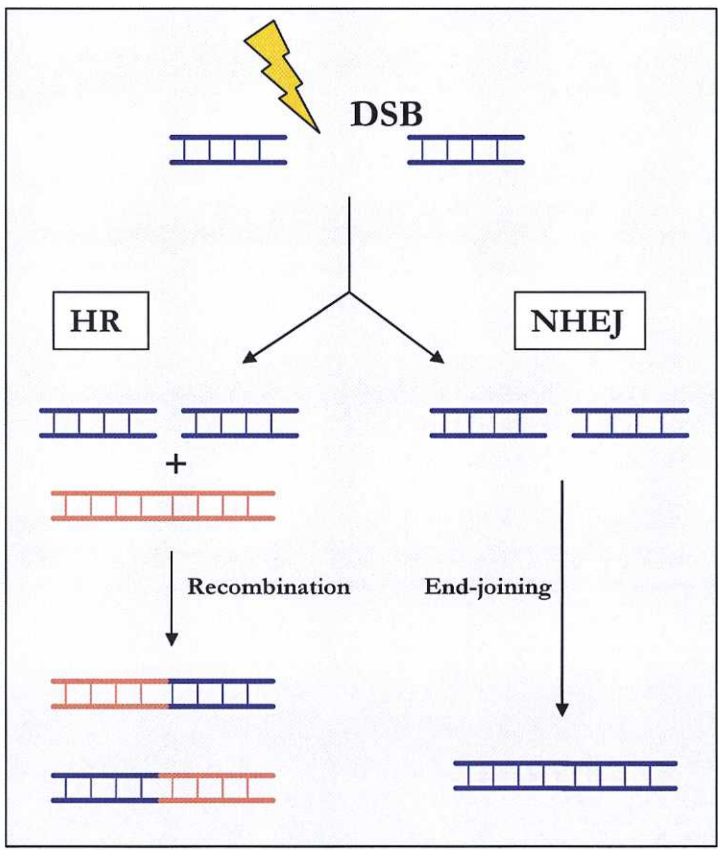

Figure 3. *HR versus NHEJ. HR requires a homologous DNA template to repair a DSB and NHEJ religates the broken DNA ends after processing of damaged nucleotides. NHEJ is error-prone, since the ends of a DSB are put together without concern for missing bases or nucleotides, both of which are taken into account in HR (picture adapted from [4]).*

in HR, has been shown to bind preferentially to DNA DSBs. Thus, RAD52 may compete for DNA ends with the DNA-binding protein Ku, involved in NHEJ, which could possibly determine whether HR or NHEJ is employed [62]. One report states that proteins involved in NHEJ interact with unprocessed ends, and when the ends have been processed, proteins involved in HR then interact with the ends [63]. There may also be proteins involved in the regulation of choice between these pathways.

NHEJ is error-prone and mutagenic. This process is unreliable when restoring the original DNA sequence, as opposed to HR, and NHEJ itself can introduce sequence changes during repair [6].

Homologous Recombination, HR

HR is the prevalent mechanism in yeast, is important for the generation of genetic diversity, and also ensures accurate chromosome segregation during meiosis [64]. It is a process for genetic exchange between DNA sequences that share homology [65]. It has been suggested that the primary function of HR is maintenance of functional replication [66], and that HR repairs DSBs that arise at broken replication forks or on DSBs occurring in segments of DNA that have already been replicated [7]. The cell also uses the HR mechanism to repair exogenous DSBs [6]. The HR pathway has three general steps: strand invasion, branch migration and Holliday Junction (HJ) formation [15]. The molecular mechanisms of HR are not entirely clear and they may also vary between different types of cells [4].

In *E. coli*, the RecA protein promotes exchange between homologous DNA sequences [4]. First, RecA binds to single-stranded DNA (ssDNA). RecA then binds to the homologous DNA molecule, and a complex is thus formed between the DNA strands, which can then base pair with each other. The exchange between the homologous DNA strands is catalyzed by RecA, and a HJ is formed (figure 3).

RAD51 is the RecA-related protein in yeast, and is required for both recombination and DNA DSB repair. In humans and other eukaryotes, RAD51-related proteins have been identified. This indicates that RecA-related proteins are important for HR in both prokaryote and eukaryote cells.

When the HJ has formed, the RuvA, B and C *E. coli* proteins become involved in the process. RuvA recognizes the HJ and recruits RuvB, which drives the branch migration and varies the positions where the crossed DNA strands are cleaved and rejoined. Subsequently, RuvC cleaves the crossed strands and the HJ is resolved. The cleaved strands are ligated, the process is complete and two recombinant molecules have been formed.

In yeast, once a DSB is encountered, the strands are resected to make ssDNA ends (figure 3) [67]. This step probably involves the MRX complex, composed of Mre11, RAD50 and Xrs2 (Nbs1 in humans) [64, 67]. RAD51 binds to the ssDNA ends, with other proteins involved in this process as well, including RPA, RAD52 and RAD54. RAD51 also binds to an undamaged, homologous DNA molecule and catalyzes strand exchange between them: the damaged DNA molecule invades the undamaged DNA, and one strand is displaced, forming a D-loop and a HJ (figure 3). DNA polymerase then comes into the picture and extends the damaged DNA strand, using the homologous DNA as a template, and the ends are ligated by DNA ligase I. Finally, the HJ is resolved by cleavage and ligation, leaving two intact DNA molecules.

HR normally repairs DNA DSBs correctly and without mutations. However, when there are direct repeats adjacent to the DSB, the DSB can be repaired by a specific HR pathway called single-strand-annealing (SSA) (described below, see figure 3). This may lead to loss of DNA, since the DNA between the homologous sequences is deleted [68].

There are mammalian homologues for all the known HR factors in yeast, but the details of HR in higher eukaryotes are much more complex. There is also a report that links the breast cancer susceptibility proteins BRCA1 and BRCA2 to HR

[69]. If BRCA1 and BRCA2 function is lost, the efficiency of correct HR is markedly reduced [70, 71].

In mammalian cells, there are two pathways involved in HR, either RAD51-dependent strand invasion, or a RAD51-independent pathway known as single-strand annealing (SSA), which is used if the DNA adjacent to the DSB has repetitive sequences (figure 4) [72].

First, as in yeast, the DNA ends at the DSB are resected to form 3'-overhangs of ssDNA. The MRN complex (Mre11, RAD50 and Nbs1) could play a role in this step, but no evidence has yet confirmed this [65].

If the DNA adjacent to the DSB has repetitive sequences, repair via SSA can occur. In this case, RAD52 and replication protein A (RPA) bind to the ssDNA [62]. The mechanism for this is still unknown. The ssDNA base pairs with homologous DNA sequences and subsequently, when they are annealed, the DNA between the repetitive sequences flip out on each side. The ERCC1/XPF endonuclease, usually involved in NER, removes the excess DNA, and remaining gaps are ligated. SSA is error-prone, since the DNA between the homologous sequences is deleted [72]. Owing to the fact that mammalian genomes contain large portions of repetitive sequences, SSA is likely to be used frequently in the DSB repair [65].

If there are no repetitive sequences available adjacent to the DSB, RAD51-dependent strand invasion occurs. RAD52 and RPA bind to the ssDNA ends at the break. RAD52 may recruit RAD51 to the site of the break, and RPA is removed. RAD51 catalyses the search for homologous sequences, strand pairing and also exchanges between the strands [65]. Strand invasion is stimulated by RAD54 [73], which opens the homologous template and supports invasion of the ssDNA. When a homologous sequence is found, the invading strand anneals to that sequence, a D-loop and a HJ are created and DNA synthesis is initiated by a DNA polymerase [65].

The next step is either synthesis-dependent strand annealing (SDSA) or a DSB-repair model including a double HJ [74], depending on whether the second DNA end invades the intact duplex or not (figure 4).

If SDSA is employed, the invading strand is displaced after DNA synthesis beyond the break site. This strand rejoins with the second DNA end and the DSB is repaired by SDSA. The homologous dsDNA strand is left intact and gene conversion (were two genes on homologous DNA have switched places) occurs.

If the second DNA end invades the homologous DNA strands as well, a double HJ is created. In *E. coli*, these steps are catalyzed by the RUVABC-complex, as mentioned above. The eukaryotic proteins responsible have not been identified, but RAD51C, XRCC3 and RAD51B may be involved [72].

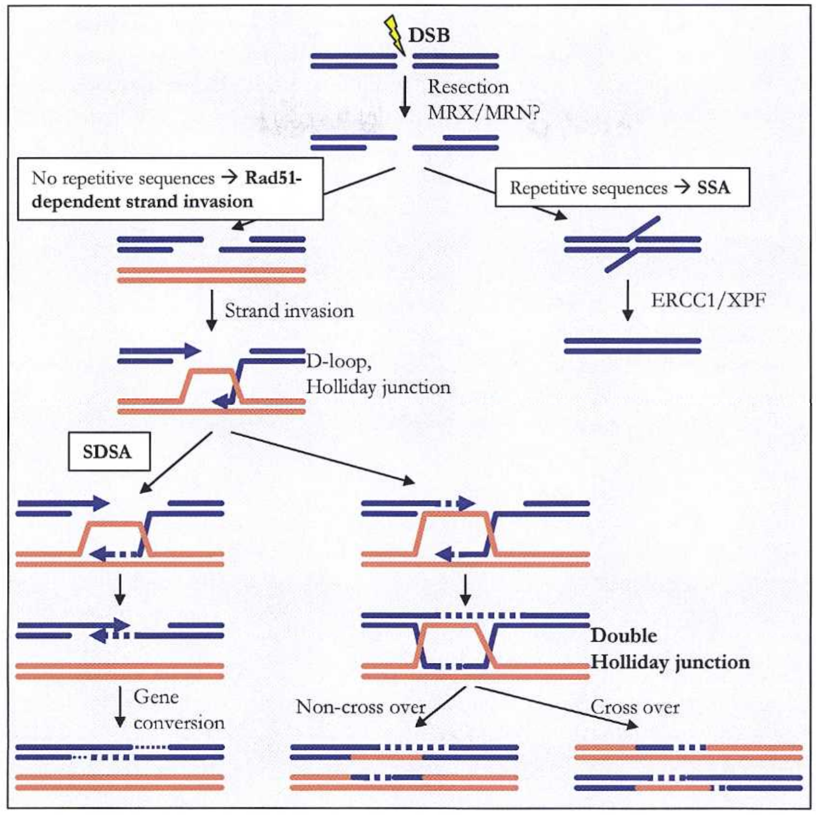

Figure 4. *Homologous recombination. The first step is resection of the DNA ends at the DSB. MRN could be involved, but no evidence has confirmed this. Single-strand annealing (SSA) is employed if the DNA adjacent to the DSB has repetitive sequences [72]. RAD51-dependent strand invasion occurs if there are no repetitive sequences available adjacent to the DSB. The homologous template is opened and invasion of the ssDNA takes place. When a homologous sequence is found, the invading strand anneals to that sequence, a D-loop and a Holliday Junction (HJ) are created, and DNA synthesis is initiated by a DNA polymerase. The next step is either synthesis-dependent strand annealing (SDSA) or a DSB-repair model including a double HJ, depending on whether the second DNA end invades the intact duplex or not. If SDSA is employed, the invading strand is displaced after DNA synthesis beyond the break-site. This strand rejoins with the second DNA end and the DSB is repaired by SDSA. The homologous dsDNA strand is left intact and gene conversion occurs. If the second DNA end invades the homologous DNA strands as well, a double HJ is created.*

Defects associated with homologous recombination

Genetic defects in HR can lead to both impaired DNA replication and enhanced IR sensitivity [75]. Cells deficient in HR are not impaired in repairing IR-induced DSBs,

and they are also less sensitive to IR than cells deficient in NHEJ [76]. Defects in HR may also result in chromosome instabilities [77], and increased mutation [78]. Patients with defective HR proteins run a higher risk of developing cancers [79]. Knockouts in mice of many of the HR proteins (for instance RAD51, RAD51B, RAD51D, RAD50, Mre11, Nbs1) are lethal, which demonstrates that HR is vital for development [65]. This is probably because HR is required to resolve the DSBs that occur during DNA replication, making HR essential to DNA synthesis [80]. RAD52 knockout mice however, are healthy [81], indicating that RAD52 plays a less important role than some of the other proteins in HR.

Non-Homologous End-Joining, NHEJ

In mammalian cells, DSBs induced by IR are predominantly repaired by NHEJ. NHEJ is active in every phase of the cell cycle, including the S phase. This pathway requires several proteins that recognize and bind the DSBs, catalyze the synapsis of the broken ends, and then process and reseal the break [27, 82].
At least five proteins participate in mammalian NHEJ [83], they include the DNA-dependent protein kinase (DNA-PK), composed of the DNA-binding heterodimer Ku (Ku70 and Ku80) and the catalytic subunit DNA-PKcs. Other proteins involved are the DNA ligase IV/XRCC4 complex. It is also likely that additional NHEJ factors remain to be discovered [84, 85], since these five known proteins alone cannot promote efficient DNA end joining in vitro [86]. The same study reported that the MRN complex restored the end-joining activity to levels of crude nuclear extracts [86].

The mechanism of Non-Homologous End-Joining

When a DSB is introduced in the DNA, Ku binds to the DNA ends (figure 5A). Ku encircles DNA as a preformed ring, translocates inwards on the DNA and recruits DNA-PKcs to the damage site [87, 88]. One report indicates that DNA-PKcs is able to bring the DNA ends together [89]. The kinase activity of DNA-PKcs is activated upon binding to the free ends of the DSB (figure 5B) [88, 90-93]. At this step there is probably also a nuclease involved in trimming the ends before ligation. A tetramer of two DNA ligase IV-molecules and two XRCC4-molecules then binds to the damage site (figure 5C). DNA-PKcs is autophosphorylated, falls off, and the DSB is ligated by DNA ligase IV/XRCC4. The proteins involved in NHEJ assemble into complexes, where DNA-PK binds to chromatin, whereas the Lig4/XRCC4 complex interacts with a nuclear structure resistant to Dnase I [94]. The appearance of NHEJ protein complex formation correlates well with the appearance of γ-H2AX following DSB introduction and, similarly, decrease at the same rate following repair [94]. However, γ-H2AX does not seem to be required for DSB-dependent mobilisation of the NHEJ proteins, since NHEJ proteins were still recruited in the absence of H2AX phosphorylation [94].

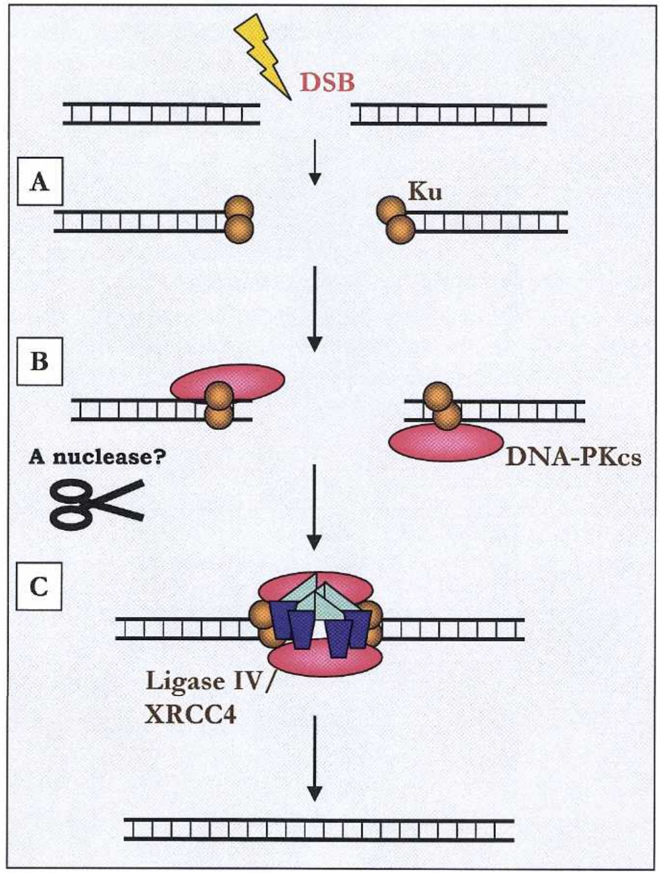

Figure 5. *A model of DSB repair by NHEJ. When a DSB is introduced in the DNA, Ku binds to the DNA ends (**A**). Ku encircles DNA as a preformed ring, translocates inwards on the DNA and recruits DNA-PKcs to the damage site. This brings the DNA ends together and DNA-PKcs is activated upon binding to the free ends of the DSB (**B**). At this step there is probably also a nuclease involved in trimming the ends before ligation. A tetramer of two DNA ligase IV-molecules and two XRCC4-molecules then bind to the damage site (**C**). DNA-PKcs is autophosphorylated, falls off, and the DSB is ligated by DNA ligase IV/XRCC4.*

DNA-PK

The DNA-PK is a serine/threonine protein kinase composed of a 465-kDa catalytic subunit (DNA-PKcs) [95], and a DNA-binding heterodimeric subunit of Ku70 and Ku86, with molecular weights of 70 and 86 kDa respectively [88, 96, 97]. The number of Ku molecules is far greater than the number of DNA-PKcs molecules in most cell types [98]. DNA-PK may be the first protein to bind to a newly generated

DSB, since it has a specificity for binding to, and is activated by, DSBs, because of its cellular abundance, and also since it constitutes the main end-binding activity in nuclear extracts from human cells [88, 99-101].

Ku

Ku binds specifically to DSBs [96, 97, 102] and encircles DNA as a preformed ring [87]. Ku does not appear to make contact with the DNA bases, and it seems only to make a few contacts with the sugar-phosphate backbone. Rather, it fits sterically to the contours of the backbone, and thus positions the DNA in the protein ring [87]. Consequently, Ku has no sequence specificity for DNA ends [87]. After binding to the DNA ends, Ku translocates inwards on the DNA strands, and allows DNA-PKcs to interact with a 10 base pair region of the terminal ends of the DNA strands, [92, 102, 103]. Ku specifically recruits DNA-PKcs via a 12-amino acid sequence that flips out from the Ku80 carboxy-terminus [104, 105]. If DNA-PKcs is not present, more Ku molecules are recruited to the DNA ends at low levels of DSBs [94]. Thus, one role for DNA-PKcs may be to limit the recruitment of Ku onto the DNA ends [106].

DNA-PKcs

DNA-PKcs is a member of the phosphatidylinositol-3-kinase (PI3-kinase)-like family. The kinase domain is found in the carboxyl terminal of the enzyme [98], which is also the part of DNA-PKcs that interacts with Ku. DNA-PKcs must bind to DNA ends to be activated, and the cooperative interaction within the DNA/Ku/DNA-PKcs complex result in the activation of the inherent kinase activity of DNA-PKcs [67, 107]. The kinase activity is required for efficient repair and radioresistance [108], but the underlying molecular mechanism of this activity is not understood [7]. Additional roles, beyond the involvement in NHEJ, have also been proposed for DNA-PKcs kinase activity, like regulation of apoptosis, and restart of DNA synthesis after DNA damage [109, 110], as well as telomere maintenance and prevention of chromosomal end fusion[111, 112].

DNA-PKcs and Ku are weakly associated when not bound to DNA ends [113], but both Ku and DNA-PKcs are required for DNA-PK to be active at physiological salt concentrations, so Ku has a role in stabilizing the binding of DNA-PKcs to DNA ends [88]. DNA-PKcs also plays an end bridging role [89].

There is evidence implying that the participation of DNA-PKcs may not be absolutely vital to the NHEJ pathway. In fact, when using a cell-free assay, Huang and Dynan made the discovery that this system was dependent on all five components of NHEJ, but as additional purified proteins were added to the system, the DNA-PKcs-dependence was decreased progressively [86]. The authors speculated about a model where DNA-PKcs acts early to regulate progression of the NHEJ pathway. Riballo et al. [11] presented evidence of a model where 90% of the IR-induced DSBs were repaired by NHEJ, where DNA-PKcs was not required for, but only facilitated the process, and where repair of a subset of DSBs requiring

some end-processing were dependent on ATM phosphorylation of Artemis to process the ends, and were also dependent on DNA-PKcs, Lig4, and H2AX, 53BP1, Mre11 and Nbs1.

DNA-PK activation

DNA-PK was first identified as the dominant DNA-activated protein kinase in extracts from mammalian cells [101]. The kinase activity of DNA-PK is specifically activated by dsDNA ends [88], and the activation involves interaction with the ssDNA ends exposed at a DSB [90]. DNA-PKcs probably also have separate binding sites for ssDNA and dsDNA [114].

SSBs have been shown to activate DNA-PK poorly [91, 101, 115]. DNA-PKcs interacts with ssDNA ends [90], but is not activated by DNA fragments only exposing one ssDNA end. Our work has shown that DNA-PK must interact with two ssDNA ends to be activated (paper I), that the bases at the terminal nucleotides are not required, and that DNA-PKcs also lacks strand polarity preference. However, interaction with the negative charges of the DNA backbone seemed to be important for the kinase activation of DNA-PKcs, although the interaction with one charged DNA strand proved to be sufficient for activation (paper I). DNA-PK recognized DSBs regardless of chemical complexity, including drug and radiation-induced strand breaks relevant for cancer treatment, as long as the DSB is freely exposed to DNA-PKcs (paper II). Our results then show that DNA-PK is activated by DNA ends containing a wide variety of modifications, indicating that DSBs that remain undetected by DNA-PK are rare (paper I and II).

Relevant DNA-PK phosphorylation targets

Up until this point, the only relevant physiological DNA-PK target is DNA-PK itself [116, 117]. DNA-PKcs have also been shown to phosphorylate Chk2 [118], and H2AX [18] in response to DSBs. Apart from these, no phosphorylation has been seen in vivo of targets that appear to be relevant in a DNA damage signaling and repair perspective.

DNA-PKcs autophosphorylation

Seven autophosphorylation sites have thus far been identified in DNA-PKcs [116, 119, 120]. Chan et al. demonstrated that DNA-PKcs is autophosphorylated in vivo at the highly conserved threonine 2609 in response to IR [120], and Chen et al. reported the same for serine 2056 [116]. Both these phosphorylation sites are important for DSB repair [116, 117, 120, 121], and mutation of the autophosphorylation sites leads to defects in IR-induced DSB repair and correlates in vitro with loss of kinase activity [122]. Autophosphorylation is also important for its dissociation from Ku [123], and possibly for deprotection of DNA ends as well, to make them accessible to subsequent gap-filling or ligation steps [106, 124].

Protein phosphatase 5 has been shown to dephosphorylate DNA-PKcs specifically, mainly at T2609 but also at S2056 [125].

A model of DNA-PKcs activation

A model of activation explaining the importance of ssDNA ends for activation of DNA-PKcs was proposed in [114], in which one ssDNA end enters a channel present in DNA-PKcs (figure 6A). However, this model does not explain why DNA-PKcs requires interaction with two ssDNA ends simultaneously. DNA-PKcs might require dimerization of two DNA-PKcs molecules, where both bind a DNA end (figure 6B) (proposed in [114]). This would allow DNA-PK to be fully activated until synapsis of the two DSB ends is complete. This model would also allow DNA-PKcs to interact with the negatively charged DNA backbone.

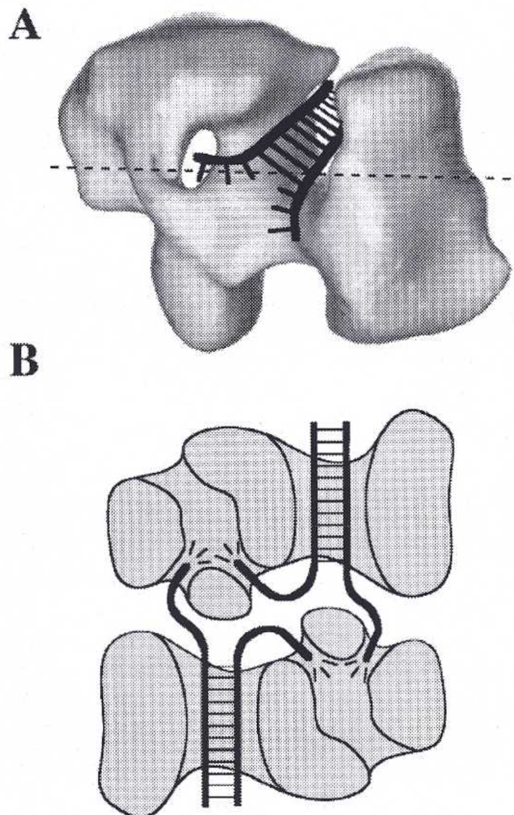

Figure 6. *A model for DNA-PKcs activation by two ssDNA ends.* ***A,*** *electron crystallography structure of DNA-PKcs with superimposed sketch of a bound DNA end [114]. DsDNA could potentially bind to an open channel in the molecule. Binding to ssDNA might occur once the ssDNA portions of the DSB reach the opening of an enclosed channel in DNA-PKcs [114]. The dashed line indicates a cross-sectional slice through the molecule used to produce the sketch shown in* B. ***B****, possible model for the DNA-PKcs activation by two ssDNA ends. Two DNA-PKcs molecules with DNA bound to them first form a complex. Kinase activation occurs simultaneously when two ssDNA ends from the two different dsDNA ends of a DSB are threaded into two openings of the enclosed cavity in each molecule.*

Processing of the DNA ends at a double-strand break

It is possible that the ssDNA ends at a DSB may need some processing before the break is sealed. Proteins that have been implicated in this step include Artemis [11, 126, 127] and the MRN complex [128].

Artemis is a 5'-3' exonuclease that has been shown to be regulated by phosphorylation of DNA-PK, thus gaining 5'-3' endonuclease activity and hairpin-opening activity [126], and might be specifically required for DSB repair [11, 126, 127]. DNA-PK-deficient cells are unable to cleave DNA hairpin intermediates formed during V(D)J recombination, which indicates that DNA-PK activates Artemis' ability to cleave hairpins in vivo [126]. Owing to this interaction during V(D)J recombination, it has been proposed that DNA PK may also recruit Artemis to the break site during DSB repair by NHEJ [126]. Artemis deficiency also leads to radiosensitivity, a consequence well known from defects in the NHEJ pathway. However, the actual involvement of Artemis in NHEJ has not yet been established.

Another candidate implicated in trimming the ends at a DSB before repair is the MRN complex, composed of Mre11, RAD50, and Nbs1 [86]. Like Artemis, the MRN complex also has exonucleolytic activity. The RAD50 protein in this complex was identified among proteins participating in HR, but may play an additional role in NHEJ. If the ssDNA ends of the DSB had extensions of RAD50, it would be easier for the two ends to find each other, since the search radius of the DNA ends would be inceased [65]. The exact role of MRN in NHEJ is still unclear, but it seems to be involved in signaling of DSBs [13].

Other candidates suggested for end processing include human terminal deoxytransferase (TdT), which colocalizes with Ku at DNA damage sites, and adds nucleotides to 3'-DNA ends without requiring a template [129, 130], the Werner syndrome protein (WRN) which interacts with DNA-PK and is phosphorylated in vitro by DNA-PKcs [131] and mammalian polynucleotide kinase (PNK), which has both 5' DNA kinase and 3' DNA phosphatase activities [132].

Resealing the double-strand break

The XRCC4 (X-ray cross-complementary gene 4)/DNA ligase 4 complex is responsible for resealing the DSB specifically during the NHEJ process [133, 134]. DNA-PK is an in vitro kinase for Lig4, and might influence the stability of Lig4 by phosphorylation [135]. Both DNA-PK subunits are required for recruitment of the XRCC4/Lig4-complex [136], and DNA-PKcs seems to be a physical necessity for this recruitment [94].

V(D)J recombination

HR can occur at any extensive region of sequence homology. In addition there is also a site-specific recombination process, which also occurs between shorter homologous DNA regions. Proteins involved in this process recognize the specific target sequences in the DNA, as opposed to complementary base pairing as in HR.

One example of site-specific recombination is V(D)J recombination, which is linked to NHEJ. This is a strictly regulated process necessary for development of antibodies in the immune system of vertebrates, and the NHEJ proteins are involved in rejoining the DSBs specifically introduced by this pathway. The antibodies recognize substances foreign to the body and provide protection from a tremendous number of infectious agents [4]. The key to the diversity is that the antibodies are encoded by different gene segments that are recombined in many ways [137]. The antibody consists of a light and a heavy polypeptide chain, each of which, in turn, includes both constant and variable regions. The variable regions of both the light and the heavy chains can be recombined in various ways. Three genes, V, J and C, encode the light chain. In mice, V contains approximately 250 regions, J contains four, and C only one constant region. These regions are combined in the chain, with one single region of each type, resulting in over a thousand different possible constellations. The heavy chain has a fourth region as well, D, in mice with 12 regions, 500 V regions, four J regions and the constant C region. This gives even more combination possibilities, and a very effective immune system. In addition to this, the NHEJ process that links the regions together is error-prone, resulting in insertions or deletions of nucleotides. This leads to frameshifts, and thus the antibody diversity is very large. Recombination between these V, D and J regions is mediated by the proteins Rag1 and Rag2 (recombination-activating genes), specifically expressed in lymphocytes. Each region is flanked by specific recombination signal (RS) sequences adjacent to gene coding sequences. The Rag proteins recognize the RS sequences, introduce DSBs between the RS and the coding sequences, and the coding and the signal ends are rejoined by non-homologous end-joining [11]. During cleavage of the gene segments, hairpins are generated at the ends of the coding regions. These hairpins are cleaved by a protein complex consisting of DNA-PKcs and Artemis [126]. Artemis associates with and is phosphorylated by the DNA-PKcs, and acquires endonucleolytic activity on 5' and 3' overhangs, and hairpins [126]. The NHEJ system subsequently rejoins the breaks, and defects in this pathway result in an inability to complete V(D)J recombination, with a consequent immunodeficiency.

Defects in non-homologous end-joining

Defects in NHEJ are generally associated with genetic instability, inefficient repair of DSBs, extreme sensitivity to IR and severe combined immunodeficiency (SCID), due to the involvement of the NHEJ proteins in V(D)J recombination.

SCID is a rare inherited disorder that may result from defects in at least 15 different proteins required for V(D)J recombination or signal transduction [138], for instance Rag1 and 2, and DNA-PKcs. Mutations in DNA-PKcs lead to SCID in horses and dogs[139-141], however no mutations in human DNA-PKcs have been found. A variant of human SCID, radiosensitive SCID (RS-SCID) results from Artemis deficiency [127, 142]. Like cells lacking DNA-PKcs, cells deficient in Artemis are also radiosensitive, but it is not clear why [143], since Artemis deficient cells still repair a majority of their DSBs efficiently [127].

DNA-PKcs deficiency does not give additional defects other than immunodeficiency, whereas defects in Ku and Lig4 also lead to growth retardation, neurological defects, premature cell senescence, and a general susceptibility to cancer development [144, 145]. In contrast, DNA-PKcs knockout mice develop cancer only from the immune system, owing to the V(D)J recombination defect [146, 147]. This shows that DNA-PKcs is not required for normal cell function in mouse cells, in contrast to Ku and Lig4. Knockout of XRCC4/Lig4 causes embryonic mortality, massive apoptosis of newly differentiated neurons, which is also seen at lower levels in Ku-deficient mice, in addition to immunodeficiency [11].

In addition to these defects, defects in ATM [28] have also been shown to result in defects in NHEJ. However, these symptoms are probably not due to repair defects, but rather to defects in the signaling response to DSBs.

The present investigation

Overall Aim

The overall aim of the present investigation was to take a closer look at the repair of DSBs in human cells, and the involvement of the DNA-dependent protein kinase (DNA-PK) in this process.

Aim of paper I

To study the activation of DNA-PK by chemically modified ssDNA ends.

Aim of paper II

To determine whether or not DNA-PK could recognize DNA strand breaks generated by agents used in cancer treatment.

Aim of paper III

To characterize the cleavage of calicheamicin γ1, in order to find ways to improve cancer treatment and DSB studies.

Aim of paper IV

To determine whether SU11752 is a selective DNA-PK inhibitor.

DNA-PK kinase assay (papers I, II, IV)

The activation of DNA-PKcs was monitored by the kinase assay, which measures DNA-PKcs kinase activation by allowing DNA-PKcs to phosphorylate a peptide. DNA-PK was mixed with substrate peptide in kinase buffer containing [^{32}P] ATP, on ice. DNA was added and the reaction was incubated at 37°C for 10-12 minutes. During incubation, DNA-PKcs is activated by the DNA, and subsequently phosphorylates the substrate peptide with [^{32}P] ATP. The reaction was stopped by transferring it back on ice, acidified by adding 50% acetic acid, and then transferred to phosphocellulose filter. The filter was washed in 15% acetic acid to remove excess [^{32}P] ATP, and the amount of radioactivity (phosphorylated peptide) bound to the filter was measured by scintillation counting. The peptide contains three lysines that allow the peptide to bind tightly to the negatively charged phosphocellulose filter under acidic conditions, since the amino group in the lysine is protonated and thus positively charged at acidic pH. The radioactivity retained on the filters obtained in the absence of added DNA or enzyme was defined as background and subtracted from each value obtained in the presence of DNA.

In paper IV, the kinase assay was used to examine the mode of inhibition of DNA-PK by the inhibitor SU11752. The assay was performed as described above, with various concentrations of ATP, and fixed concentrations of inhibitor added last. The results were plotted in a Lineweaver-Burk plot, with 1/substrate concentration (ATP) on the x-axis, and 1/kinase activity on the y-axis. The point of interception on the y-axis shows the enzymatic activity at infinite ATP concentration. If all ATP titrations at fixed SU11752 concentrations intercepts at the same point, this indicates that infinite ATP concentration can remove the inhibitory effect of SU11752, and that SU11752 is a competitive inhibitor to the ATP-site in DNA-PK.

Electromobility shift assay (EMSA) (papers I, II)

The binding of DNA fragments to DNA-PK was monitored by the electromobility shift assay, where radioactively labelled DNA was mixed with increasing amounts of unlabeled competitor DNA. DNA-PKcs (paper I) or Ku (paper II) was added last. After 10 min incubation at room temperature, the binding of DNA-PKcs or Ku to the DNA fragments was monitored by polyacrylamid gel electrophoresis. The gels were dried on Whatman 3MM paper and subjected to autoradiography, and the difference in migration between DNA fragments with or without DNA-PK bound to them could be monitored.

Purification of Damaged Plasmid DNA (paper II)

In paper II, we studied the activation of DNA-PK by supercoiled (undamaged), nicked (containing one SSB) and linear (at least one DSB) plasmid DNA. Cleaved plasmid DNA was resolved on agarose gels at 16 V/cm. To better resolve linear and nicked plasmid DNA, we also employed a high-voltage separation technique [148]. At 80 V/cm, nicked plasmid DNA is specifically trapped in agarose gels and cannot migrate, resulting in effective separation from linear and supercoiled plasmid DNA. To minimize heat formation at this high voltage, the agarose gels were run in a vertical gel apparatus designed for SDS-PAGE with ice-cold running buffer. The DNA was visualized using ethidium bromide staining under long-wave UV light, and bands corresponding to linear or nicked plasmid DNA were excised from the gel and electroeluted.

The gel purification was repeated one to three times until the contaminating linear plasmid was below 0.1% in preparations of nicked plasmid DNA. However, a small amount of linear plasmid DNA (0.1%) was always present in the nicked plasmid DNA preparations even after five rounds of gel purification. We concluded that the linear DNA was probably generated during the purification procedure. Experiments in which supercoiled plasmid DNA was mixed with 0.1% linear plasmid DNA showed that this level of contaminating linear plasmid DNA did not significantly activate DNA-PK within the DNA concentration range used in paper II.

After the last electroelution, plasmid DNA was extracted twice with phenol/chloroform, ethanol-precipitated and dialyzed extensively with TE buffer. Bands were visualized under long wave UV light and quantified with a video-based imaging system (Chemi Doc (Bio-Rad)), using Quantity One 4.2 software.

The FAR (fraction of activity released) assay (papers III, IV)

The FAR assay was used to measure the amount of DSBs (paper III), and also rejoining of DSBs (paper IV). Cells were grown in monolayers and harvested after treatment with DSB-inducing agents. The cells were suspended in 1xPBS, mixed with agarose and transferred to a plug mold. The cells in the plug were lysed, and then subjected to constant field agarose gel electrophoresis for 17 h. The gels were stained with ethidium bromide and analysed by a fluorescent scanner. The gel was cut in lanes and wells, the activity of ^{14}C in each sample was measured in a liquid scintillation counter, and the fraction of activity released (FAR) from the well into the lane was calculated (paper III). Alternately, the FAR value was quantified using the Image Quant software (paper IV), after ethidium bromide staining.

In the case of the rejoining experiment (paper IV), treated cells were washed twice with ice-cold PBS, pre-warmed medium was added, and the monolayers incubated for different time at 37°C, before analysis with the FAR assay.

The comet assay (paper III)

The comet assay was used in paper III to measure the amount and distribution of strand breaks in cells subjected to the DSB-inducing drug caliceamicin. A microscopic slide was layered with 5,000–10,000 pre-treated cells mixed with low-melting point agarose, held at 37°C. The agarosecell suspension was spread into a thin layer on microscopic slides pre-treated with a small amount of agarose, and air dried. The slides were left on a chilled plate for 5 min, the cells were then lysed for 1 h, the DNA denatured with alkaline treatment, and thereafter rinsed for 1 h. Finally, electrophoresis was run at 0.8 V/cm for 15 min. The slides were then neutralized, rinsed in deionized water, and air dried. The slides were subsequently fixed in methanol, stained with ethidium bromide for 5 min, and rinsed briefly in TAE buffer. Analysis of DNA that migrated from the nucleus, the tail moment, was done using a fluorescence microscope.

H2AX phosphorylation (paper III, IV)

H2AX is phosphorylated by ATM in response to DSBs [17, 19]. We examined the cellular response to DSBs induced by calicheamicin in paper III, by measuring H2AX phosphorylation. Monolayers with human fibroblasts were harvested after treatment with calicheamicin, and lysed. The histones were acid extracted, separated on SDS-PAGE, and transferred to PVDF-membrane. The membranes were incubated with an antibody specific for the phosphorylated form of H2AX (γ-H2AX), and after incubation with secondary antibody, the membrane was developed with a Dura ECL kit and analyzed on a digital camera based system. In paper IV, γ-H2AX was used to examine the inhibition of ATM by SU11752.

Paper I

Previous studies had not clearly shown the specific DNA structure required for DNA-PK-activation, until Hammarsten et al. conducted a systematic study where it was concluded that a DNA break must contain free ssDNA ends to fully activate DNA-PK [90]. These ssDNA ends often display altered chemistry when DSBs are introduced by ionizing radiation, and it was still unclear if such ends would be able to activate DNA-PK. We therefore wanted to study the activation of DNA-PK by ssDNA ends with chemical modifications. IR introduces a variety of damage in DNA, mostly base damage and SSBs, but also DSBs. The majority of the DNA ends at these lesions expose nucleotides with altered chemistry, such as thymidine glycol, 8-hydroxyguanine, and phosphoglycolate that preclude direct repair [3]. Since DNA-PK is required for efficient and effective repair of IR-induced DSBs, the enzyme must be able to recognize these altered DNA ends. Indeed, it has been shown that DNA-PK is activated by DSBs introduced by IR, and DNA fragments that end with 3'-phosphoglycolates [91]. However, a subset of the DSBs produced by IR might not be recognized by DNA-PK, and will subsequently be inefficiently repaired. Several studies have also shown that SSBs activate DNA-PK poorly [91, 101, 115]. Since an SSB might expose ssDNA ends by fraying, the requirement of ssDNA ends does not provide an explanation of how DNA-PK discriminates between SSBs and DSBs.

The properties of the ssDNA-binding site were examined by using DNA fragments with modified ssDNA extensions. DNA fragments with a wide range of defined ssDNA modifications at the terminal nucleotides were synthesized and assayed for their ability to activate DNA-PKcs.

DNA-PKcs binds to and is activated by dsDNA ends in the absence of Ku [92, 149]. In fact, under low salt conditions and using short DNA fragments containing unpaired ssDNA at the ends, the kinase activity of DNA-PKcs is actually higher without Ku in the reaction [90, 92]. Therefore, we characterized the interaction of DNA-PKcs with ssDNA ends without Ku, since DNA-PKcs interacts directly with ssDNA ends [90, 103, 114], and also to make the experimental system more amenable to Michaelis-Menten kinetics and determination of binding constants. When synthesizing the DNA fragments, we used a fragment that was 12 nucleotides long, with single stranded portions of three thymidines on each end, called f12-3T (Figure 7). This fragment bound and activated DNA-PKcs efficiently. In contrast, a blunt-ended fragment with only 12 nucleotides with no single stranded portions on the ends (figure 7) was too short to bind and activate DNA-PKcs [114]. Thus, the binding and activation of DNA-PKcs to DNA was dependent on the three nucleotides on each single stranded end. This difference between f12 and f12-3T was used to explore the question of which part of ssDNA is responsible for DNA-PKcs activation and DNA binding. Subsequently, f12-3T was the fragment we modified to find out what type of ends activated DNA-PKcs.

		DNA-PKcs binding and activation
f12-3T	3 12 3	+ + +
f12	12	–

Figure 7. *f12-3T with three unpaired nucleotides on each single stranded end binds and activates DNA-PKcs efficiently and effectively, whereas the blunt-ended f12 is too short to bind and activate DNA-PKcs.*

Symmetrical DNA fragments with the structure of f12-3T were synthesized, and the three unpaired bases at the ssDNA ends were exchanged with normal or modified bases. These DNA fragments were then tested with the kinase assay to assess the activation capacity.

By removing the bases completely from the ssDNA ends, and also exchanging the bases for the RNA base uracil, we showed that the bases in the ssDNA extensions were not required for activation or binding. The importance of the DNA backbone was examined by studying the interaction of DNA-PKcs with DNA fragments containing spacers ending with the DNA base thymidine (T), and the results indicated that interaction with a correct base at the end of a spacer was not sufficient for DNA-PKcs activation, and that DNA-PKcs activation also required interaction with the DNA backbone. This was further examined by replacing the hydroxyl group of the DNA backbone with methylphosphonate linkage. This modification gives the backbone a neutral charge instead of a negative one, and did not activate DNA-PKcs, showing that the negative charge of the backbone was important for DNA-PKcs activation, and that DNA-PKcs interacts with the DNA backbone as well. If the methylphosphonate modification was present on only one of the DNA strands, DNA-PKcs was still activated.

The fact that the RNA base uracil on the ssDNA extensions still activated DNA-PKcs made us wonder whether dsRNA activates DNA-PKcs as well. Others had shown that this was not the case [88, 101]. To test this we constructed a double-stranded RNA fragment with precisely the same structure as f12-3T and the hybrid fragment with uracil on the ssDNA extension. The dsRNA fragment did not activate or bind DNA-PKcs, indicating that DNA-PKcs discriminates RNA ends from DNA ends by interaction with the double-stranded portion of DNA ends.

At an SSB, the DNA strands become flexible and may expose ssDNA ends by fraying. DNA-PK may be able to assemble on SSBs, since the DNA binding subunit Ku binds to them. A SSB could then, in theory, activate DNA-PKcs. It was therefore unclear how DNA-PK distinguishes SSBs from DSBs. However, SSBs cannot expose two ssDNA ends at the same time, at the correct distance found at a

DSB. So one way for DNA-PK to distinguish between SSBs and DSBs, would be if DNA-PK were dependent on simultaneous interaction with two ssDNA ends. To investigate this, we constructed a series of fragments exposing one or two ssDNA ends, and it was clear that one ssDNA end, as exposed by an SSB, was not sufficient to activate DNA-PKcs. This suggested that activation of DNA-PKcs involved interaction of two ssDNA ends simultaneously, as exposed at a DSB.

The conclusions drawn from paper I were that DNA-PKcs recognizes DNA ends containing a wide range of modified nucleotides, and also that DNA-PKcs allows for activation even if one strand is incompatible with activation. Therefore, it is probably extremely unusual for DSBs not to be detected by DNA-PK. Also, DNA-PKcs probably distinguishes between SSBs and DSBs in that it requires simultaneous interaction with two ssDNA ends for activation. These properties potentially explain how DNA-PKcs can be specifically activated by DSBs, but still recognize the diverse chemical structures exposed when DSBs are introduced by ionizing radiation.

Paper II

The results in paper I indicated that a wide range of modified ssDNA ends could activate DNA-PK. The next step was to determine whether DNA-PK could recognize DNA strand breaks generated by agents used in cancer treatment. To study this, we damaged purified plasmid DNA with the anticancer drugs bleomycin, caliceamicin and etoposide, as well as with γ-radiation.

Caliceamicin induces high relative levels of DSBs, and contains two radical centers that become positioned close to the DNA backbone when calicheamicin binds to dsDNA. Activation of the radical centers is cooperative, and occurs after reduction of calicheamicin, resulting in efficient DSB induction [150, 151]. The DSBs often display abnormal nucleotides on both strands, which probably add to the extreme toxicity of caliceamicin [152]. Bleomycin cleaves DNA by binding simultaneously to DNA and a ferrous ion (Fe^{2+}), through two separate binding sites in the molecule. The ferrous ion mediates formation of hydroxyl radicals that can cleave the DNA phosphate backbone. Since radicals are produced in the proximity of DNA, effective cleavage occurs.

We also irradiated plasmid DNA with high-LET (^{137}Cs γ-rays) and low-LET (N^{7+} ions) radiation. LET stands for Linear Energy Transfer, and is the energy deposited per length unit of particle track. When DNA is irradiated with high-LET radiation, the number of ionization events in the DNA is higher than when it is irradiated with low-LET radiation. High-LET radiation is known to produce more complex DNA strand breaks and more DSBs than low-LET radiation. This is probably the reason why high-LET radiation is more toxic to cells than low-LET radiation.

The most frequently used drug that induces DSBs in cancer therapy is etoposide. Etoposide induces DSBs indirectly by interfering with topoisomerase II (topo II). Topo II is a homodimer that generates protein-linked DSBs through which an undamaged DNA helix can pass, and then religates the breaks rapidly [153]. By doing so, topo II can unknot tangles of DNA that would otherwise form

during meiosis, when long parental DNA strands unwind and daughter strands are synthesized. This mechanism functions at several steps in replication. For instance, moving of the replication fork induces positive supercoils ahead that needs to be removed. Topo II is also required for the separation of the two daughter chromatids resulting from a single round of DNA replication. When topo II cleaves DNA, the monomers form a covalent bond to each 5' DNA end generated. The complexes of topo II and DNA are usually very transient intermediates, owing to the rapid religation of the DSB, and are only present in low steady-state concentrations in the cell. In the presence of etoposide, religation is inhibited, resulting in a dramatic increase of topo II-linked DSBs and extreme cell toxicity (figure 8). Thus, etoposide converts topo II to a DSB inducing agent. If SDS is added to the reaction, the topo II-cleaved DNA complex is denatured and the DSB will have a topo II monomer linked to each 5' end. The topo II moieties can be removed by proteolytic degradation with proteinase K.

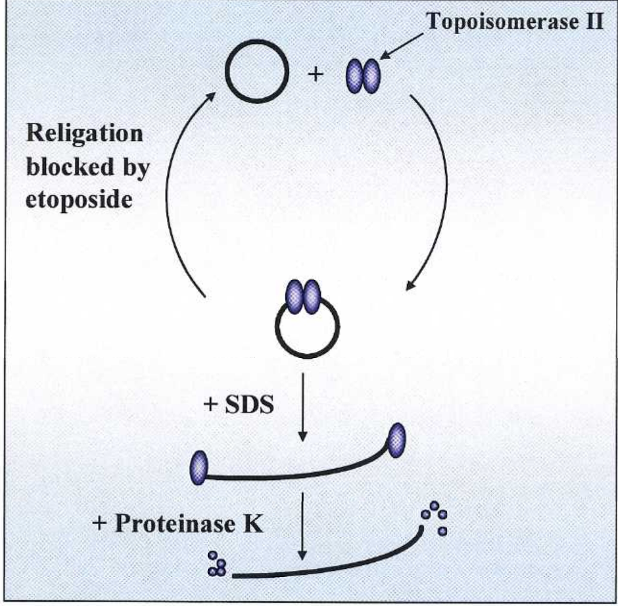

Figure 8. *Induction of DSB by topoisomerase II (topo II) and etoposide. A homodimer of topoII binds and cleaves plasmid DNA (circle), generating a protein-linked DSB. The topo II/DNA complexes are present in low steady-state concentrations in the cell, owing to the rapid religation of the DSB. In the presence of etoposide, religation is specifically inhibited, resulting in a dramatic increase of topo II-cleaved DNA and cell toxicity. Thus, etoposide converts topo II to a DSB inducing agent. If SDS is added to the reaction, the topo II-cleaved DNA complex is denatured and the DSB will have a topo II monomer linked to each 5' end. The topo II moieties can be removed by proteolytic degradation with proteinase K.*

The damaged plasmid DNA was purified and tested for its ability to activate purified DNA-PK. By measuring the relative levels of linear and nicked plasmid, we found that 5% of the strand breaks generated by bleomycin contained DSB, whereas at the same level of total DNA damage, 32% of the strand breaks generated by calicheamicin were DSBs. This confirms that calicheamicin is an effective DSB-inducing agent [154]. At a dose of 1,000 Gy, when close to 70% of the plasmid DNA is damaged, 1.5% of the strand breaks generated by low-LET radiation, as compared with 4% when plasmid DNA was damaged by high-LET radiation. This indicated that high-LET radiation induced DSBs slightly more efficiently than did low-LET radiation.

To allow for direct comparison of the different data sets in this paper, we related the DNA-PK activity in each experiment to the maximum DNA-PK activity obtained with blunt-ended, linear plasmid DNA generated by the restriction enzyme SmaI. The results showed that when we mixed the damaged plasmid DNA preparations with purified DNA-PK and measured the resulting kinase activation, plasmid DNA damaged with calicheamicin activated DNA-PK more effectively than plasmid DNA damaged with bleomycin, probably reflecting the differences in the DSBs produced by the two drugs. When we compared plasmid DNA damaged with high and low-LET radiation, DNA-PK was more effectively activated by high-LET radiation-induced strand breaks. This is probably also attributable to the higher levels of DSBs produced by high-LET radiation.

We purified supercoiled (undamaged), nicked (containing one SSB) and linear (at least one DSB) plasmid DNA separately from the damaged preparations, and analyzed them with the kinase assay. These results showed that DNA-PK was not activated by nicked plasmid DNA from any of the preparations, whereas linear plasmid DNA produced by bleomycin, calicheamicin and both high and low-LET radiation activate DNA-PK to levels matching the kinase activation obtained with simple restriction endonuclease-induced DSBs. This indicates that DNA-PK recognizes DSBs regardless of chemical complexity. In paper I, we found that both the 3' and the 5' ends of a DSB must contain an incompatible modification to prevent DNA-PK activation. It was, therefore, interesting to find that calicheamicin-induced breaks modified on both 3' and 5' ends [151, 154], activate DNA-PK effectively. Another interesting result was that DSBs induced by high and low-LET radiation activated DNA-PK to similar levels. Previous reports have shown that low-LET radiation-induced DSBs activate DNA-PK effectively [91, 93], and our data extend these observations to include strand breaks induced by high-LET radiation as well.

We also studied the DSBs induced by etoposide. In contrast to bleomycin and calicheamicin induced DSBs, the protein-linked DSBs produced by etoposide and topo II failed to activate DNA-PK. This indicates that DNA-PK cannot recognize the protein-linked DSBs produced by etoposide and topo II. Our data also show that Ku proteins were unable to bind topo II-induced DSBs, as shown by using the electromobility shift assay (EMSA), indicating that the topo II moieties must be removed from the DNA ends for these ends to be detected by DNA-PK. Alternately, DNA-PKcs is not part of the repair of etoposide-induced DSBs, which would explain why DNA-PKcs-deficient cells, in contrast to Ku-deficient cells, are not hypersensitive to etoposide, despite being sensitive to other DSB-inducing

agents [155]. There could thus exist a Ku-dependent, DNA-PKcs-independent, repair pathway for etoposide-induced DNA breaks [147, 156], where the Ku proteins may bind the DSB after removal of topo II.

The conclusions drawn from paper II were that DNA-PK recognizes DSBs regardless of chemical complexity, including drug and radiation-induced strand breaks relevant for cancer treatment, as long as these ends are not protein bound.

Paper III

Classical DSB-inducing agents, such as ionizing radiation and bleomycin mostly induce single-strand breaks (SSBs), while only a few percent of the DNA damage is DSBs [157]. The cellular response to these agents therefore includes a complex mixture of signals to SSBs, base damage, DSBs and oxidative damage to other molecules in the cell. In addition, evidence indicates that radiation therapy kills cancer cells by inducing DSBs [158]. To find ways to improve cancer treatment and DSB response studies, it will be important to identify agents that preferentially introduce DSBs in cells. One candidate drug in this class is the enediyne antibiotic calicheamicin γ1 (CLM) [159, 160]. CLM contains two radical centers that are positioned close to the DNA backbone when it binds to dsDNA. Activation of the radical centers is cooperative, and occurs after reduction of a tri-sulfide in the molecule. This gives a change in the structural geometry, and a radical is generated. When CLM then binds to DNA, hydrogen atoms are abstracted from double-stranded DNA, and both strands are cleaved, resulting in effective DSB induction. The DSBs often display abnormal nucleotides on both strands, which probably contributes to the extreme toxicity of CLM. CLM is inactivated when it cleaves DNA, and can only cleave once.

It is assumed that the efficient tumor-reducing effect of CLM is mediated by its ability to introduce DNA DSBs in cellular DNA. To test this assumption we compared CLM-mediated cleavage of cellular DNA and purified plasmid DNA. We found that neither excess tRNA nor protein inhibited cleavage of purified plasmid DNA, indicating that CLM specifically targets DNA. This is an important finding, since this selectivity decreases the extent of damage to other cellular structures, and increases the DNA-cleaving effect of CLM. Cleavage of plasmid DNA was not affected by incubation temperature. In contrast, cleavage of cellular DNA was 45-fold less efficient at 0°C as compared to 37°C, probably owing to poor cell permeability at low temperatures. We also found that permeabilization enhanced induction of DSBs at 0°C. The ratio of DNA double-strand breaks (DSB) to single-strand breaks (SSB) in cellular DNA was 1:3, close to the 1:2 ratio observed when CLM-cleaved purified plasmid DNA [154], and the DNA strand breaks introduced by CLM were evenly distributed in the cell population, as measured using the comet assay. This assay monitors DNA strand breaks by electrophoresis of cells molded into agarose. The amount of DNA migrating out of the nucleus (the tail moment) correlates with the amount of DNA strand breaks in individual cells.

The repair of CLM-induced breaks was studied with fibroblasts, treated at 0°C. X-ray treated fibroblasts were used as controls. The cells were allowed to rejoin at 37°C, and the remaining level of DSBs was analyzed at different time points.

CLM-induced DSBs were repaired slowly, probably reflecting the complex DSBs produced by this drug [152, 161]. However, they were eventually repaired completely.

The cellular response to CLM was also examined by measuring the phosphorylation of H2AX in calicheamicin-treated cells [49]. H2AX is phosphorylated in a DSB-dependent manner [19, 49], and CLM treatment resulted in high levels of H2AX phosphorylation, as well as effective cell cycle arrest. In addition, the DSB-repair deficient cell line M059J, defective in DNA-PKcs, was found to be hypersensitive to CLM.

In this study we identified several parameters important for reproducible cleavage of cellular DNA by CLM. The temperature has to be controlled, since cleavage was 45-fold less effective at 0°C as at 37°C. This could have implications for CLM treatment. Hyperthermia has been used to enhance the effects of IR, and the same might also apply to the effects of CLM. We also found that when cells were treated with CLM at 0°C, significant cleavage of cellular DNA occurred after the CLM-solution had been thoroughly washed away from the cells. This may be attributable to the existence of a micro-precipitate of CLM on the cellular membranes, specifically deposited there at low temperatures. The addition of sheared herring-sperm DNA to the washing solution alleviated this problem. CLM was stable in normal growth medium for at least 24 h, but addition of thiols quickly inactivated CLM, providing an effective means of detoxifying solutions containing CLM.

Conclusions drawn from paper III are that DSBs are the crucial damage after CLM, and that CLM-induced DSBs are recognized normally and also induce a normal DSB response. The high DSB:SSB ratio (about 30% of the strand breaks are DSBs), the specificity for DNA and the even damage distribution all make CLM a superior drug for studies of the DSB response, and emphasizes its usefulness in treatment of malignant diseases.

Paper IV

Radiation therapy is used in more than one third of all cancer patients [162], although it is often associated with severe side effects. If the tumor cell could be sensitized to radiation, this would allow for modulation of the radiation dose required to obtain tumor control, and might also limit the side effects of the treatment. Evidence indicates that IR kills cancer cells by inducing DSBs [158], and consequently that cells and animals with DSB repair defects are hypersensitive to IR [163]. Interfering with DSB repair might result in cells made more sensitive to IR.

DSBs introduced by IR are predominantly repaired by NHEJ, and require DNA-PK and XRCC4/Lig4 [88, 133, 163, 164]. Loss of the DNA-dependent protein kinase (DNA-PK) results in increased sensitivity to ionizing radiation owing to inefficient repair of DNA double-strand breaks [164]. Overexpression of DNA-PK in tumor cells conversely results in resistance to ionizing radiation [165, 166]. DNA-PKcs deficiency does not result in additional defects other than immunodeficiency, while deficiencies in Ku and Lig4 result additionally in growth retardation, neurological defects, premature cell senescence, and general

susceptibility to cancer development [144, 145]. This shows that DNA-PK is not required for normal cell function. It is therefore possible that transient inhibition of DNA-PK might selectively sensitize tumor cells to IR, making DNA-PK a relevant target for development of radiosensitizers. Available inhibitors of DNA-PK, such as wortmannin, are cytotoxic and stop the cell cycle, since they inhibit phoshatidylinositol-3-kinases (PI3-kinases) at 100-fold lower concentrations than those required to inhibit DNA-PK. PI3-kinase activity is required for many cellular processes, including growth factor signaling [167, 168].

In an effort to develop a specific DNA-PK inhibitor we have characterized SU11752, derived from a 3-substituted indolin-2-ones library. By measuring the DNA-PK activity at different ATP concentrations, at a fixed concentration of SU11752, we characterized the mode of inhibition by SU1172. In a Lineweaver-Burk plot, results indicated that SU11752 is a competitive inhibitor for the ATP-site in DNA-PK. This was confirmed by a direct assay for ATP-binding, where we used the PI3K-inhibitor wortmannin. SU11752 and wortmannin were equally potent inhibitors of DNA-PK. However, inhibition of the PI3-kinase p110 required 500-fold higher concentrations of SU11752, indicating that SU11752 was more selective against DNA-PK than was wortmannin.

Cells with no DNA-PK repair only a fraction of the DSBs introduced by X-rays, and they are hypersensitive to IR [156]. Thus, a DNA-PK inhibitor would be expected to reduce DSB repair and sensitize cells to IR. We used human cells with normal DNA-PK level, known as M059K, and a matching cell line from the same patient with no DNA-PK expression, known as M059J. The cells were first pre-incubated with SU11752 or wortmannin and then treated with CLM to introduce DSBs. The initial level of DSBs and the DSB level remaining after 4.5 hours of repair were measured using the FAR assay. This experiment showed that cells preincubated with wortmannin repaired less than 5% of the DSBs, and the cells lacking DNA-PK expression repaired less than 10% of the DSBs. In comparison, the control cells with normal expression of DNA-PK, treated with DMSO instead of inhibitor, repaired 50% of the DSB. SU11752 inhibited DSB repair to the same extent as wortmannin even at the lowest concentration used, indicating that SU11752 inhibited DNA-PK in cells. The same experiment was repeated with IR instead of CLM, and showed that a slightly higher concentration of SU11752 was needed to inhibit the repair of IR-induced strand breaks, as compared with the repair of CLM-induced strand breaks. This probably reflects the larger amount of DSBs produced by CLM.

SU11752 also inhibited DNA double-strand break repair in cells, resulting in a 5-fold sensitization to ionizing radiation. At higher doses of IR, the inhibition was even more pronounced. At concentrations of SU11752 that inhibited DNA repair, cell cycle progression was normal, ATM kinase activity was not inhibited at SU11752-concentrations that resulted in inhibition of DSB repair, and it was only slightly affected at concentrations resulting in radiosensitization. Importantly, this together with the fact that inhibition of the PI3-kinase p110 required 500-fold higher concentration of SU11752, shows that SU11752 was not a general inhibitor of PI3-kinase-related kinases.

However, at the present stage, SU11752 is unlikely to be clinically useful, owing to its relatively weak binding to DNA-PK.

The conclusion drawn from paper IV is that SU11752 defines a new class of drugs that serves as a starting point for development of specific DNA-PK inhibitors that can be used in combination with radiotherapy to improve tumor control.

Future directions

To better understand how drugs and ionizing radiation kill tumor cells, we must know how our cells handle DSBs. This knowledge can be used to improve current cancer treatment (i.e. radiosensitizers) and to enable development of new treatment modalities (i.e. DSB response-triggering drugs). It is very important to understand the mechanisms by which current cancer treatment kills cancer cells, in order to optimize the treatment, and to achieve greater efficiency and fewer severe side effects.

We are currently studying etoposide-induced DNA breaks. To date it has been believed that the mechanism of etoposide is to induce DSBs by inhibiting the religation step of topo II. Etoposide does this by binding to the topo II-molecules (Figure 8), thus creating a DSB with topo II bound to the cleaved ends [169]. Eukaryotic topo II is homodimeric, with one monomer binding to each DNA strand in the DNA molecule. In 2003, Bromberg et al. concluded that in order for etoposide to induce DSBs, one etoposide molecule must bind to each topo II monomer [170]. If the concentration of etoposide is too low, only one of the two topo II monomers is inhibited in the religation step and an SSB is created, which is not nearly as toxic to the cell as a DSB. Results from this report and other in vitro experiments [171, 172] suggest that at clinically relevant drug concentrations, etoposide is only able to inhibit one of the topo II molecules and, consequently, most of the breaks are SSBs. In this case, the result would be the same as for camptothecin, which is a topoisomerase I (topo I) inhibitor. Topo I only cleaves one of the strands in double-stranded DNA, and passes the other strand through the break. The function of topo I in eukaryotic cells is unclear, since topo II is present to relax the DNA strands, but in *E. coli* topo I it is essential to viability.

We have previously studied DSBs induced by etoposide (paper II), and found that the protein-linked DSBs produced by etoposide and topo II failed to activate DNA-PK. This indicates that DNA-PK cannot recognize the protein-linked DSBs produced by etoposide and topo II. Our data also showed that Ku proteins were unable to bind topo II-induced DSBs, indicating that the topo II moieties must be removed from the DNA ends for these ends to be detected by DNA-PK.

It is possible that etoposide induces solely SSBs at clinically relevant concentrations. However, no experiments have yet been made at clinically relevant concentrations to test this hypothesis. We intend to explore this hypothesis for cells and in patients, at etoposide concentrations used in cancer treatment. The cells will be treated with etoposide, to monitor the amount of DSBs and SSBs induced at different concentrations used to treat patients in the clinic. We have also received blood samples from patients treated for cancer with etoposide. These patient blood samples will also be examined for DNA strand breaks.

The DSB induction in the cells will be monitored using the neutral FAR (Fraction of Activity Released) assay (described in the method section) and the SSB induction in the cells will be monitored using the alkaline FAR assay, which is a modification of the neutral FAR assay. In this method, the cells will be lysed under alkaline conditions, allowing denaturation of the DNA strands, which provides a possibility to analyze the SSB-induction. H_2O_2, which only induces SSBs, will be used as a control to ensure that we are indeed looking at the amount of SSBs and DSBs. All cells will be labeled with a four hour pulse of ^{14}C-thymidine, making it possible for us to look specifically at the DSBs produced in the S phase. Etoposide seems to be an S-phase specific drug, since experiments performed on chondrocytes blocked in G1 phase, and treated with etoposide, does not contain DNA strand breaks, whereas cycling cells do (unpublished results). This is probably owing to the involvement of topo II in resolving positive supercoils ahead of the DNA replication fork [153].

Another approach is to measure the amount of topo II covalently bound to DNA with the TARDIS (Trapped In Agarose DNA Immunostaining) assay [173]. The TARDIS assay measures topo II/DNA complexes in individual cells after treatment with topo II targeting drugs. The cells are embedded in agarose, layered onto microscopic slides and lysed. DNA and covalently bound topo II from each cell remain trapped in the agarose. The topo II can then be detected *in situ* using immunofluorescence. This assay requires a small number of cells, which makes it suitable for analysis of patient samples. Using isoform-specific antibodies, we can also distinguish between the two isoforms of topo II (α and β). It may also be possible to investigate the resistance status of etoposide-treated patients using this technique, and to use it as a prognostic marker.

The cellular response to DSBs induced by etoposide and calicheamicin will also be examined and related to ATM and DNA-PKcs autophosphorylation. This will enable us to determine whether or not DSBs induced by topo II and etoposide activate the DSB response, as do calicheamicin-induced DSBs. Since DSBs introduced by topo II and etoposide have topo II-moieties bound to the DNA ends, it is possible that these DSBs will not activate the DSB signal as quickly as calicheamicin-induced DSBs, which are not protein-bound.

By combining these techniques, we hope to be able to discover the mechanism explaining why etoposide is such an effective anti cancer agent, and also to relate the amount of DSBs to the concentrations actually used in the clinic.

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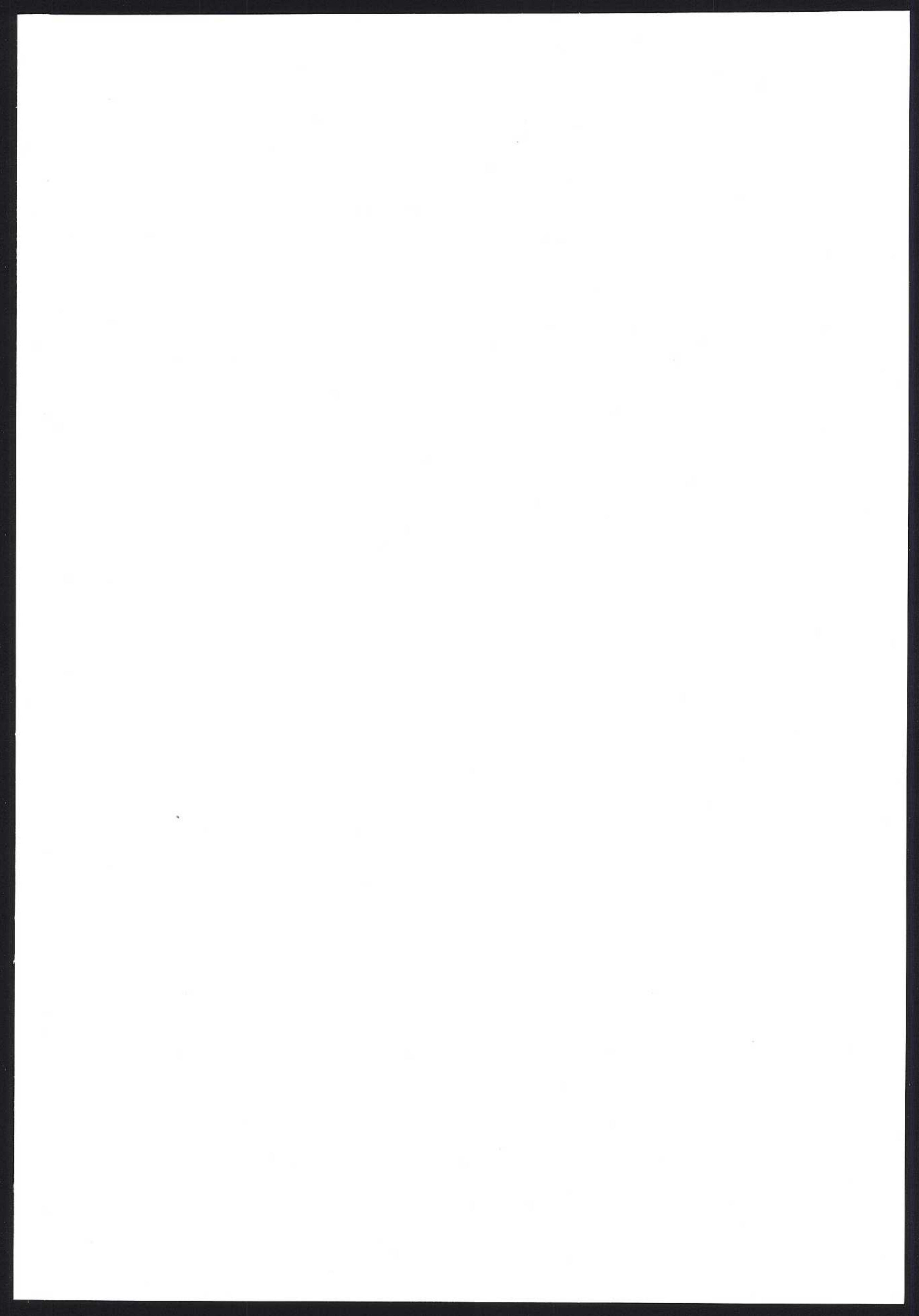

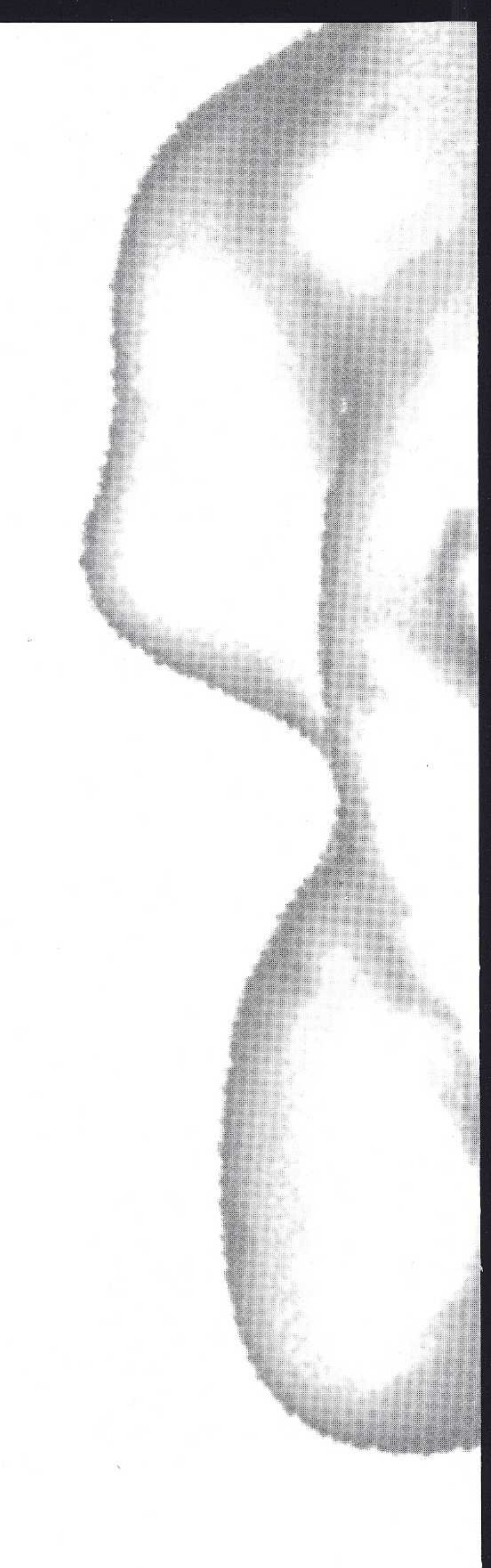

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