Final Report Summary - DDRESPONSE (The DNA damage response and breast cancer)

Executive Summary:
The DDResponse consortium has worked towards clinical application of PARP inhibitors and the development of novel treatment options using the concept of synthetic lethality. The consortium started from the observation that cells deficient in the BRCA1 or BRCA2 genes are extremely sensitive to PARP inhibition. Also the first clinical studies were very hopeful. This project identified several biomarkers of PARP inhibitor sensitivity. For example, low levels of PARP1 expression correlated with relatively low sensitivity. Furthermore, the consortium also contributed to our knowledge of specific resistance mechanisms, such as loss of Rev7 expression in BRCA1 deficient tumors. Taken together, our understanding of these mechanisms has helped to refine the combination of biomarkers to be analyzed.

Especially in the first years, consortium members have carried out several screening efforts to obtain additional combinations of genetic defects in tumour cells and small molecule inhibitors that lead to synthetic lethality. The various screens have resulted in many possible genetic interactions that have been followed up in the later stages of the project.

PARP inhibitors in combination with alkylating agents (such as carboplatin) cause severe toxicity to normal tissue, particularly to hematopoietic stem cells. The consortium found that a different dosing schedule, in which the PARP inhibitor treatment was started two days after the carboplatin dose, could alleviate these deleterious effects, providing a novel way of optimizing therapy. This will now be tested in clinical trials.

DDResponse also worked towards development of novel technology that will help analyze the effects of treatment and to select patients for PARP inhibitor treatment. Novel, high resolution microscopy techniques have been developed to analyze DNA repair complexes in cells at the single molecule level. In a different effort, culture conditions for tumor tissue specimens have been optimized, such that various DNA damage response assays can now be tested directly on fresh tumor tissue from the patient (such as biopsies).

In an effort to bring these novel assays to the clinic, DDResponse has analyzed panels of breast and ovarian tumors. Several tumors deficient in the first steps of homologous recombination have been identified and their genetic and epigenetic defects have been analyzed. We found that 13% of the unselected primary breast tumors and 30% of the ovarian tumors appeared to be deficient in this process, implying that this would be the patient population to be treated with PARP inhibitors.

Consortium members have contributed to the scientific, as well as the public discussion of PARP inhibitors, as evidenced by frequent visits to scientific meetings (often as invited speakers) and the popular media (newspaper interviews, television programs, etc.). With the approval of Olaparib/Lynparza as the first PARP inhibitor by the EMA and FDA in December 2014, this consortium witnessed the successful journey from scientific idea to clinical application.

Project Context and Objectives:
One of the most important developments in biomedical genetics over the last decade is the realisation that preservation of genetic integrity mediated by the DNA damage response (DDR) is both of enormous fundamental and medical importance. This holds most notably for cancer and many aging-related diseases, which have strong connections with DNA damage and genome maintenance. As these diseases represent the main causes of mortality and morbidity in many countries world-wide, this DDResponse project directly addresses the prevailing medical needs of all modern societies. The principal objective of this collaborative project is to unlock the full potential of genome maintenance / DNA damage response mechanisms to derive new...
therapies, assess individual susceptibility and predict individual responses to anti-cancer therapies that exploit DDR deficiencies, which appear universal in most, if not all, cancers. The project focused on, but was not limited to, breast cancer. The recent use of specific inhibitors to down regulate particular DDR pathways in cancer therapy is exemplified by the highly successful clinical trials with PARP1 inhibitors on BRCA1/2-deficient breast/ovarian tumours. This case illustrates the power of combined interference with different DDR processes to combat cancer: Homologous Recombination, which is selectively deficient in the tumour due to the BRCA defect, and base excision repair of single strand breaks, which is blocked by the administration of PARP inhibitors. The huge therapeutic potential of cancer treatment with the aid of selective DDR interference was recognised by ‘Nature’ to belong to the 10 most important breakthroughs in the entire spectrum of sciences in 2009 and the most pertinent development in the wide field of Cancer Research (Highlight in Nature 462, p. 961, 2009). It is the treatment par example of tailor-made, very effective cancer therapy, with comparatively minimal side effects. In this regard, the selective action of PARP inhibition on BRCA1/2 deficient tumours appears to be a ‘magic bullet’ therapy for this type of cancer. DDResponse brought together the main founders of the PARP inhibitor treatment and other world-leading European laboratories in the field of DDR in a truly joint effort to uncover the full potential of this novel approach. Three major, specific objectives based on this concept have been studied, in toto fully addressing the premise of the FP7 call:

a. Systematic assessment of normal tissue consequences of PARP inhibitors using an arsenal of specific and high-throughput (‘-omics’) approaches on mouse models and clinical samples. These investigations helped optimise and customise the use of drugs that target the DDR.

b. Design of novel types of ex vivo assays using viable tumour tissue, which identify the subpopulation of cancer patients eligible for application of PARP inhibitor protocols. We developed a novel generation of rational-based laboratory tests assessing response parameters of viable tumour tissue samples, which will enable diagnosis and treatment in a tailor-made fashion.

c. To expand the powerful concept of synthetic lethality based on compromised DDR of tumours as a basis for novel, highly selective cancer treatment modalities for subtypes of other tumours. This is carried out by exploring the realm of new possible synthetic lethal combinations of DDR defects by extensive systematic screenings in relevant model systems, such as S.cerevisiae C. elegans and mammalian cell lines.

As most, if not all tumours have acquired defects in the DDR, this presents hitherto untapped opportunities for therapies that target the specific vulnerability of tumour subtypes. Thus, the main aim of this proposal is to exploit our extensive knowledge of the DDR to optimise and further personalise cancer therapies, while minimising side effects.

DNA damage induction

Due to the complex chemical composition of DNA a perplexing diversity of lesions is continuously induced in our genes, which threatens proper functioning. DNA lesions are derived from three main sources:

i) environmental agents such as ubiquitous UV and ionising radiation, as well as numerous natural or man-made genotoxic chemicals;

ii) (by)products of normal cellular metabolism, notably reactive oxygen species (ROS) generated by respiration, lipid peroxidation, endogenous alkylating agents, estrogen and cholesterol metabolites and reactive carbonyl species. These compounds generate a diversity of lesions, e.g. ROS alone generate already several different kinds of single strand breaks (SSBs) and >70 oxidative base- and sugar-products in DNA;

iii) spontaneous disintegration of chemical bonds in DNA, including hydrolysis of nucleotide residues, inducing abasic sites and deamination of C, A, G or 5me-C.

Estimates of daily SSBs and spontaneous base losses in nuclear DNA run up to 104 per cell per day. Together with other spontaneous damage, the total may amount to >104-105 lesions per cell per day. Also inflammation may locally cause high levels of oxidative damage. This indicates the magnitude of the DNA damage problem for higher organisms containing large genomes, including mammals.

The DNA damage response

Mammals have developed a highly sophisticated machinery of complementary genome-stabilising systems to cope with the fundamental DNA damage problem and limit its deleterious consequences. The elaborate DDR apparatus consists of three main types of components.
1. The heart of genome protection is comprised of an intricate network of DNA repair mechanisms that are highly conserved and as a whole cover most of the wide range of damages induced in the vital genetic information.

2. To solve the problem of lesions interfering with replication, the DDR machinery has acquired a recently discovered class of translesion DNA polymerases that temporarily take over from the blocked regular replication machinery to allow lesion bypass. However, these aberrant polymerases permit translesion DNA synthesis at the expense of an increased mutation rate.

3. To avoid the deleterious consequences of DNA damage, the genome protection apparatus has inserted built-in mechanisms in the cell cycle machinery to sense genome injury and arrest at specific safe points in G1, S, G2 and M phases to allow repair of lesions that otherwise would be converted into permanent mutations or chromosome aberrations.

The DDR and cancer

The DDR is highly relevant to all aspects of cancer. First, numerous cancer predisposing syndromes are attributed to mutations in genes in the DDR pathways such as TP53 (Tumour suppressor P53, mutated in Li Fraumeni syndrome and mutated in more than 50% of all sporadic cancers), ATM (mutated in Ataxia-Telangiectasia), BRCA1/2 (mutated in 50% of familial breast and ovarian cancer patients), XP-A to -G and XP-V (defective in Xeroderma Pigmentosum (XP)), numerous FANC genes (deficient in patients with Fanconi’s Anaemia), etc. Second, DDR is important for the onset of carcinogenesis and thereby also for prevention, since most carcinogens are genotoxic, targeting the DNA in a direct or indirect manner. Third, DDR impinges on the evolution to a malignant tumorigenic state, which is driven by mutations and chromosomal instability. The latter can lead to inactivation of tumour suppressor genes, activation of proto-oncogenes and bypassing telomere attrition. Fourth, DDR mechanisms are also relevant to the effectiveness of classical therapeutic treatments, such as radio- and chemotherapy, because these therapies are strongly based on DNA damage induction, which triggers cell death particularly in proliferating cells. Fifth, the DDR also affects therapy resistance, due to attained genetic or epigenetic alterations that prevent cell death (e.g. by apoptosis), which undermines effective cure in most cancer treatments. Finally, hyper- and hyposensitivity of the normal tissue of patients to these classical anti-cancer modalities may at least in part be caused by inter-individual differences in repair and response systems. Thus, the DDR is central to the cancer problem. Due to the intimate links between DDR and carcinogenesis, most tumours have acquired one or more compromised aspects of the DDR in order to reach the number of oncogenic changes required for malignancy and/or to avert cell death despite ongoing DNA-damage induction. Therefore, assessment of the DDR status in tumours is very valuable not only for prevention, diagnosis and prediction of individual cancer susceptibility, but also for predicting individual response to treatment and for counteracting side effects, including long-term consequences and development of therapy resistance. Interestingly, compromised DDR mechanisms may represent an ‘Achilles heel’ of specific tumours as target for therapy. The following section demonstrates the value of this concept.

The case of synthetic lethality as the basis for effective tumour eradication

An illustration of the power of the approach proposed here is provided by the success of potent inhibitors of the single strand DNA break (SSB) repair protein poly(ADP)ribose polymerase (PARP), such as the oral drug Olaparib pioneered by partners 2, 4 and 6 of our consortium. Based on specific defects in the DDR, particularly in homologous recombination (HR), the first of a new class of very promising drugs (for BRCA-deficient breast, ovarian and prostate tumours) was developed, which selectively targets the tumour, while leaving normal cells and tissues in the patient relatively untouched. The specific deficiency in the BRCA1 or 2 genes, as an obligate step in tumorigenesis in familial breast cancer, renders tumour cells exquisitely sensitive to PARP inhibitors, while HR provides a back-up repair system for the repair of spontaneously induced single strand breaks in normal cells in the body. This is an example of synthetic lethality, where the lack of one pathway of genome maintenance in the tumour (HR in this case) in combination with the subsequent inhibition of another (SSB repair involving PARP1) promotes selective tumour cell death, because of functional redundancy.

Olaparib and several other PARP inhibitors are now in clinical trials for BRCA-defective tumours and others with remarkable results. In view of the complexity of the DDR and the frequent occurrence of partial redundancy, it is to be expected that more therapeutically exploitable cases of synthetic lethality are to be discovered, which may be applied to specific tumours and patients. As described below some new examples of synthetic lethality are emerging from current research.

It is important to mention that the clinical impact of application of PARP inhibitor therapy may well exceed the cases of carriers...
of inherited BRCA1/2 mutations as indications have been obtained for the occurrence of silencing of BRCA genes in several sporadic cancers. To accomplish the ambitious long term goal of finding novel DDR inhibitors with similar potential as PARP inhibition, with potentially a very high pay-off, and to improve current therapeutic options we have defined the following set of objectives:

• Discover DDR biomarkers that predict response to PARP inhibitors in Triple Negative (ER-negative, PR-negative, HER2-negative) Breast Cancer (TNBC) and extend this knowledge to other types of (breast and ovarian) cancer.
• Define how different DNA repair and DDR pathways/components interact with one another, regulate one another and in some instances compensate for one another. Understanding such relationships – and in particular redundancies – in combination with specific impairment in tumours may suggest how drugs that target one DDR component could specifically kill cancer cells.
• Analyse the status of DDR pathways in bone marrow (BM) stem cells to better manage BM toxicities when combining DDR inhibitors with DNA-damaging chemotherapies and investigate DNA damage sensitivities of other tissues in (repair-deficient) mouse models.
• Identify and validate markers indicative of functionality of DNA damage signalling and repair pathways in (fresh) tumour biopsies.
• Develop strategies to select patients for personalised treatment with PARP inhibitors.

Overall strategy and general description

The call “Predicting individual response and resistance to cancer therapy” is very timely and suited with regard to the major discovery on which this proposal is based: the selective killing of tumours, carrying a defect in the homologous recombination (HR) repair genes BRCA1 and 2, because of their exquisite sensitivity to inhibitors of the single strand break repair protein PARP, which by itself leaves normal tissue virtually untouched. This is the most desired way of combating cancer but also a prime example of personalised cancer therapy. This project intended to further develop the use of this specific inhibitor by generating tools that identify patients eligible for this very promising treatment, avoid undesired effects of normal tissue and optimise the use in combination with other treatments. At the same time this project aimed to expand the concept of synthetic lethality to other combinations of DDR pathways and anti-cancer treatments to identify other patient populations which may equally benefit from highly selective and effective targeted therapies. To accomplish these goals our overall strategy involved 5 defined approaches described in highly integrated work packages.

1. In depth analysis of high-throughput ‘-omics’ databases of numerous highly defined breast tumours to identify DDR biomarkers that reveal the BRCA1/2 status and in a broader perspective the functioning of the HR repair pathway as a whole in order to predict sensitivity of tumours to PARP inhibitors and other therapeutic interventions targeting the DDR. The consortium has one of the most extensive breast cancer biobanks available, including data on mRNA and miRNA expression profiles and proteomics, combined with detailed clinical follow-up information. In addition, we used experimentally versatile cell lines.

2. Genetic (synthetic lethality) and proteomic screens of model organisms (C. elegans, S. cerevisiae) and mammalian cell lines have been carried out to discover novel therapeutic approaches that exploit individual tumour-specific DNA repair deficiencies. These approaches extended the spectrum of tumours targeted by PARP inhibitors.

3. To improve the therapeutic ratio of PARP and other DDR inhibitors (with or without additional anti-cancer treatments) normal bone marrow stem and progenitor cells were used to establish normal tissue toxicity profiles. Conversely, we explored predictive biomarkers that indicate subsets of human breast and ovarian tumours resistant to standard genotoxic chemotherapy, in order to avoid overtreatment of such patients.

4. We developed innovative protocols that use viable organotypic slices of normal tissue and tumour specimens such that their DDR can be quantitatively and functionally determined ex vivo following exposure to genotoxic agents and DDR inhibitors. This will enable diagnosis and treatment in a tailor-made fashion.

5. Building upon the methods for ex vivo DDR assays, we performed a comprehensive analysis of breast and ovarian tumour specimens and correlated the ex vivo DDR parameters with relevant clinical observations and pathology results.

Project Results:
WP1 – Biomarkers
As PARP inhibitors are now moving from clinical studies to the status of approved anti-cancer drugs (with the official approval of Olaparib/Lynparza in Europe and the USA), biomarkers for patient selection (positive and negative) are of major importance. Several strategies have been pursued in DDResponse. We highlight the most important findings from the project. Interestingly, a low PARP-1 level predicts olaparib response. Cancer cells with high levels of PARP-1 can either be responders or non-responders, whereas cancer cells with low levels of PARP-1 are not sensitive to olaparib treatment. This observation provided the novel insight, that trapping of enzymatically inactive PARP-1 protein on DNA lesions may be the trigger for synthetic lethality, rather than the absence of PARP-1. Furthermore, defects in DNA-damage sumoylation or sumo-dependent ubiquitylation cause hypersensitivity to Olaparib and other agents causing DNA damage in S-phase. Furthermore, changes in the ubiquitylation and sumoylation status of DDR components in certain cancers could be valuable prognostic markers as well as being potential therapeutic targets. For example, SUMO-directed RNF4 ubiquitination events are essential for efficient HR. Neddylation pathway proteins may also be useful as a biomarker since components of this pathway are over expressed in ovarian cancer and linked with resistance to “standard of care” platinum therapy that is also reported to correlate with PARP inhibitor sensitivity.

One of the challenges in selecting patients for olaparib response is the fact that any one of a number of HR-associated genes could be deficient, making any single gene/protein unlikely to have sufficient utility as a patient selection tool. We have generated extensive data that both the selectivity and specificity of olaparib biomarkers increases as multiple biomarkers are combined. For example, a combination of several biomarkers, including BRCA1/2 and MRN status, PARsylation, Rad51 focus formation, and 53BP1 can be predictive for PARP-1 inhibitor response.

Furthermore, we have significantly expanded the numbers of potentially predictive markers by carrying out a hypothesis-free genome wide mRNA expression analysis. This work has led to the identification of over 400 genes that correlate with olaparib response in cell lines and where two major biological networks have been identified. One network or cluster contains genes associated with cell cycle, replication and proliferation, the second a DNA repair cluster that includes PARP-1 and other BER genes, HR genes such as BRCA1 and RAD51 and additional DNA repair pathway genes such as the mismatch repair genes MSH2 and MSH6.

A similar analysis in materials from clinical trials has been completed with the 155 refined candidate gene set having been demonstrated to have predictive value for both platinum response in the MDACC data set where 5 novel cluster genes correlate with overall survival and with olaparib response in Study 19 where FEN-1 and MUTYH have been shown to correlate with progression free survival following olaparib maintenance therapy. The increased confidence resulting from the predictive value identified for the 155 gene set means these genes will be selected to be taken forward for additional analysis of future olaparib clinical trials in breast cancer.

One of the main challenges in validating response signatures of gene expression is the availability of clinical trials with outcome data based on the response to the targeted therapy of interest i.e. in our case the number of trials where clinical samples are available from olaparib treated patients. An assessment of different chemotherapies in the breast cancer cell line panel identified platinum response as a potential surrogate for olaparib response. This correlation means that we may also benefit from testing our potentially predictive olaparib response gene signatures in clinical samples from patients where platinum response is available, which in turn will increase the confidence that we have in our patient selection tools.

In addition to biomarkers of PARP inhibitor sensitivity, we also investigated biomarkers of resistance to PARP inhibitors in BRCA1 deficient cells. In collaboration with Sven Rottenberg at the Netherlands Cancer Institute, we investigated the mechanism of PARP inhibitor resistance in BRCA1 deficient cells after depletion of REV7. We found that REV7 depletion led to increased resection at DSBs to create single strand DNA overhangs that can initiate homologous recombination. This resistance mechanism appears similar to the previously found function of 53BP1. This REV7 function was at least partially dependent on interaction with the DNA polymerases REV3 (in the pol-zeta complex) and REV1, suggesting that DNA synthesis is important for this function.

Conclusions on biomarkers

Together, the data generated in this reporting period have provided several important insights into patient selection biomarkers that can be used to predict olaparib or other PARP inhibitor sensitivity:

- In platinum sensitive HGSOC where there is already a high incidence of BRCA mutations, the gene expression ‘signature’ approach appears to have limited value – however, this may still have utility in other tumour indications such as TNBC and this
can be tested in the future

• For the IHC biomarkers BRCA1 and RAD51 failed to accurately identify BRCA1m tumours in Study 19 samples so these won’t be taken forward

• The 53BP1 IHC did not show any correlation of low levels of staining with BRCA1m tumour olaparib resistance (based on poor PFS response). Future work could still involve additional analyses of 53BP1 levels with olaparib outcome in additional clinical samples, although the most relevant will be matched pre- and post olaparib dosed samples from the same trial

• The γH2AX levels in Study 19 showed that higher levels of γH2AX correlated with better PFS in the olaparib treated arm compared to placebo. These interesting data will be followed up with future analyses of samples from olaparib trials where outcome data is available

• The ATM IHC assay has been validated in the Study 39 Gastric cancer Phase II trial and is now being used as an integral part of the olaparib GOLD Phase III trial and will be developed as a companion diagnostic for olaparib in this and potentially other tumour settings

• The Myriad HRD test aimed at identifying a ‘genomic scar’ associated with the loss of homologous recombination repair was very effective at identifying cancer cell line BRCA1 and BRCA2 mutations but failed to predict responses in all other genetic backgrounds including suspected non-BRCA HRD mutations and cell lines with various levels of detectable BRCA1 promoter methylation – future work will assess the Myriad HRD test in Study 19 and 39 clinical samples

• The RAD51 foci analysis by immunofluorescence was a more accurate predictor of olaparib response in the cancer cell lines. Although it may be useful in a research setting, there are drawbacks as a patient selection approach: failure to detect olaparib sensitivity resulting from ATM/MRE11 and possibly other mutations and the technical difficulty to turn this assay into a companion diagnostic for PARP inhibitors

• Biomarkers of resistance in a BRCA mutated situation need to be pursued further in clinical samples. DDResponse has added to the list of possible resistance mechanisms by identifying REV7 loss as a novel cause for olaparib insensitivity. At present, the most likely way forward for patient selection biomarkers as companion diagnostics is either selected IHC, such as for ATM, or for a broader panel of HRR-associated genes through Next Generation Sequencing of tumours, CTC’s or plasma born tumour DNA.

WP2 - Genetic and proteomic screens for Synthetic Sickness and Lethality

PARP inhibitors and Homologous Recombination deficiency is the first example of synthetic lethality that can be used for therapeutic purposes. The DDResponse consortium has invested significantly in identifying other combinations that could be taken forward towards clinical application in other subsets of tumours. We have identified combinations of PARP inhibitors with other genetic defects that cause synthetic lethality or sickness (SSL) of the (tumour) cells. We also invested in finding synthetic lethal combinations with other DNA damage response inhibitors, enlarging the population of tumours that can be targeted.

We have designed and optimized a genetic screening system to identify DNA mismatch repair synthetic lethal targets. Using these tools, we identified a number of hits that confer synthetic lethality with the mismatch repair system. We found various interesting candidates, including multiple components of complexes I-V of the electron transport chain, suggesting that drug interventions targeting mitochondrial function will be possible.

A fast, robust and quantitative screening pipeline has been created for C. elegans SSL screens. This has been combined with an image-based high throughput screen toolkit for survival assessment in C. elegans, a method that makes use of fluorescently engineered nematodes that can be analyzed by microscopy with automated image acquisition and processing. Additionally we set up a fast and automated data analysis tool to facilitate quality control, data sorting and hit identification for high throughput screens. This set up has been used in screens to identify genetic interactions in the DDR. We have further characterized the mechanism of action of one hit (PolQ) from this analysis. We obtained a detailed mechanistic understanding of the role of PolQ in DNA double strand break repair, which can explain why mutations in polq-1 (the gene encoding PolQ) are synthetic lethal with Polη.

Several SILAC-based differential proteomics studies were carried out to systematically analyze how protein levels, acetylation and phosphorylation are affected after treating cells with the anti-cancer DNA damaging agents ionizing radiation (IR) and...
etoposide. This work identified new potential substrates of the ATM, ATR and DNA-PK enzymes as well as additional phosphorylations likely mediated by other protein kinases. Moreover, through validation studies and further exploration, this work established that the protein phosphatase PPM1G is recruited to sites of laser-induced DNA damage and revealed that the RNA processing factor THRAP3 is a functionally important new DDR target protein.

The DDR involves a complex network of proteins that are tightly regulated through post-translational modifications. We have carried out a series of screens to identify the role of ubiquitin-like proteins in the DDR. This includes all known E2 ligases, and additional ubiquitin-like proteins and we have demonstrated that a number of these post-translational modifications e.g ubiquitination, SUMOylation and neddylation provide an additional layer of DDR regulation.

Many proteins are known to be ubiquitylated following DSB generation and consequently require deubiquitylases (DUBs) to reverse this process once DNA damage has been repaired and the DDR is turned off. We have carried out a systematic siRNA screen of all known human DUBs to identify those that have a role in the DDR. These screens have identified several DUBs that function in DNA damage responses. We identified a number of DUBs with previously unknown links to double-strand break (DSB) repair, the G2/M DNA damage checkpoint and genome integrity maintenance. Furthermore, we established that the DUB UCHL5 regulates DSB resection and homologous recombination through protecting its interactor, NFRκB, from degradation. This work extends the list of DUBs linked to the DDR and highlights their potential as cancer therapy targets.

Three siRNA screens have been carried out:

i) Given the emerging importance of 53BP1 as a potential biomarker for PARP inhibitor sensitivity in BRCA1-deficient cells (see WP1), we first screened for factors involved in regulation of the 53BP1 abundance/size of the 53BP1 nuclear bodies. In this way we identified two ubiquitin ligases, TRIP12 and UBR5.

ii) Another screen for SSL with the PARP inhibitor olaparib use micronuclei as the read out. One of the strongest candidates was the E3 ubiquitin ligase, CBLC. We validated the finding by demonstrating that silencing of CBLC causes increased sensitivity to olaparib in breast cancer cell line models and that defective homologous recombination (HR) DNA repair is the likely cause. This data provides an example of how defects in the ubiquitin machinery have the potential to influence the response of tumour cells to PARP inhibitors.

iii) The third siRNA screen has been conducted to identify SSL interactions with ATM inhibitors. Several candidates have been identified and are currently studied in more detail. This may provide a starting point for developing biomarkers for ATM inhibitor treatment of cancer patients.

We have functionally validated some top hits from the screens, such as the DDR-related protein TOPBP1and the JMJD1C demethylase, which we found to operate upstream of the BRCA1 in response to DNA double strand breaks (its loss enhances resistance to PARP inhibitor, especially in the BRCA1-defective human cancers).

Conclusions on screens
Functional screens have uncovered many synthetic lethality interactions in various settings. Some hits from these screens have been validated and studied in more detail, but the majority of the information is still to be taken forward. The outcomes of the screening efforts will be a valuable source of candidate genes to be investigated in more detail in order to get a better understanding of the intricate genetic interactions in the DNA damage response. The outcome of this WP is indispensable for finding good biomarkers (WP1) and also feeds into identifying new synthetic lethal interactions, involving other inhibitors, such as ATM or DUB inhibitors. In the years to come, this repository of genetic and physical interactions will provide a starting point for many detailed investigations into mechanistic studies, as well as identification of a robust set of biomarkers.

WP3 - Normal tissue toxicity

Counteracting excessive bone marrow toxicity
Building on our previous work where we showed differences in DNA damage repair kinetics in bone marrow versus tumour cells in vitro, we developed a new assay to monitor bone marrow DNA damage response after olaparib and carboplatin in vivo treatment. We demonstrated that olaparib (100 mg/kg) induced DNA damage is resolved by 24h after initial dose and was not associated with bone marrow cell loss. Carboplatin (40 mg/kg) caused greater induction of DNA double strand breaks and significant bone marrow cell loss, especially in multi-potent and erythroid progenitor populations.
To assess long-term treatment effects of olaparib, carboplatin and their combination (concurrent and scheduled) on hematopoietic system, we conducted a 7-cycle study in rat and analyzed the impact on bone marrow cellularity and function, and expression of selected DDR genes in the bone marrow cells.

This study showed a significant hypo-cellularity and changes in bone marrow populations' distribution after 7 completed cycles of carboplatin and combination treatment. There was no such impact observed in animals treated with olaparib alone. Comparison of mouse and rat models led to the conclusion that rat resembles the human situation much more closely than mouse. Therefore, we decided to focus exclusively on rat models. Recent data suggests that the carboplatin response in rat peripheral blood components indeed mirrors that of humans in the clinic. Modelling of peripheral blood cell counts from a broad population of patients who had received various doses of olaparib in combination with carboplatin revealed that olaparib dosing together with carboplatin causes delay in absolute neutrophil count (ANC) and slower recovery to the baseline level. We therefore developed a model to reproduce the increased BM toxicity seen in the clinic with concurrent olaparib and carboplatin treatment. We used this to differentiate between schedules, such as gapped sequencing of chemotherapy and olaparib. Firstly, we ran a pilot study and used PK-PD (pharmacokinetics – pharmacodynamics) modelling to established clinically relevant doses of olaparib and carboplatin and cycle duration for the rat model. Consistent with the clinical toxicities seen with carboplatin, the bone marrow response in this preclinical model confirmed the major impact on multipotent progenitors and erythroid populations. Importantly, the concurrent combination of carboplatin and olaparib identified a statistically significant and greater impact on the CD90+/Lineage- and CD71+ populations. These biomarkers were then used to investigate a gapped schedule between the platinum and olaparib treatment of one, two, three and four days. We demonstrated that with only one day gap there was no statistically meaningful improvement in the levels of the CD90+/Lineage-, and CD71+ biomarkers. However, a two day gap (or greater) was sufficient to bring the levels of these markers back to that seen with carboplatin alone. This benefit of a 2 day gap schedule on bone marrow cell’s recovery, also translated into an improved peripheral blood effect. These finding were consistent with the γH2AX bone marrow data that were generated showing that carboplatin-induced DNA damage was resolved within 48 hours of treatment. Importantly, we also assessed the γH2AX kinetics in tumour models and demonstrated that carboplatin DNA damage repair kinetics in tumour cells lasts longer than 72 hours, suggesting that there is a potential therapeutic window and provide a clear rationale for taking a 2-day gapped schedule of platinum and olaparib into clinical trials.

Gene expression after DNA damage induction
In order to get a better understanding of gene expression changes upon genotoxic exposure, mouse ES cells have been used to define a gene expression signature that is characteristic for exposure to DNA damaging agents. Several known DNA damage response pathways were up-regulated in these cells, but we also noticed differences between exposures to different DNA damaging agents. Both wild type and DNA double strand break repair deficient mice were exposed to a low dose of ionizing radiation (0.2 Gy gamma radiation) and gene expression patterns from liver were determined. Careful analysis of the gene expression patterns revealed a set of known DNA damage response genes that were all regulated in a similar pattern: increased expression after ionizing radiation exposure and also increased expression in repair deficient mice compared to repair proficient mice. This provided a good starting point to define a ‘DNA damage response signature’. Similar approaches also defined RNA expression changes of small and long non-coding RNAs.

Conclusion on normal tissue toxicity
Our results demonstrate for the first time that the haematopoietic cell markers can be used to predict bone marrow toxicity for targeted agents and also to generate data that can prospectively help in the design of clinical combination schedules for DNA repair inhibitors. Following on with the olaparib and carboplatin combination we have started using this preclinical bone marrow model to investigate the effects of other DDR agents such as the Wee1 inhibitor (AZD1775) and ATR inhibitor (AZD6738) which will help their clinical development both in combination with chemotherapies and with olaparib or other DDR agents.
The impact of the olaparib and carboplatin preclinical studies have been supported by Hillary Calvert, one of the world’s leading experts on platinum chemotherapy and its use in the clinic. Following his support and the data generated from this project, AZ is now planning a new Phase Ib olaparib and carboplatin combination trial using the gapped schedule approach in a neo-adjuvant breast cancer setting.

Gene expression changes upon DNA damage induction are a sensitive way to investigate the effects of exposure to genotoxic agents. The methods developed by DDResponse will be helpful to analyze the short and long term effects of treatment with PARP inhibitors alone or in combination with genotoxic agents.

WP4 – Ex vivo assays

Novel technology to study the DNA damage response
We developed novel techniques to monitor the DNA damage response in single cells. Following irradiation, multiple DNA damage responsive proteins rapidly redistribute into microscopically visible sub-nuclear aggregates, termed ionizing radiation induced foci (IRIF). How the enrichment of proteins on damaged chromatin actually relates to DNA repair remains unclear. We have used three-dimensional structured illumination microscopy (3D-SIM) to better dissect the relationships between DSB responsive factors in damaged chromatin.

We have also developed a novel method that utilizes super-resolution microscopy and a novel extraction method to visualize previously unresolvable DNA repair complexes. Our knowledge of the repair of DNA double-strand breaks (DSBs) has been technically hampered due to limitations of resolution and detection methods currently available. One of the main sensors of DSBs is the protein complex Ku that binds to DNA ends. Until now it has not been possible to visualize Ku at single DSB sites in cells by fluorescence microscopy. We have developed a method that combines RNase- and detergent-based pre-extraction with high-resolution microscopy allowing detection of Ku and other non-homologous end joining (NHEJ) proteins at DSBs in single cells. This method has allowed us to detect and quantify Ku at DSBs induced not only by IR but also with chemotherapeutic agents. We also applied this technique to investigate the role of neddylation in the DNA damage response. We found that neddylation is required for normal removal of the Ku heterodimer from DNA after the repair reaction has been completed.

DNA damage responses in tumour specimens
We determined the optimal conditions for collection and culturing of fresh breast and ovarian tumour specimens. We have adapted procedures to study the DDR in breast and ovarian tumours. For the reliable performance of the DDR assays, it is essential that tumour cells maintain their viability and replicative potential. We have optimized (A) thickness of the tumour slices (optimum around 0.3 mm), (B) culture media (with addition of several additional growth factors) and (C) diffusion of media components into the tumour slices (by culturing on a rotating platform or a support in the culture dish). Using these optimized protocols, we are able to keep cell viability and proliferative characteristics constant for 4-7 days ex vivo. We also optimized the thawing of viably frozen tumour tissue. Both thawed breast and ovarian tumours were shown to retain morphology and still contained replicating cells that form RAD51 foci upon ionizing radiation.

Ovarian tumour specimens have also been dissociated using enzymatic dissociation. Similar methods were also successful for dissociation of bladder tumours. However, only 10% of dissociated breast tumours provided sufficient numbers of cells for analysis after a similar protocol. We therefore conclude that this technology is only suitable for easily dissociable tumours, such as ovarian and bladder tumours.

To reliably assess the DDR in tumour cells we optimized conditions so that the following prerequisites were met:

a. Presence of sufficient numbers of replicating tumour cells (assessed by incorporation of the nucleotide analogue EdU)
b. Distinguish tumour cells from normal cells (based on tissue morphology and/or cytokeratin expression)
c. Possibility to apply ionizing radiation or genotoxic agents (ensured by using thin slices of tumour material)

Ionizing radiation, radiomimetic agents (zeocin or bleomycin) or PARP inhibitors have been used to induce DNA damage in fresh tumour tissue. After fixation and embedding of the tumor tissue in paraffin, the efficacy of HR has been assessed by the presence of γ-H2AX or 53BP1 foci (indicative of the presence of double strand breaks) and RAD51 foci (indicative of HR
We adapted current staining protocols for the analysis of these foci in paraffin embedded-formalin-fixed tissue sections. These assays have been optimized in such a way that they can be used to screen a larger panel of tumours. They have been validated in BRCA1 proficient and deficient xenograft tumours and in BRCA deficient breast and ovarian tumour material. We also adapted procedures such that needle biopsies can be assayed for RAD51 foci formation, which will facilitate the use of this assay in patient studies in the metastasized or neoadjuvant setting.

Cytotoxicity of the treatment has been assessed by analysis of general tissue morphology after treatment and measurement of apoptosis (by immunohistochemical staining for cleaved-Caspase-3 or TUNEL staining). We also discovered and characterized a novel DDR-related biomarker of replication stress: formation of 53BP1 nuclear bodies, formed in very early G1 phase, symmetrically in both daughter cells that suffered replication stress in the previous cell cycle.

A major difficulty is tumour heterogeneity. Therefore, one has to take into account that the relative contributions of stroma and tumour cells is not the same in different areas and that not all tumour areas are equally proliferative or prone to undergo apoptosis. The relative contribution of tumour cells and stroma was assessed by co-staining with keratin to detect the tumour area and calculate the fraction of tumour in the image. The number of EdU or TUNEL positive cells is then divided by the area occupied by tumour cells to compensate for this heterogeneity. The heterogeneity between tumour areas cannot be compensated completely. However, we minimize this problem by taking 10 microscopic fields randomly. This appears to be a valid way to assess tumour sensitivity to a treatment.

We also adapted the tumour tissue slice cultures for pancreas, prostate and head and neck tumours. Bladder tumours were analyzed after dissociation and culturing of the tumour cells as monolayers on coverslips. RAD51 IRIF formation could be investigated in all samples.

Conclusions on Ex vivo assays

New methodology has been developed to assess the DNA damage response in more detail in cells and tissue slices. The advanced microscopy methods have been optimized to allow visualization of single DNA repair protein molecules, which will in the future greatly increase our ability to study the effects of drug treatment in cells at the molecular level. Especially the competition between the two main pathways of DNA double strand break repair, Homologous recombination and Non-homologous end-joining, is of major importance to understand the effects of PARP inhibitor treatment in various genetic backgrounds.

Direct assessment of DNA damage responses and cytotoxicity of treatments will be important to investigate tumour response to PARP inhibitor treatment and may be used to select patients, at least in a study setting, for inclusion in PARP inhibitor studies. The methodology will also be useful for investigation of the effect of other treatments and may eventually even be developed into a general testing to predict efficacy of various treatments. In the DDResponse project these methods have been used to correlate genetic and epigenetic factors with RAD51 IRIF formation and PARP inhibitor sensitivity (WP5).

WP5 – Towards clinical applications

In order to move functional assays to the clinic, the first step is to show robustness of the assays and the proof of principle that known genetic defects can indeed be identified. We therefore collected over 150 fresh primary breast tumour specimens, of which a conclusive RAD51 Ionizing Radiation Induced Foci (IRIF) assay could be determined in 126 tumours. A total of 17 tumours turned out to be Homologous Recombination (HR) negative, judged from the RAD51 IRIF assay (13%), suggesting that these tumours might benefit from PARP inhibitor treatment. Especially in triple negative breast cancer (TNBC), the prevalence of HR deficiency is high, over 50% of all TNBC showed a clear RAD51 IRIF defect. In ER/PR positive breast tumours, on the other hand, the prevalence was much lower (approximately 7%). The number of ER/PR negative, HER2 positive tumours was too small to give reliable percentages, but the fraction of RAD51 IRIF deficient tumours appears to be intermediate in our analysis.

Although RAD51 IRIF deficiency correlates highly with TNBC, approximately 40% of all HR deficient tumours were found in the ER/PR positive group. Therefore, we conclude that determination of HR status should not be restricted to TNBC or ER/PR negative tumours.

In the first panel of 45 tumours, we also investigated the correlation of RAD51 IRIF formation with several other parameters,
including age, tumour size, tumour subtype and grade. We did not find clear correlations of any of these parameters with RAD51 IRIF formation, except a correlation of RAD51 IRIF formation deficiency with undifferentiated carcinoma and a trend towards higher grade. All defects were associated with a BRCA gene defect: three disease causing mutations in BRCA2 and two tumours with epigenetic BRCA1 gene silencing caused by promoter methylation. We did not find a BRCA gene defect in the RAD51 IRIF positive TNBC or in the one tumour with intermediate RAD51 IRIF formation ability in this panel. The rest of the tumours are currently under detailed investigation, which has already yielded at least one more definite BRCA gene mutated tumour.

We also performed the RAD51 IRIF assay on a panel of 27 ovarian cancer tumour samples obtained from patients that underwent ovarian cancer surgery (n=13) or ascites drainage (n=14). In total, 7 RAD51 IRIF deficient samples were identified (26%).

The majority of the ovarian tumours collected were serous ovarian tumours (21/27). Five out of the 21 serous ovarian tumours were RAD51 IRIF deficient (24%) whereas two out of six other subtype ovarian tumours were RAD51 IRIF deficient (33%). Although the number of tumours is still limited, this indicates that HR deficiency is not restricted to serous ovarian tumours. Three patients in whom no RAD51 IRIF could be detected in the tumour carry a pathogenic germline mutation in BRCA1 or BRCA2. In four other patients, BRCA1 and BRCA2 germline mutation analysis was negative, including one patient with a RAD51 negative tumour. The possible genetic or epigenetic defect in this tumour, as well as the 14 tumours for which BRCA gene mutation status was not known, is currently under investigation.

Conclusions on Clinical Application

The ex vivo assays have identified several BRCA deficient tumours that would be eligible for PARP inhibitor treatment. A major hurdle towards the clinic is the technical difficulty of performing the RAD51 foci formation assay in a regular pathology laboratory. We envision that the assay should be carried out in a specialized and controlled setting. Therefore, samples would have to be sent to central locations. We found that tumour specimens can be shipped on ice without loss of RAD51 IRIF formation ability in the first 24 hours. Alternatively, tumour tissue can be frozen in dedicated freezing medium. This would allow analysis on samples from hospitals outside the academic setting.

Even though ex vivo assays are a possible option for patient selection, it will be difficult to handle the very large numbers of specimens that can be expected if it would be used as a companion diagnostic test. Therefore, the genetic and epigenetic alterations that we identify in our RAD51 IRIF deficient samples will guide us to develop a panel of genetic and epigenetic alterations that can be determined in formalin fixed or frozen material. The ex vivo assays will then mainly help to identify additional factors that cause a defect in RAD51 IRIF formation and/or sensitivity to PARP inhibitors. The ex vivo assay may also be used as a tool to assess whether BRCA mutated tumors may have acquired resistance to PARP inhibitors (i.e. show RAD51 IRIF), which would prevent treatment of patients that would not benefit from PARP inhibitor treatment.

Potential Impact:

PARP inhibitors make their way into clinical practice

At the start of the DDRresponse project, there was a proof of concept that PARP inhibitors could be used as a therapeutic agent to kill BRCA1 and BRCA2 deficient tumour cells. The first clinical trials also showed encouraging results. During the course of the project, PARP inhibitors first experienced some serious setbacks. The first problem was a failed phase III trial with Iniparib, which had been advertised as a PARP inhibitor. It soon became clear that the mechanism of action of this drug was not primarily via PARP inhibition, but it took time and effort to convince officials that this trial should not be interpreted as evidence that PARP inhibitors are ineffective in the clinic. Subsequently, definition of the best patient inclusion criteria was an issue to be solved. Research in DDRresponse has contributed to this discussion. Although the final word has not been said about this, a reasonable start is now that BRCA gene mutated tumours should be considered for PARP inhibitor therapy.

At this moment several PARP inhibitor studies have been concluded and several phase III clinical studies are currently running. Mainly on the basis of study 19 (Astra Zeneca) on ovarian cancer patients, EMA and FDA have now approved the PARP inhibitor Lynparza/Olaparib for (second line or maintenance) treatment of cisplatin sensitive ovarian cancer. Approval for other cancer types can be expected soon. Also other PARP inhibitors will probably be approved in the next few years.
In conclusion, DDResponse has been a very timely project that gained valuable insights that will be indispensable in the near future for more accurate definition of patients that are eligible for PARP inhibitor treatment, identification of possible resistance mechanisms (and how to counteract them), as well as providing insight into normal tissue toxicity of combination treatments and how to reduce these negative effects as much as possible.

Dissemination
The DDResponse consortium has been particularly active in providing information to the general public. We made a brochure explaining the principle behind PARP inhibitor therapy. This brochure can be downloaded from our website. We also made an animation explaining this same point, which can be watched on YouTube, with a link on our website. Together with interviews on the website and several articles in the local press with consortium members explaining how this treatment works, DDResponse has contributed considerably to the dissemination of knowledge among patients and the interested public. In addition to dissemination activities targeting a lay audience, consortium members have also contributed considerably to dissemination of knowledge at scientific meetings, often as invited speakers at conferences on DNA repair, cancer or drug discovery. The large number of papers in top rated journals can be taken as evidence for the impact of this project.

Exploitation of knowledge
DDResponse has yielded several biomarkers of PARP inhibitor sensitivity, as well as resistance mechanisms in BRCA mutated tumours. This knowledge could be developed into a signature for selection of patients for PARP inhibitor treatment. The set of genes needs to be evaluated more rigorously, but many candidate genes have been identified over the past four years. Studies on normal tissue toxicity have provided important insights into possible strategies to implement better scheduling of combination treatments involving PARP inhibitors and carboplatin. This will directly impact clinical practice. The observation that starting PARP inhibitors two days after carboplatin administration leads to much less severe bone marrow toxicity is of vital importance for finding the right scheduling.

Development of functional assays that predict treatment response may have broad implications for the prediction of therapy outcome. RAD51 foci formation is most probably a good read out for PARP inhibitor sensitivity. Furthermore, direct assessment of PARP inhibitor response is also possible, which could be developed into a standardized assay. Furthermore, the technology is also available for other treatments ex vivo, which may help to avoid unnecessary ineffective treatments. We expect that this can be implemented at least in a research setting.

List of Websites:
www.ddresponse.eu

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