Targeting DNA in therapies: using damages to design strategies on cell sensitisation

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Abstract. The unequivocal role of DNA in driving nearly all cell function designates it as one strategic target in several therapeutic anti-cancer protocols. The understanding of the structural effects of the induced DNA damage and the finding of the correspondent sensor/repair proteins are both important issues in improving therapeutic success for a given agent. Model damages and organisms can be used for such purposes. One way to achieve successful cell death derives from covalent adduction of chemical agents to nitrogen bases causing their bonding to either one or both DNA strands (monoadducts or cross-links, respectively). We present in this communication the connections between DNA repair phenotypes and other elicited responses with the structure of damages induced by chemotherapeutic agents in E. coli strains. The results outline two new experimental approaches designed to improve the impact of radiation-induced DNA strand breaks: tracking key sensor/repair proteins and finding radiosensitising drugs.

1. BACKGROUND

DNA-targeted chemotherapeutic agents belong to a group of chemical substances able to covalently bind to and modify the coding properties of the genetic material. DNA repair mechanisms can handle such modifications to avoid cell killing and mutant survivors whenever possible. If damages are too extensive to be repaired, or somehow too obstructive to prevent their processing by repair proteins, then they may critically render cells unable to continue growing. DNA-targeted anti-cancer protocols are based on such grounds [1].

2. BASIC STUDIES WITH MODEL DAMAGES IN E. coli

Despite all the complex molecular mechanisms comprised within a cell network response to a given treatment [2], survival of a cell population results from one simple “biological equation”

\[ \text{CELL KILLING} = \sum [\text{INDUCED DAMAGE}] - \text{REPAIR} \]

We selected one well-known model organism, E. coli, and just one DNA repair mechanism to counteract the induced damages, the manifold UvrABC-mediated Nucleotide Excision Repair (NER). Thus our unknown variable consisted of defining the damage profile induced by a given chemotherapeutic agent. The most common chemotherapeutic agents used in cancer therapy were
selected including, Psoralen plus UV-A; photochemotherapy (PUVA); cisplatin (cis-Pt); nitrogen mustards; and mitomycin C (MitC). The purpose was to screen for their lethal potentiality in terms of inactivation a DNA repair deficient strain.

3. Testing the ability of repair mechanisms to remove adducts from genomic DNA

*E. coli* Nucleotide Excision Repair (NER) has been pointed out as a backup mechanism for removing a broad range of DNA damaged substrates. Sancar and Rupp [3] determined the events leading to the recognition of a lesion site *via* the UvrA2B complex and bilateral strand incisions by UvrB and UvrC endonucleases, followed by removal of the damage-containing fragment. On the basis of the *in vitro* reactions performed by those authors with UvrA, UvrB and UvrC proteins acting on a UVC-induced cyclobutane pyrimidine dimer (CPD) DNA substrate, other non-UVC damages were also demonstrated *in vitro* to be repaired by the three proteins [4]. The ubiquitous action of the UvrABC mechanism on such a variety of damages was suggestive of a topological rather than chemical recognition of a given lesion within the DNA structure [4]. This assumption was evaluated by our group *in vivo*, by phenotyping the sensitivities of isogenic wild type, *uvr* A-, *uvr* B- and *uvr* C-deficient bacterial strains against PUVA, MitC, nitrogen mustards HN2 (mechlorethamine) and HN1 (2-dimethylaminomethyl-chloride hydrochloride), and cis-Pt.

4. Material and Methods

Cultures of *E. coli* wild-type and *uvr* A, *uvr* B and *uvr* C deficient strains were grown up to the exponential phase at 37°C in Lysogeny Broth (LB) medium. After that, they were washed by centrifugation to remove the medium and resuspended in salts solution, incubated with different concentrations of the above indicated agents (supplied by Sigma-Aldrich). Aliquots were taken, diluted and seeded onto the same agar-solidified LB medium to allow surviving colonies to develop after overnight incubation at 37°C. The survival ratio was obtained by dividing the number of surviving colonies after each dose by the initial number of viable cells.

5. DNA damage and repair induced by Psoralen plus UV-A (PUVA) photochemotherapy

PUVA is a well established first-line therapy for selected patients with cutaneous T-cell lymphomas (CTCL), a group of skin homing non-Hodgkin’s T-cell lymphomas and their variants [5]. This therapy is mediated by a photosensitising agent that can be taken orally prior to UVA exposure, being safe and effective for the treatment of CTCLs.

Psoralens comprise a subclass of photoreactive furocumarins, planar 3-ringed heterocyclic compounds, which have been used in PUVA therapy [6]. They intercalate between adjacent base pairs in double-stranded DNA. The two extreme rings of the molecule (pyrone and furan) are photoactive and the intercalated psoralens can photoreact with the adjacent pyrimidine bases, mostly thymines, upon near UVA irradiation (320-400nm). Monoadducts appear as psoralens bind to pyrimidines on a single DNA strand. Then a second photon can further bind the free psoralen side to the other strand if there is an available pyrimidine in the appropriate position on the opposite strand, causing part of the monoadducts to become inter-strand cross-links [7]. In the first photoreaction step either a furan-side C4,C5 double bond or the pyrone-side C3,C4 reacts with the C5,C6-double bond of the target pyrimidine base. A second photon may thus convert the furan-side monoadducts into cross-links [8]. After treatment, the two types of adducts are found in genomic DNA, monoadducts being the prevalent ones. The lesser frequent cross-links are nevertheless thought to critically impair cell function and survival, but are otherwise implicated in mutagenic consequences if the cell somehow survives.

PUVA adducts had already been described as being removed by NER in *E. coli* [9]. When modified psoralens were employed instead of the original psoralen, e.g., the clinically used 8-methoxypsoralen, we unexpectedly found a high sensitivity of the *uvr*B-deficient *E. coli* strain with no significant sensitivity inflicted to *uvr*A and *uvr*C ones. Such killing of the *uvr*B strain alone by PUVA became even greater when the monofunctional 3-carbethoxypsoralen analog was employed [10].
Different damage recognition processes were thus supposed to occur within the predicted UvrABC mechanism, depending on the structural deformations caused by a particular psoralen on the DNA substrate [10]. Besides the suggested differences in the way different psoralens could affect the DNA structure, other novel damages were shown to be induced by PUVA by means of mass spectrometry analysis of the different DNA samples [11].

6. DNA damage and repair induced by mitomycin C

Mitomycin C (MitC) is a natural antitumor drug discovered in the 1950s by Japanese microbiologists in fermentation cultures of *Streptomyces caesapisosus* [12] and has been used in cancer chemotherapy since 1974 after being approved by the Food and Drug Administration [13]. It is largely used against solid tumors such as breast, stomach, lung and prostate cancer, being especially effective against colorectal cancer [14].

MitC damages DNA by mono- and bifunctional alkylations at N² positions of guanines, resulting in both MitC-guanine monoadducts and MitC-guanine diadducts, which can occur as intra-strand diadducts or inter-strand cross-links [15]. Both the mono- and diadducted DNA lesions occur only after reduction of MitC molecules, since those that are not reduced cannot bind to DNA [16]. Upon enzymatic or chemical reduction of the quinine, however, a cascade of spontaneous transformations ensues, culminating in the opening of the aziridine ring to produce the unstable vinylogous quinine methide C¹ carbon which has high alkylating reactivity with the N² amino groups of guanines. Additional intra- or inter-strand cross-links may also form by means of an extra reaction with another available guanine with the MitC C¹⁰ position [17]. The reactivity of MitC with guanines depends on their sequence context; the monoaalkylation reactions towards 5'-d(CpG)-3' sequences [18] are preferred because they allow the second crosslinking reaction with the opposite strand. Cross-links do not occur unless guanines are in such sequence orientation, while monoadducts MitC-guanine can be detected elsewhere [19]. The removal of MitC-induced cross-links has been previously suggested as depending on a complex pathway involving incisions by NER followed by homologous recombination repair [20].

The same approach detailing the *in vitro* repair of CPD was reproduced by other authors to check whether MitC-induced adducts could be repaired by the UvrABC complex. This appeared to be the case. The similarity between other bacterial responses arisen from both UVC and MitC treatments caused the latter to be considered a “UVC mimetics” agent [4]. We detected the same UvrB-dependent phenomenon while assaying survival to MitC in *uvrA*-, *uvrB*-, and *uvrC*-deficient *E. coli* strains *in vivo* [21] and also by measuring gene expression after either MitC or UVC treatments [22]. Taken together, our MitC results reinforce the existence of an NER-uncoupled role for UvrB in the repair of particular DNA adducts, like those derived from PUVA with the 8-methoxyl or 3-carbethoxyl psoralen analogs [10].

7. DNA damage and repair induced by cisplatin

Platinating compounds, such as, cis-diamminedichloroplatinum (cisplatin), carboplatin and oxiplatin, represent an important class of chemotherapeutic agents used alone or together with radiotherapy against a broad spectrum of tumors including head and neck, testicular, ovarian, cervical, lung, colorectal and relapsed lymphoma [23]. Cis-Pt is intracellularly activated by displacement of its chlorine ligands by water to form a positively charged aquated platinum complex. The platinum atom is allowed to stably bind to DNA, RNA, proteins or other critical macromolecules [24], although it is by far accepted that DNA is the main target of cis-Pt induced cytotoxic effects. Monoadducts can be induced by covalent binding of cis-Pt at N⁷ positions of guanines and adenines in the DNA. The initially formed monoadducts at N⁷ positions in 5'-d(GpG)-3' or 5'-d(ApG)-3' sequences can result in either intra- or inter-strand DNA cross-links after reaction with another adjacent purine. Platinated sites cause the DNA to bend, with loss of critical functions such as DNA synthesis, in which case may trigger cell death by apoptosis [25]. Intra-strand diadducts (1,3 d(GNG), 1,2-d(ApG) and 1,2-d(GpG)) are efficiently induced by cis-Pt and seem to account for its pronounced lethal effects [26].
NER has been pointed out as the cell’s main tool for removing platinated adducts recognized by means of the remarkable conformational changes they introduce within the DNA structure. The importance of NER was experimentally attributed to the full recovery of cis-Pt damaged plasmids if they were subjected to prior action of UvrABC proteins \textit{in vitro} [27]. Additionally, Beck \textit{et al}. [28] revealed the striking sensitivity of DNA repair-deficient bacterial strains after exposure to cis-Pt as measured by loss of colony forming ability when compared to wild-type cells. Inter-strand cross-links are supposed to depend on the action of homologous recombination proteins, as was the case for MitC [20,29].

We have tested the ability of \textit{E. coli} NER deficient strains to survive cis-Pt-induced challenge. Particular strains were constructed for this purpose, either single or double \textit{uvr}-deficient strains, among others, following their expression of the different \textit{uvr} genes after cis-Pt treatment. The entire UvrABC protein set is key for surviving cis-Pt-induced damage, as evaluated by the similar sensitivity of single and double \textit{uvr}A \textit{uvr}B recognition-deficient, and \textit{uvr}B \textit{uvr}C endonuclease-deficient strains [30]. Importantly, cis-Pt appears to challenge cells heavily, as the expression of the \textit{uvr}A gene increases even after 4h post-treatment when compared to the same amount of UVC-induced damage [30]. Taking into account that the 1,2 intra-strand diadducts are repaired less efficiently than the 1,3 diadducts [31], it seems reasonable to suggest that they may be responsible for the observed response.

8. DNA damage and repair induced by nitrogen mustards

The search for alkylating agents to be used in tumor chemotherapy relies on the supposition that inter-strand cross-links can efficiently induce death of malignant cells through blockage of DNA replication [4]. Mustard compounds emerged as radiomimetic chemical agents in terms of the elicited modifications observed within the cell nucleus [32]. Mechloretamine (HN2) was the first developed anti-cancer agent [33] and it is presently used against Hodgkin’s Disease alone or together with other anti-cancer drugs in a therapeutic cocktail [34]. Other mustard derivatives are used against cutaneous T-cell lymphomas [35] and brain tumors [36].

Upon reaching the intracellular medium, HN2 loses its chlorine atoms, becoming reactive towards N7 positions in guanines. Reactions may occur with a single guanine base (monoadducts), or with guanines in the same (intra-strand diadducts) or in opposite DNA strands (inter-strand cross-links) [37]. Quantitative analysis reports HN2 to efficiently induce monoadducts and intra-strand diadducts, with inter-strand cross-links consisting of about 4% of the total induced damage [38]. Monofunctional mustard HN1 is considered a non-therapeutical chemical analog of HN2, because it harbors a single reactive site capable of generating monoadducts [38].

Repair of HN2-induced damage in \textit{E. coli} was shown to require the entire set of Uvr proteins [39]. Interestingly, a previous \textit{in vitro} reference suggesting increased lability of glycosidic bonds by HN2-guanine induced adducts [37] was further reinforced by our \textit{in vivo} results [39]. Weakened binding of the base with the DNA backbone may cause its loss (abasic sites), with great impact on genome stability and cell survival. This harmful damage may account for a meaningful killing effect in whatever DNA repair-deficient background and opens up the prospective development of new protocols to improve HN2 reactivity with DNA.

There is lack of information concerning possible metabolic activation of mustards in the cellular milieu, as is fairly well-known for MitC. This line of evidence prompted us to investigate the influence of the pro-oxidant status on lethality induced by HN2, assuming that increased respiratory rates might add extra damage to HN2-treated \textit{E. coli} cells. As expected, that indeed appeared to be the case [40].

9. Concluding remarks on DNA damage and repair of chemotherapeutic-induced damage in \textit{E. coli}

The phenotypes obtained for each agent appeared to require either a UVC-like UvrABC mechanism (cis-Pt and HN2 nitrogen mustard) or an uncoupled UvrB-dependent mechanism (PUVA and MitC). Consequently, one can infer that, in the absence of a functional UvrB enzyme, there may be no backup
system to manage and to repair even the smallest number of lethal events [10]. Of note, is the fact that gene expression in UVC-, MitC- or cis-Pt-treated cells is connected with the observed correspondent phenotypes [22,30]. Altogether, these results suggest the possibility of new forms of damage recognition/repair of non-UV damage by E. coli Uvr proteins. In silico analysis of the above-mentioned DNA damages are being carried out in molecular dynamics calculations to provide structural hints that might explain the collected biological data.

The panel summarised in Table 1 compares the DNA reactive targets, induced damages and repair for each of the studied agents. An overview of the collected data reveals a general connection between the occurrence of DNA intra-strand diadducts with repair by the entire set of Uvr proteins (Table 1). Particular features of the structures of psoralen and MitC may bypass the requirement for recognition by UvrA, prompting direct access to repair by UvrB.

Table 1. Summary of newly found evidence for repair of DNA adducts in E. coli.

| Agent                    | Predicted targets in DNA | Predicted induced damage | Involvement of NER proteins |
|--------------------------|--------------------------|--------------------------|-----------------------------|
| UVC                      | C5′-C6′ double bonds in thymines | Intra-strand diadducts (CPD) [4] | Requirement of the full set UvrA, UvrB and UvrC proteins* |
| PUVA (psoralen)          | C5′-C6′ double bonds in thymines | Monoadducts and cross-links [4] | Requirement of the full set UvrA, UvrB and UvrC proteins [10] |
| PUVA (8-methoxypsoralen or 3-carbethoxypsoralen) | C5′-C6′ double bonds in thymines | Mainly monoadducts, cross-links [41] | UvrB plus unidentified protein(s), non-UvrA and non-UvrC [10] |
| Cisplatin                | N7-Gua, N7-Ade           | Intra-strand diadducts, Monoadducts and cross-links [23] | Requirement of the full set UvrA, UvrB and UvrC proteins [30] |
| Mitomycin C              | N2-Gua                   | Monoadducts and intra-strand diadducts, cross-links [15] | UvrB plus unidentified protein(s), non-UvrA and non-UvrC [21] |
| Bifunctional nitrogen mustard HN2 | N7-Gua, N3-Ade | Monoadducts and intra-strand diadducts, abasic sites cross-links [38] | Requirement of the full set UvrA, UvrB and UvrC proteins [39] |
| Monofunctional nitrogen mustard HN1 | N7-Gua | Monoadducts, abasic sites [38] | No requirement for UvrA, UvrB and UvrC proteins [39] |

* according to [4]

Table 2 indicate the degree of toxicity from each treatment under conditions of the ineffective removal of the induced damage, a situation similar to what occurs in malignant cells.

According to the data, PUVA photochemotherapy is by far the most lethal treatment, followed by MitC and cis-Pt, and then by mustards. Altogether these results indicate potential reactivity of the agent with its DNA target bases, as previously shown in Table 1. Excluding the UVA-requiring PUVA treatment, alkylating agents MitC and cis-Pt seem to promptly react with their target purines, with high yields of DNA damaged sites per unit concentration.
Table 2 Comparative killing effect by DNA-damaging agents based upon analysing the survival curves obtained for a uvrA-deficient E. coli strain.

| DNA-DAMAGING AGENT       | DOSE TO 10% SURVIVAL | KILLING RATEb |
|--------------------------|----------------------|---------------|
| UVCa                     | 2.0 J.m⁻²            | 1x10⁻¹        |
| PUVA (psoralen)          | 0.25µM               | 1x10⁰         |
| MITOMYCIN C              | 1.48µM               | 3x10⁻¹        |
| CISPLATIN                | 3.30µM               | 1x10⁻¹        |
| BIFUNCTIONAL MUSTARD     | 250µM                | 5x10⁻⁴        |
| MONOFUNCTIONAL MUSTARD   | Above experimental feasibility | 4x10⁻⁶ |

a the killing effects obtained by the UVC-induced model damage CPD are shown here for comparison.
b calculated as the angular coefficient a from survival curves obtained after treatment of the uvrA-deficient strain, according to the linear function \( y = ax + b \)

In conclusion:

Toxic effects of DNA damaging agents can be comparatively measured by using a well-known organism such as E. coli. DNA repair proteins are envisaged as highly conserved entities from bacteria to man [42] to allow preliminary inferences of their impact on human cells;

The removal of particular damages from the genome may require a specific DNA repair function, with no backup activity from other available mechanisms. The knowledge about such key DNA repair proteins may help find candidates for future gene silencing protocols to increment cell lethality induced by the correspondent chemotherapeutic agent.

10. USING DNA DAMAGES TO DESIGN STRATEGIES ON CELL SENSITISATION

The success of anti-cancer treatments relies on their ability to eliminate malignant cells with minimal, if any, irreversible toxic effects inflicted on adjacent normal tissues. Our main concern about cell killing effects focuses on DNA-targeted chemotherapy, as seen by the acquisition of data from a model organism like E. coli. It is the present goal to apply some of these promising results to anti-cancer therapy. The most important aspects of this issue are briefly presented below.

10.1.1. Current radiotherapy protocols and perspectives. Radiosensitive solid tumours may have their growth significantly slowed down by the use of ionising radiation due to its blocking effect on cell cycle progression [1,2]. Nevertheless, counteracting mechanisms ensue. Huge amounts of DNA strand breaks originating from the irradiation can be sealed by the complex activity of DNA Ligases [43]. Radioresistance in tumours also benefit from hypoxic conditions leading to lower amounts of indirect DNA damage [44], and this subject has been also addressed carefully [45].

10.1.2. Current chemotherapy protocols and perspectives. They are based upon the premise that malignant cells are genetically unstable as a consequence of failure in DNA repair mechanisms [46]. Such desirable cytotoxicity caused by chemotherapeutic agents may be overcome by the induction of multidrug resistance in malignant neoplasms [47], and overexpression of DNA repair functions [48]. In the first case, multidrug resistance is promptly expressed in chemically-treated cancer cells allowing extrusion of any toxic molecule delivered to the intracellular medium [49] causing dramatic tumour resistance to chemotherapy. Chemoresistance can be minimised by employing chemotherapeutic cocktails, in which the DNA-targeted drug is administered together with other multi-targeted toxic drugs. In the latter case, as tumour cells are continuously exposed to successive doses of a given drug and DNA damage accumulates, repair mechanisms are required for their removal. For instance, resistance to cis-Pt, appears as tumor cells overexpress human NER analog proteins XPA and ERCC1-XPF [50], while responsiveness in testicular cancer is attributed to
low expression of the same proteins [51]. Therefore, massive amounts of work must be done by cancer researchers to develop more efficient drugs to overcome tumour chemoresistance mechanisms [46,52].

10.1.3. Current radiosensitising approaches. Chemically-induced DNA damage may add to radio-induced damage by either enhancing strand breaks or by blocking access to DNA repair enzymes [53]. From the chemotherapeutic agents studied here, only cis-Pt has been largely used in radiosensitisation protocols against solid tumours [54,55]. Cisplatin is thought to block repair of strand breaks by causing structural hindrances to approaching DNA repair enzymes [56]. Accordingly, base analogs can be useful sensitisers to increment radioinduced DNA breaks [53,55]. Systemic toxicity, arising from the combination of two highly genotoxic agents, is the imposed price to the patient [57].

10.1.4. New perspectives in the search of radiosensitising drugs. Efficient chemical sensitisation is implied in finding adequate drug structures with an affinity for interacting with the DNA. High affinity means target specificity and can potentially contribute to enhance the radiation impact on the genome. A picture of such an idea can be drawn from the lethal effects of PUVA (see Table 2) due to the high affinity of psoralen for the DNA environment. Additionally, important physiological features must be considered for promising candidates: low toxicity to healthy tissues, selectivity towards malignant cells, and easy turnover by normal cells. Additionally, the drug must be pharmacologically available to the target tissue at the moment of irradiation [58]. We are currently investigating an often forgotten class of DNA-interacting drugs, the intercalants [59]. They comprise a class of fused planar rings, with great affinity towards the inner base pair environment within the double helix, without any covalent binding to DNA. Such a property may diminish toxicity to normal cells. Besides their potential helix destabilising effects [60] capable of improving access to radiolytic radicals to the very inner regions within DNA, they may be positively charged, a great advantage in terms of clustering radiation-induced negatively charged ions and radicals. DNA radioinduced damage would presumably be enhanced if these properties were available in the same molecule. A variety of candidates are currently under in vitro investigation by our group.

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