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ORIGINAL ARTICLE

Enhancing the activity of platinum-based drugs by improved inhibitors of ERCC1–XPF-mediated DNA repair

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Abstract

Purpose The ERCC1–XPF 5′–3′ DNA endonuclease complex is involved in the nucleotide excision repair pathway and in the DNA inter-strand crosslink repair pathway, two key mechanisms modulating the activity of chemotherapeutic alkylating agents in cancer cells. Inhibitors of the interaction between ERCC1 and XPF can be used to sensitize cancer cells to such drugs.

Methods We tested recently synthesized new generation inhibitors of this interaction and evaluated their capacity to sensitize cancer cells to the genotoxic activity of agents in synergy studies, as well as their capacity to inhibit the protein–protein interaction in cancer cells using proximity ligation assay.

Results Compound **B9** showed the best activity being synergistic with cisplatin and mitomycin C in both colon and lung cancer cells. Also, **B9** abolished the interaction between ERCC1 and XPF in cancer cells as shown by proximity ligation assay. Results of different compounds correlated with values from our previously obtained in silico predictions.

Conclusion Our results confirm the feasibility of the approach of targeting the protein–protein interaction between ERCC1 and XPF to sensitize cancer cells to alkylating agents, thanks to the improved binding affinity of the newly synthesized compounds.

Keywords DNA repair · Protein–protein interaction · Chemical synthesis · Cancer

Introduction

A well-functioning DNA repair apparatus naturally allows cells to be protected from endogenous and exogenous damages, preserving health status and integrity of tissues. Although the DNA repair mechanisms are physiological in cells, they can be self-defeating in cancer therapy, as they can interfere with the DNA damage inflicted by therapies in tumour cells. The heterodimer ERCC1–XPF is a 5′–3′ endonuclease formed by ERCC1, which is involved in DNA–protein and protein–protein interactions, and XPF, which retains the endonuclease active site. This enzyme belongs to

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structure-specific endonucleases, since its mechanism of action involves the cleavage of hanging single strand portion of DNA from double stranded filaments. ERCC1–XPF is part of the nucleotide excision repair (NER) pathway, responsible for repairing lesions such as bulky helix distortions like cyclobutane pyrimidine dimers induced by UV irradiation. Other DNA repair mechanisms in which this enzyme is involved are inter-strand crosslinks repair (ICL) and double-strand breaks repair (DSB) [1–3]. NER and ICL are the mechanisms primarily involved in development of resistance to DNA damaging agents such as cisplatin, mitomycin C and cyclophosphamide; therefore, the inhibition of these mechanisms may overcome cancer resistance and increase effects of chemotherapy on tumours [4, 5]. Downregulation of ERCC1 and XPF by siRNA has been shown to decrease DNA repair and enhance the sensitivity of several cancer cell lines to cisplatin [6–8].

One approach to inhibiting ERCC1–XPF is through the use of small molecules that target ERCC1–XPF interaction [9, 10]. Properly executing this strategy allows one to employ combination cancer therapy using genotoxic chemotherapeutic agents with DNA repair inhibitors. Indeed, the co-administration of the two agents to patients may improve the efficacy of widely used DNA crosslinking drugs such as the platinum based agents [11, 12]. Cancer chemotherapy outcomes for patients treated with DNA crosslinking drugs depend on the tumoral stage and type, but above all on the cancer cell biology. Indeed, the outcome of platinum-based chemotherapy is influenced by different cellular processes including those upstream of DNA damage/repair mechanisms such as cellular uptake and efflux and detoxification by cytoplasmic proteins, and in cell death-triggering processes such as DNA damage detection and apoptosis [13, 14].

In previous studies we designed and synthesized various ERCC1–XPF inhibitors aiming to improve effects of widespread DNA crosslinking drugs like cisplatin and mitomycin C [9, 10]. Preliminary in vitro assays confirmed that F06 shows promising inhibitory effect against ERCC1–XPF endonuclease activity and acts synergistically with cisplatin and mitomycin C [9]. However, the activity of F06 is suboptimal in terms of clinical properties, including its potency and pharmacokinetic profile, and a derivatization strategy was adapted to optimize the action of the compound [10, 15, 16]. After a successful synthesis of the top in silico screened compounds, they were subjected to several cell-free and cell-based assays. The results yielded two potent compounds, A4, previously named as compound 4, and B9, that showed a significant sensitization of colorectal cancer cells to cyclophosphamide and UV radiation [10, 15, 16]. Here, we continue our effort to evaluate these improved molecules intended for combination cancer therapy. The influence of the new compounds on the action of traditional DNA crosslinking drugs has been investigated in the present study through synergy studies between the DNA crosslinking drugs and the ERCC1–XPF inhibitors.

Materials and methods

Overview of in silico design strategy for ERCC1–XPF inhibitors

Since the design and screening of F06 analogues were performed as described previously [10, 15], we only provide a very brief outline. The chemical structures were obtained by modifying different F06 sites using an in-house collection of molecular fragments [10] and Molecular Operating Environment (MOE) MedChem transformations (Chemical Computing Group Inc, 2015, Molecular Operating Environment, MOE, 2015). Molecules were docked on the XPF surface to a pocket involved in key interactions with ERCC1 using Pharmacophore docking in MOE Dock, and scored using generalized Born Volume Integral/Weighted Surface Area (GBVI/WSA) function [17]. Rescoring was performed using 2 ns of molecular dynamics simulations of the ligand–receptor complexes, and Molecular Mechanics/Generalized Born Surface Area (MM/GBSA).

Synthesis and characterization of ERCC1–XPF inhibitors

Synthesis of **F06**, **A2** and **A4** has been previously described [15]. The synthesis of **B9** (4-((6-chloro-2-methoxyacridin-9-yl)amino)-2-((4-(2-(dimethylamino)ethyl) piperazin-1-yl) methyl) phenol) was previously reported as **B5** [10]. General synthetic route of **D7** was performed through nucleophilic aromatic substitution reaction by mixing 6,9-dichloro-hydroxyacridine and 2-amino-4,5-dimethoxybenzonitrile.

MTT cytotoxicity and synergy assays

Cytotoxicity assays were performed as described before [10], using human lung cancer (A549) and human colon cancer (HCT-116) cell lines purchased from American Type Culture Collection (Manassas, VA). Cells (3000 per well) were seeded in 96-well plates in 100 μL media and allowed to adhere before different concentrations of compounds were added. After 72 h in culture, MTT (1 μg per well) was added and replaced by 100 μL isopropanol/H_2O/HCl 90/9/1 v/v/v after 2 h incubation. Finally, absorbance was determined at 570 and 690 nm with a Multiskan EX bench-top microplate reader (ThermoFisher, Waltham, USA). For synergy assays, ERCC1/XPF inhibitors and alkylating agents were added in fixed ratios (close to ratios of the IC_{50}). Values for IC_{50} and combination index 95 (CI95) were calculated using CompuSyn software 1.0 (ComboSyn, Inc., Paramus, NJ, USA). Effect of associations was indicated as synergy if $CI95 < 0.9$, additive if $0.9 < CI95 < 1.1$, and antagonistic if $CI95 > 1.1$.

Apoptosis assay

A549 and HCT-116 cells were seeded in 24-well plates at a density of 50,000 cells per well. Cells were incubated in complete media and left overnight to adhere before adding compounds at indicated concentrations. Upon a further incubation of 45 h, cells were washed with PBS and stained with AnnexinV-Fluos Staining kit (Roche) as indicated by the manufacturer. Cells were analysed by FACS (Fortessa, BD Biosciences, Franklin Lakes, NJ, USA) and the percentage of living cells (AnnexinV negative and propidium iodide negative), was used to measure the activity of drugs and combinations.

Proximity ligation assay

A549 cells were seeded in an 8-well Nunc Lab-Tek Chamber Slide system at a density of 30,000 cells per well. The cells were left to adhere for 24 h before adding the compounds alone or in combinations as indicated in the Results section. The plate was incubated for 24 h and then processed for protein proximity analysis using the Duolink assay (Olink Bioscience, Uppsala, Sweden) with an ERCC1 antibody (FL-297, 1/100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an XPF antibody (LS-C173159, 1/100; LifeSpan BioSciences, Seattle, WA). The samples were then fixed and stained with DAPI. Cells were observed using a ZEISS Axio Scan.Z1 slide scanner (ZEISS, Oberkochen, Germany). Images of the red dots representing the interaction of ERCC1 and XPF were analysed using the ImageJ software (LOCI, University of Wisconsin, USA). Both cell number and dots quantity were assessed by automatic counting in each microscopic field. A total of eight different microscopic fields and more than 1400 cells were analysed per condition. Results are expressed as mean values from two experiments conducted independently.

Results

Synthesis of F06-based analogues

A2, **A4** and **B9** were top-ranked analogues, with MM/GBSA scores of -11.60, -13.12, and − 12.44 kcal/mol, respectively, and thus were selected for synthesis and testing. For comparison, the corresponding value for **F06** was − 17.78 kcal/mol. **D7** was manually designed as a non-active compound, screened in the in vitro ERCC1–XPF endonuclease assay (data not shown). Synthesis of compounds **F06**, **A2** and **A4** was achieved through a one-pot sequential addition reaction in three steps as reported before [15]. In summary, this Mannich-type reaction of *p*-acetamidophenol with formaldehyde and the appropriate secondary amine in 2-propanol was carried out under reflux for 12 h. The solvent and the excess of unreacted formaldehyde from the resulting mixture were removed under vacuum, and without isolating the compound, the resulting viscous residue was treated with 6 M HCl to deacetylate the acetamido group and furnish the primary amine. Afterwards, an equimolar amount of 6,9-dichloro-2-methoxyacridine was added, affording, after heating, compounds **F06**, **A2** and **A4** in moderate to good yields after isolation. The synthesis is general, easy, and reproducible. All synthesized compounds were characterized by ^{1}H NMR, ^{13}C NMR, HRMS, IR, and the purity of compounds **A4** and **B9** was determined by HPLC ($\geq 95\%$ purity) as reported before [15]. The synthesis of **B9** was accomplished through the same synthetic route as **A4** except the last step, where 1 eq of the unprotected aniline intermediate reacts with 1 eq 6,9-dichloro-2-hydroxyacridine. Nucleophilic aromatic substitution reaction was carried out to synthesize **D7** by reacting 6,9-dichloro-hydroxyacridine and 2-amino-4,5-dimethoxybenzonitrile. Figure 1 shows the chemical structures of these studied compounds.

Synergy analysis for the inhibitors with cisplatin and mitomycin C

The classic MTT assay was chosen to perform synergy studies with the conventional chemotherapy drugs cisplatin and mitomycin C in association with the new inhibitors of the ERCC1–XPF interaction. Studies were performed on A549 and HCT-116 cell lines, which were chosen as models for lung and colon cancer, respectively, as these pathologies are often treated using DNA crosslinking agents and that they have already been shown to be sensitive to such an approach [9].

The intrinsic activity (IC50 for antiproliferative activity), as determined by MTT assay, was similar for the new compounds, although a little bit lower for compound **D7** ($p < 0.01$ for comparisons with all other compounds on both cell lines), and in the low micromolar range (Table 1).

Furthermore, we assessed the synergistic activity between ERCC1/XPF inhibitors and the DNA damaging agents cisplatin and mitomycin C. CI95 values obtained from the synergy experiments performed on A549 and HCT-116 (Fig. 2), indicate that the optimized compounds **A4** and **B9** exhibit a synergistic behaviour with cisplatin, comparable with the one displayed by **F06**. Synergy of the inhibitors with mitomycin C is less pronounced compared with the same results for cisplatin, even if a slight difference is evident comparing the synergistic compounds and the negative controls.

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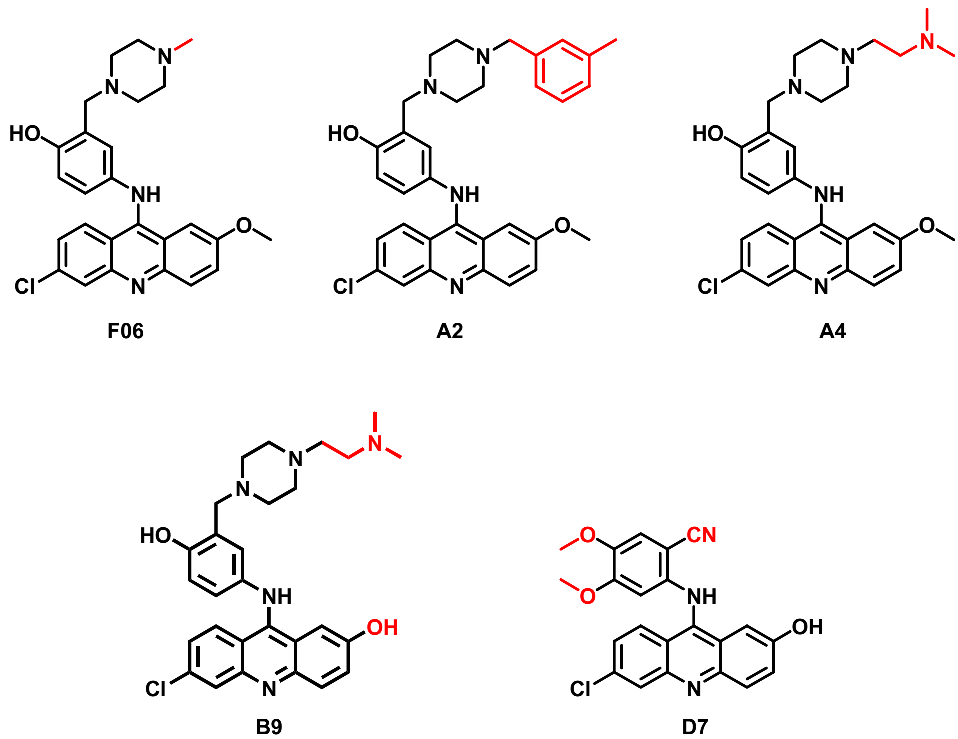

Fig. 1 Structures of ERCC1/XPF inhibitors studied. Structural differences with **F06** are highlighted in red

Table 1 Intrinsic antiproliferative activity of new compounds

Compound	A2	D7	A4	B9	F06
A549 (IC50 (μM))	4.3 ± 1.1	7.9 ± 0.8	2.0 ± 0.3	4.2 ± 0.7	3.2 ± 0.5
HCT-116 (IC50 (μM))	4.6 ± 0.9	12.0 ± 1.4	1.0 ± 0.3	1.3 ± 0.2	1.3 ± 0.3

Data are mean IC50 ± SEM (μM) of seven independent experiments

Synergies were either moderate (0.7 < CI95 < 0.85) or firm (0.3 < CI95 < 0.7). In contrast, strong antagonism between **D7** and cisplatin was observed in both A549 and HCT-116 cells. Compound **A2** was found to have a slight synergistic or additive behaviour, and this was evident mostly in the A549 cells if used together with mitomycin C (CI95 0.46–0.61). **A2** displays an additive effect also in the A549 cells, together with cisplatin, with an estimation of CI95 in the interval of 0.50–1.5.

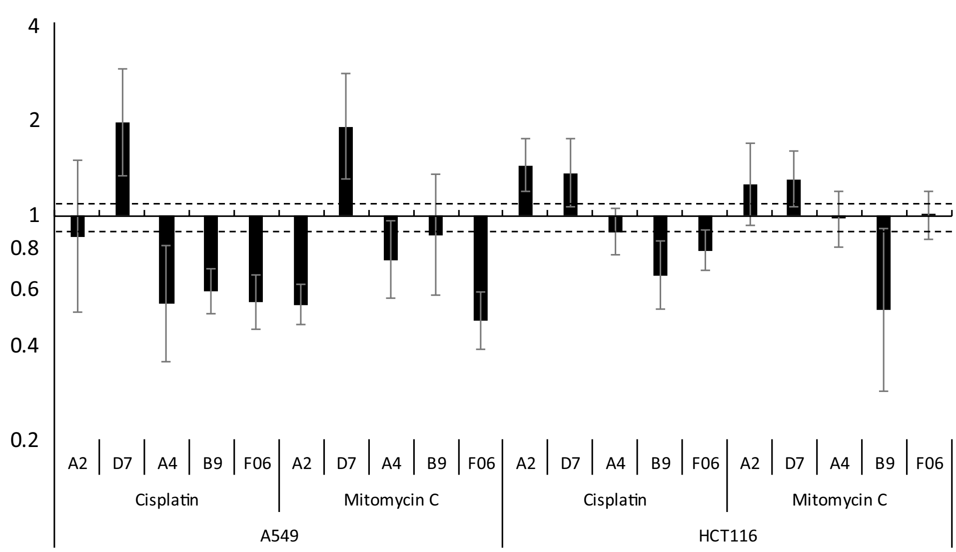

Fig. 2 CI95 values detected in A549 and HCT-116 cells exposed to cisplatin or mitomycin C in association with ERCC1–XPF inhibitors. Results are means and error bars are SEM from at least five different experiments. Dotted lines indicate values of CI95 = 0.9 and 1.1

B9 potentiates apoptosis induced by alkylating agents

To confirm the results obtained from the synergy experiments with the MTT assay, we determined the cell survival after exposure to **A4** or **B9** together with cisplatin and mitomycin C using AnnexinV and propidium iodide staining. Data clearly shows that **B9** in combination with both cisplatin and mitomycin C strongly enhances the cytotoxic action in both A549 (4.8% ± 4.7% vs 71.0% ± 2.0% live cells for 10 µM cisplatin with and without **B9**, 4.5% ± 2.3% vs 69.1% ± 2.4% live cells for 1 µM mitomycin C with and without **B9**) and HCT-116 cells (38.6% ± 11.6% vs 78.7% ± 4.4% live cells for 5 µM cisplatin with and without **B9**, 33.0% ± 3.8% vs 70.2% ± 14.2% live cells for 1 µM mitomycin C with and without **B9**), as compared to the effects of each compound alone (Fig. 3). **A4** did not show any potentiating effect in these experiments.

Interaction between ERCC1 and XPF is disrupted by ERCC1–XPF inhibitors in cells

Our hypothesis is based on the inhibition of the protein–protein interaction between ERCC1 and XPF, resulting in decreased NER activity and subsequently in a better activity of alkylating agents. To confirm that our compounds are able to disrupt the interaction between these proteins in cells, we performed a proximity ligation assay using A549

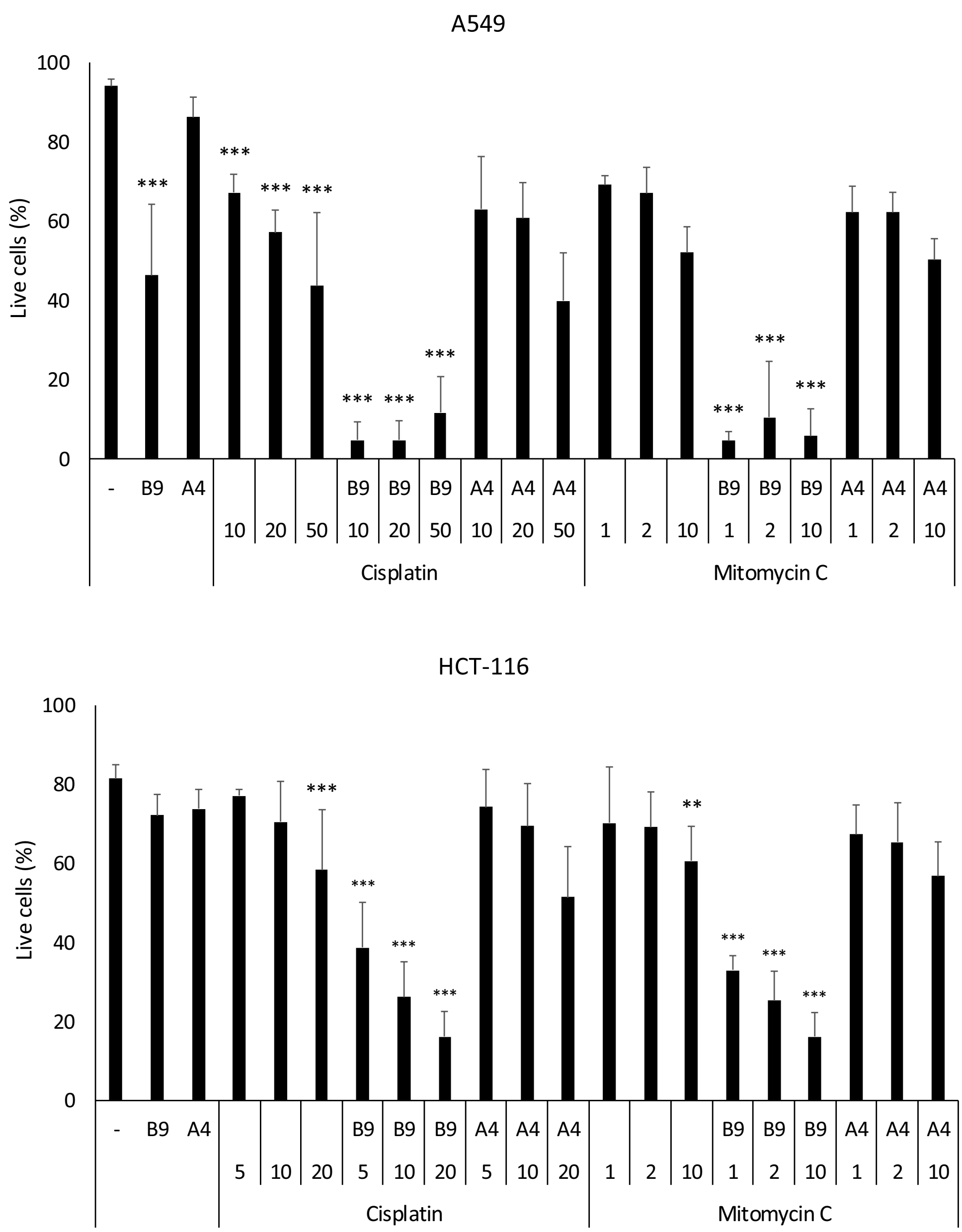

Fig. 3 Survival of A549 and HCT-116 cells exposed to cisplatin or mitomycin C (µM) alone or in combination with 1 µM **A4** or **B9**. Graphs show mean values of cell survival from three independent experiments performed in duplicate, and error bars are standard deviation. Statistical analysis was performed using one-way ANOVA tests. $^{**}p < 0.01$ and $^{***}p < 0.001$ as compared either to cells without cisplatin or mitomycin C or to cells without B9

cells exposed to compounds alone or in combination. Upon addition of cisplatin, an increase of ERCC1 and XPF interaction as shown by the foci is observed, going from 15.6 foci per cell in the unexposed cells to 56.6 foci per cell (Fig. 4 and Table 2). This enhanced interaction was compromised by **F06** as we only observed 18.2 foci per cell when cisplatin was combined with F06. Even stronger results were obtained with **A4** and **B9** for which we observed 13.8 and 2.2 foci per cell after exposure together with cisplatin, respectively. The important decrease of interaction between ERCC1 and XPF by **B9** was also observed in the absence of cisplatin. Altogether, these results show that both **A4** and in particular **B9**, possess improved efficiency in inhibiting the formation of the ERCC1–XPF complex in cells as compared to the first-generation compound **F06**. This is in line with their better biological activity and strengthens our hypothesis for the mechanism of action of the association between the new compounds and the alkylating agents.

Conclusions and discussion

Cisplatin and mitomycin C are widely used cancer chemotherapy agents and constitute the elective therapy for many tumour types [10, 17]. However, discontinuation of platinum-based therapies is common among patients because of the development of drug resistance and the high toxicity and side effects brought about by the therapy [18, 19]. Therefore,

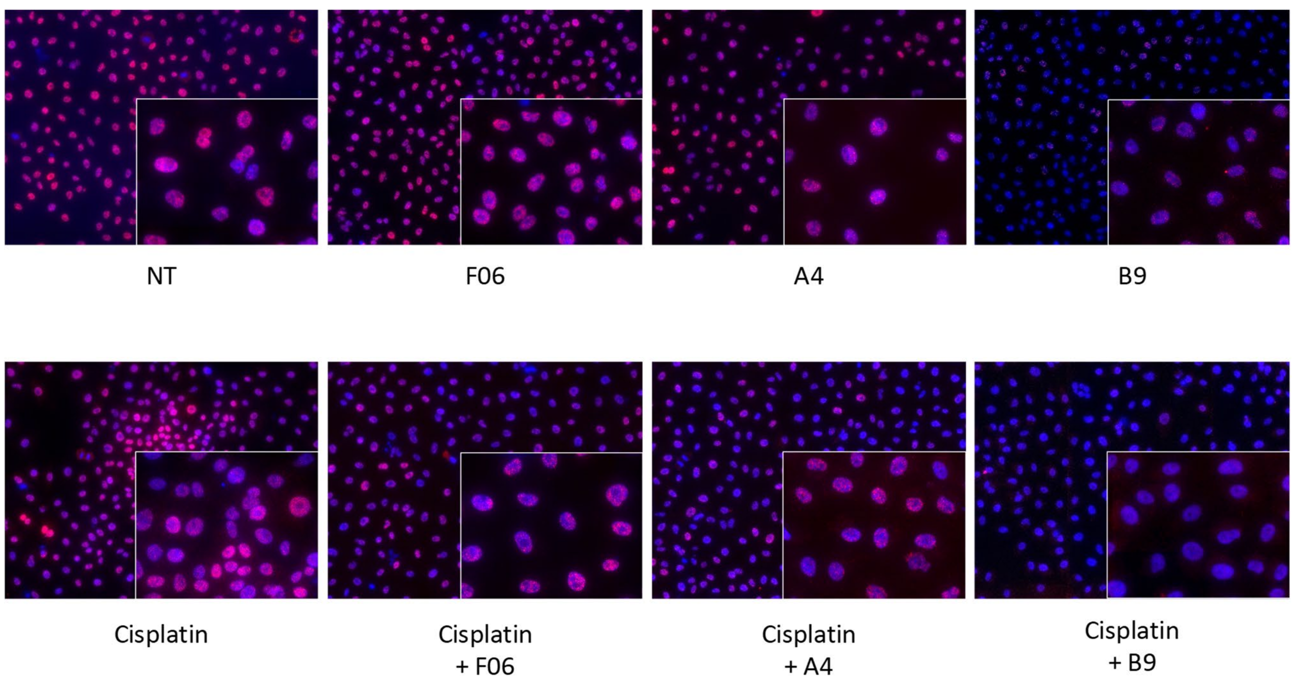

Fig. 4 Representative PLA images on A549 cells exposed to F06 (1 µM), A4 (1 µM), B9 (1 µM) and cisplatin (20 µM) alone or in combination for 24 h. Images were obtained at 40X magnification, analysed fields are shoved on the background of each square. On the lower-right zones, zoomed images show the ERCC1–XPF interaction complexes, visible as red dots, and cellular nuclei in blue, by DAPI staining

Table 2 ERCC1/XPF interaction in A549 cells exposed to cisplatin or ERCC1–XPF inhibitors alone or in combination at indicated concentrations for 24 h

Condition	NT	Cisplatin 20 µM	F06 1 µM	Cisplatin 20 µM + F06 1 µM	A4 1 µM	Cisplatin 20 µM + A4 1 µM	B9 1 µM	Cisplatin 20 µM + B9 1 µM
Mean number of foci per cell	15.6 ± 7.2	56.6 ± 19.8**	13.7 ± 5.0	18.2 ± 4.7**	13.5 ± 3.5	13.8 ± 3.6**	5.1 ± 3.9*	2.2 ± 2.0**
Total number of cells counted	2097	1455	2268	1831	1949	1701	2122	1506

Data represent mean number of foci per cell ± SD counted in eight different microscopic field, belonging to two independent experiments

*$p < 0.05$ and **$p < 0.0001$ as compared to condition without ERCC1/XPF inhibitor (or as compared to NT for cisplatin) using one-way ANOVA test

finding compounds able to significantly increase sensitivity to cisplatin in cisplatin-resistant tumours has a great potential in therapy and could lead to clinical advantages such as increasing the efficacy of chemotherapy at lower dosages, which would be better tolerated by the patient. Cisplatin and mitomycin C are DNA intrastrand and interstrand crosslinking agents, and the lesions they induce in DNA can be repaired by the NER or ICL DNA repair pathways, respectively. The use of cisplatin and mitomycin C has allowed us to test the inhibition of the ERCC1–XPF complex acting on these two different DNA repair pathways [9]. In this paper we report on our investigations of the activities of earlier published improved ERCC1–XPF inhibitors and their capacity to potentiate the cytotoxic activity of cisplatin and mitomycin C.

ERCC1–XPF interaction and activity are mainly dependent on the dimerization of the two C-terminal regions of the dimer as well as the endonuclease activity of XPF [5]. The formation of the heterodimer is principally due to the interaction of double helix–harpin–helix motifs, HhH2, which are present at the C-terminus dimerization interface of the two monomers. This domain is a promising target for inhibitors [5, 9] able to disrupt the interaction and, therefore, the activity of the enzyme. Despite the importance of this mechanism as a potential therapeutic route, few suitable inhibitors have been identified so far [9, 20–23]. McNeil et al. [22] investigated the dimerization HhH2 domain of XPF as a pharmacological target. By employing in silico screening techniques, they identified different inhibitors of the dimer, discovering a small NER inhibiting compound, able to improve efficacy of cisplatin in melanoma cells, even though the IC_{50} and K_d values of this molecule were suboptimal [22]. They also identified inhibitors of the active site that were able to sensitize cancer cells to cisplatin when used at low micromolar concentrations. In addition, Arora et al. [21] and Chapman et al. [24] were able to identify and optimise several inhibitors of NER activity with IC_{50} values within the nanomolar range that also improved the cytotoxicity of platinum-based chemotherapeutic agents in cancer cells. Compounds from Arora's paper did not alter the DNA binding of ERCC1/XPF and are, therefore, supposed to target the endonuclease activity. For compounds in Chapman's paper, the target is not described, although a clear inhibition of NER is observed. Although in these previously described works the specificity of inhibitors towards ERCC1–XPF endonuclease was assessed, a detailed knowledge of the molecular structure of the inhibitor-XPF complexes was not provided. Indeed, rationally designed inhibitors are specifically studied to adapt to the enzymatic binding pocket, and this was the approach we used for targeting the ERCC1/XPF interaction site. They are usually more specific, may exhibit lower off-target interactions due to similarities among binding pockets of other endonucleases, helping to increase efficacy and possibly reducing toxic effects. Recently, Thomas et al. [23] designed and optimized a fluorescence-based technique able to assess enzyme activity. Their aim was to generate a robust assay for high-throughput screening, based on the florescence signal generated by the enzymatic cleavage of specially tagged oligonucleotide substrates upon binding with the full-length enzyme ERCC1–XPF.

In our work, compounds tested were designed through docking-based virtual screening, then optimized in silico by further functionalization. This has resulted in improved binding affinity, specificity and activity of the new compounds **A4** and **B9** [15, 16], even if their pharmacological properties can be compared to the previously discovered compounds [21, 24] due to their chemical similarity based on similar scaffolds. However, because the design of **A4** and **B9** was based on precisely targeting the structure of the dimerization HhH2 domain of XPF, it most likely renders these compounds more specific for the ERCC1–XPF complex, and less likely to interfere with other proteins. However, we do not have any experimental proof of specific targeting of the ERCC1–XPF interaction in the cells. This could eventually be obtained using ERCC1- and/or XPF-deficient cells for antiproliferation assay or synergy experiments.

In the past, we have extensively demonstrated how **F06** interacts with XPF, using different techniques such as fluorescence quenching, immunoprecipitation and surface plasmon resonance assays. Moreover, proof of synergy between **F06**, cisplatin and mitomycin C has been provided, together with the ability of **F06** to interact with ERCC1–XPF in vitro, impair DNA repair, and disrupt the protein–protein interaction in cells [9]. Previous characterization and synthesis of F06 has allowed us to use this compound as benchmark in the study of the new generation of improved molecules able to target and suppress ERCC1 and XPF interaction.

In this work, we further investigated whether our top-ranked compounds, **A4** and **B9**, display a synergistic effect with cisplatin and mitomycin C in different cancer cell lines. Our MTT assay indicates that **A4** and **B9**, like **F06**, show synergy with both cisplatin and mitomycin C. The synergy appears to be slightly stronger with cisplatin than with mitomycin C. This may be because mitomycin C DNA monoadducts, which may not be repaired by NER or ICL pathways, can contribute to mitomycin C cytotoxicity [25]. We further examined the synergistic interactions to see if they induced an apoptotic response. Intriguingly, we observed a strong apoptotic response for **B9** with both cisplatin and mitomycin C compared with the crosslinking agents when used alone, but a substantially weaker response for **A4** and the crosslinking agents. This will require further exploration.

We also showed, using the proximity ligation assay, that the compounds markedly reduce interaction between ERCC1 and XPF in the assayed cell lines. The new generation inhibitors were found to be strikingly more effective in disrupting the ERCC1–XPF interaction compared to **F06**. **B9**, in particular, reduced the level of interaction, as measured by the assay, to almost zero in cells treated or untreated with cisplatin. This is, to our knowledge, the most efficient compounds reported thus far.

The most important finding of this work is that the newly developed compounds exhibit synergistic properties and appear to improve the cytotoxicity of both mitomycin C and cisplatin in the cancer cell lines tested. The reported data also confirm improved inhibitory activity towards the complex ERCC1–XPF, as shown before [15]. Therefore, we believe the new inhibitors, and in particular **B9**, show excellent promise to impede NER and ICL repair processes in cancer cells and thereby address drug resistance issues associated with well-known chemotherapeutic agents such as cisplatin and mitomycin C.

In conclusion, the new inhibitors have been shown to exhibit the predicted mode of action and can be adopted as a new route to target cancerous pathologies. They address drug resistance issues, paving the way for the development of innovative combination treatments, which may be clinically applied in combination with existing well-known chemotherapy agents such as cisplatin and mitomycin C. The next step in the development of this strategy will involve the in vivo validation of our culture-based experiments.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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