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Inhibition of nucleotide excision repair and sensitisation of cells to DNA cross-linking anticancer drugs by F 11782, a novel fluorinated epipodophylloid

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Abstract

F 11782, or 2′,3′-bis-pentafluorophenoxyacetyl-4′,6′-ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin 2-*N*-methyl glucamine salt, a novel dual catalytic inhibitor of topoisomerases I and II, was identified as a potent inhibitor of nucleotide excision repair (NER) by screening procedures using the *in vitro* 3D (DNA damage detection) assay. F 11782 was then shown predominantly to inhibit the incision rather than the repair synthesis step, using two new methodologies derived from this 3D assay, effectively ruling out any inhibition of polymerases δ/ε. Moreover, data from two other *in vitro* assays showed an absence of any effect of F 11782 on: (i) the DNA damage binding of the XPA–RPA complex, and (ii) on SV40 large T-antigen helicase activity. Therefore, the inhibitory activity of F 11782 on NER may involve an inhibition of the ERCC1-XPF or XPG endonuclease activity. Moreover, inhibition of DNA repair by F 11782 was confirmed in human A549 cells by monitoring unscheduled DNA synthesis following mechlorethamine treatment. Such an inhibition provides an explanation for the highly synergistic cytotoxicity observed against cultured A549 lung tumour cells, when F 11782 was combined with cross-linking agents, such as cisplatin or mitomycin C. These results emphasise the unique mode of action of this novel molecule in inhibiting NER and provide a basis for its evaluation in clinical trials in combination with DNA cross-linking agents.

Keywords: F 11782; Nucleotide excision repair; Drug combination; Catalytic topoisomerase inhibitor; DNA cross-linking agent

1. Introduction

During cancer chemotherapy, resistance frequently develops, thereby limiting its efficiency. Different molecular pathways have been implicated [1], including enhanced DNA repair activity in tumour cells resistant to, for example, cross-linking agents [2]. Consequently, DNA repair processes represent potential new targets for combating cellular resistance to certain classes of anticancer drugs [3]. Most DNA damage incurred by exogenous agents that cause bulky adducts is repaired by excision repair processes, and especially by NER which recognises DNA adducts induced by a range of chemical treatments [4]. These events, now fairly well characterised, are known to consist of two distinct major steps: (i) the incision reaction involving damage recognition and excision of the damaged oligonucleotide; (ii) repair synthesis of new DNA using the complementary strand as a template and its subsequent ligation to restore strand continuity [5]. At the molecular level (for details see reviews: [5,6]), the damage is first recognised by the XPC-hHR23B complex. This complex first recruits XPA and RPA and then TFIIH to form the preincision complex. TFIIH possesses both 3′–5′ and 5′–3′ helicase activity, respectively, through its XPB and XPD proteins, and unwinds DNA by about 20 base pairs around the damage. XPG and ERCC1-XPF bind to the preincision complex, while XPC dissociates leading to a more stable excinuclease complex. XPG makes

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Abbreviations: AgT, large T-antigen; ATCC, American type culture collection; BSA, bovine serum albumine; CI, combination index; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid; FCS, foetal calf serum; IC_{50}, concentration of drug corresponding to an inhibition of 50% in comparison with the solvent alone; MTT, 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyltetrazonium bromide; NER, nucleotide excision repair; PBS, phosphate-buffered saline; RLU, relative luminometric units; SPA, scintillation proximity assay; UDS, unscheduled DNA synthesis; UVC, ultraviolet light C (peak wavelength = 254 nm).

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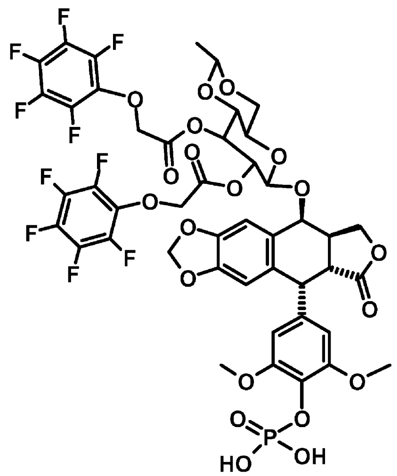

Fig. 1. Structure of F 11782 or 2′,3′-bis-pentafluorophenoxyacetyl-4′,6′-ethylidene-β-D-glucoside of 4′-phosphate-4′-dimethylepipodophyllotoxin 2-*N*-methyl glucamine salt.

the 3′ incision, whilst ERCC1-XPF makes the 5′ incision. The excised damaged DNA fragment is released and the gap generated is filled by DNA synthesis carried out by polymerases δ and ε, DNA ligase and accessory factors.

A methodology based on the *in vitro* excision repair assay initially developed by Wood *et al.* [7] has been adapted to a solid-phase assay and termed the 3D (DNA damaged detection) assay [8]. Using this technique, it is straightforward to screen for NER inhibitors and to check for any effects of various anticancer agents or antibiotics on NER [9]. During the course of such screening, F 11782 (Fig. 1), a novel fluorinated lipophylic epipodophylloid [10] with a unique mode of interaction with topoisomerases I and II [11], was identified as an active agent. Subsequently, derivative procedures of this 3D assay were used to identify and characterised *in vitro* the inhibitory activity of F 11782 against NER. Moreover, inhibition of NER by F 11782 was confirmed in cultured A549 human non-small cell lung cancer cells by monitoring UDS after treatment with mechlorethamine. Such an inhibitory activity of F 11782 should influence the cytotoxicity of DNA cross-linking agents, since their damage is repaired predominantly by NER. Indeed, using cultured A549 cells and the combination effect method [12], the cytotoxicity of F 11782 combined with either cisplatin or mitomycin C, was defined as highly synergistic.

F 11782 with marked antitumour activity in various experimental tumour models [13–15] has now completed phase I clinical trials in Europe. The effects of F 11782 against NER identified in this study provide a rationale for evaluating F 11782 in combination with DNA cross-linking agents, such as cisplatin, in subsequent phase II/III clinical studies.

2. Materials and methods

2.1. Chemicals and drugs

F 11782 (Fig. 1) and etoposide were provided by Pierre Fabre Médicament. Camptothecin was purchased from Cipla, whilst doxorubicin, aphidicolin, mechlorethamine, 5-fluoro-2′-deoxyuridine, hydroxyurea, thymidine, cisplatin and mitomycin C were obtained from Sigma. [^{3}H]-Thymidine (25 Ci/mmol) were purchased from Amersham. Cisplatin was solubilised in 0.9% sodium chloride solution prior to use, whilst etoposide, camptothecin and aphidicolin were solubilised in DMSO (SDS), used in the assay at a maximal final concentration of 0.1%. All the other drugs were solubilised in water (maximal final concentration of 1%).

2.2. Cell line and cell culture

The A549 human non-small cell lung tumour cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultivated as monolayers in a CO_2 incubator (37°, humidified atmosphere, 5% CO_2 in air) and were split twice a week in minimal essential medium (MEM) supplemented with 10% heat inactivated foetal calf serum (FCS). Cell culture media were complemented with fungizone (final concentration of 1.25 μg/mL), penicillin–streptomycin (final concentration of 100 IU–100 μg/mL) and glutamine (final concentration of 4 mM). MEM, FCS, fungizone and penicillin–streptomycin were purchased from Gibco. RPMI 1640 medium, L-glutamine and trypsin-EDTA were purchased from Seromed (Polylabo).

2.3. Detection of NER inhibition using the 3D assay

The 3D assay was performed as described previously [9]. Fifty μL of 1 μg/mL of UVC-damaged or undamaged pBS plasmids were distributed into each sensitised well and incubated for 30 min at 30° in a microplate incubator (IEMS, Labsystems) to permit adsorption. Any non-adsorbed DNA (around 20% of total DNA) was eliminated by three washes with PBS-T (phosphate buffer saline containing 0.1% Tween 20). Plasmids were then co-incubated for 3 hr at 30° with a reaction mixture containing the test drug and all the constituents necessary for the *in vitro* DNA repair reaction: 120 μg of a whole-cell extract of HeLa cells, 70 mM KCl, 0.4 μM of each dGTP, dCTP, dATP and biotinylated-dUTP (Bio-21dUTP, Boehringer Mannheim) in reaction buffer containing 40 mM Hepes-KOH (pH 7.6), 5 mM $MgCl_2$, 0.5 mM dithiotreitol, 2 mM EGTA, 10 mM phosphocreatine, 50 μg/mL of creatine phosphate and 360 μg/mL of bovine serum albumin (BSA). During this reaction, DNA damage was recognised and the excised patches were replaced by neo-synthesised DNA fragments. Throughout this step of DNA synthesis,

biotinylated-dUMPs were incorporated into DNA. The DNA repair reaction was stopped by three washes with PBS-T. Finally, a solution containing an extravidin conjugated with peroxidase (diluted 1/10,000 in PBS plus 0.025% acetylated BSA and 0.1% Nonidet P40) was distributed into each well so that during a 15 min incubation at 30° the biotinylated-dUMP incorporated during the DNA repair reaction could be recognised. After five washes with PBS-T, a solution of luminol (Aldrich) was added for a 5 min incubation at 30°. The light emitted was measured using a luminometer (Luminoskan, Labsystems) and expressed in relative luminometric units (RLU). Under these experimental conditions, the luminometric signal was proportional to the incorporated biotinylated-dUMP, and thus, to DNA repair activity.

Control experiments carried out with undamaged pBS plasmids provided the background signal. These values were subtracted systematically from the corresponding values obtained using UVC-damaged plasmids. The resultant data were then defined as the *in vitro* DNA repair activity.

2.4. Detection of incision repair or repair synthesis inhibition using the 3D assay

The protocols of these assays were adapted from those originally described by Calsou and Salles [16], and Salles and Provot [17], and were similar to that described above for evaluating NER inhibition, except that the DNA repair step was fragmented into two stages, an incision step followed by that of repair synthesis. To evaluate incision activity, 5 μM aphidicolin was co-incubated with the test compound for 2 hr at 30° with a reaction mixture containing all the constituents necessary for the *in vitro* DNA repair reaction: 120 μg of a whole-cell extract of HeLa cells, and 70 mM KCl in reaction buffer, described above. During this reaction, DNA damage was recognised and excised. Neo-synthesis of the excised DNA fragments cannot occur since the presence of aphidicolin leads to a complete and specific inhibition of DNA-polymerases δ and ε involved in repair synthesis. The incision reaction was stopped by three washes with PBS-T. This step was followed by the repair synthesis reaction: in the absence of aphidicolin, 0.5 U Klenow fragment were incubated for 20 min at 30° with 0.4 μM each of dGTP, dCTP, dATP and biotinylated-dUTP (Bio-16dUTP, Boehringer Mannheim) in reaction buffer containing 90 mM Hepes-KOH (pH 6.6), 5 mM $MgCl_2$ and 3 mM $MnCl_2$. Throughout this step, biotinylated-dUMPs were incorporated. The DNA repair reaction was stopped by three washes with PBS-T.

To evaluate repair synthesis, the incision reaction was performed as described above for the incision activity, except for the absence of test compound. As specified earlier, damaged DNA was only recognised and excised during this step. This was then followed by the repair synthesis reaction: In the absence of aphidicolin, each test compound was incubated for 30 min at 30° with 120 μg of a whole-cell extract of HeLa cells, 70 mM KCl, 0.4 μM of each of dGTP, dCTP, dATP and biotinylated-dUTP (Bio-21dUTP) in reaction buffer described above. Throughout this step, biotinylated-dUMPs were incorporated. The DNA repair reaction was stopped by three washes with PBS-T.

For each of these two derivative assays, control experiments were carried out with undamaged pBS plasmids, as mentioned for the classical 3D assay, and the resultant data were then defined as, respectively, the *in vitro* incision repair and the *in vitro* repair synthesis activity.

2.5. Detection of DNA damage recognition by XPA–RPA complex

The 3D assay was recently modified so as to detect DNA repair proteins bound to DNA damage [18]. The adsorption of damaged or undamaged plasmids onto the microplate was performed as described previously for the classical 3D assay. The plasmids were then co-incubated at 30° with the cell extract in the presence of the test compound, but for only 30 min. Two washes with PBS-T were performed, between each of the following steps. The XPA–RPA complex bound to DNA damage was detected by a 30-min incubation at 30° with 0.1 mg/mL of rabbit anti-XPA (a generous gift from M. D'Incalci, Istituto di Recerche Farmacologiche Mario Negri, Italy) diluted in 100 mM Tris, 0.2% BSA or with 0.2 μg/mL of murine anti-RPA/p32 (Neomarkers) diluted in PBS pH 7.4, 0.2% BSA. The secondary biotinylated antibody (Pierce), respectively, anti-rabbit (diluted 1/50,000 in PBS) or anti-mouse (diluted 1/25,000 in PBS), was added and incubated for 30 min at 30°. Quantification was performed by the addition of extravidin-peroxidase, then luminol, followed by luminometric measurements, as detailed above for the detection of NER. In parallel, similar experiments were performed using undamaged plasmids so as to define the non-specific background signal. This was then subtracted systematically from the signal corresponding to the quantity of XPA–RPA complex bound to damaged plasmids. Data were expressed as a percentage of the amount of complex bound to damaged plasmids in the absence of drug.

2.6. Detection of helicase inhibition

Inhibition of the helicase activity of 1 μg SV40 large T antigen (AgT; molecular biology resources) was evaluated after 45 min of incubation at 33° in the presence of the test compound, by measuring the displacement of a [^{3}H]-labelled DNA fragment from a partial duplex with single strand phage M13. The free [^{3}H] DNA fragments were quantitated using a scintillation proximity assay (SPA) system following Amersham's instructions. Helicase activ-

ity was then determined as a percentage of the control obtained by testing with the solvent only.

2.7. Unscheduled DNA synthesis

UDS detection was carried out according to the method of Bootsma *et al*. [19]. A cell suspension (3×10^4 cells/mL of growth medium) was incubated on slides at 37°, in 5% CO_2 in air for 18 hr. Medium was then aspirated and after a 4-hr incubation at 37° with 0.1 mM mechlorethamine, cells were incubated in the growth medium containing F 11782, 10 μM 5-fluoro-2′-deoxyuridine and 10 mM hydroxyurea to inhibit replication and labelled with 10 μCi/mL [^{3}H]-thymidine. Cells were washed three times for 20 min with growth medium containing 200 μM non-radiolabelled thymidine, then fixed with methanol–acetic acid (3:1) for 15 min and air dried. Slides were dipped for 10 s into an autoradiographic emulsion (EM-1, Amersham). After 5 days exposure in the dark at 4°, slides were developed (Kodak) and the nuclei were counterstained with Mayer's haemalum solution 1/5 diluted (Merk). Cells were viewed using a Zeiss Axioplan microscope combined with a KY-F50 camera (JVC) using a 40× objective. For each image, nuclei were detected and numbers of nuclear grains were counted using Histolab software (Microvision). Each experiment was performed at least three times, with 100 cells being analysed for each experimental point. Values corresponding to the background, which was obtained using cells not treated with mechlorethamine, were subtracted from each experimental point and for each the mean number of nuclear grains per cells (±SD) was determined. Data are presented as a percentage of cellular NER activity using as 100% control, the value determined for cells treated with mechlorethamine alone.

2.8. Cell growth inhibition and combination index method

Drug-induced cytotoxic effects were determined in 96-well microtiter plates after a 48-hr incubation with each drug alone or in combination (co-incubation), using a 3-(4,5-dimethyl-thiozol-2-yl)-2,5-diphenyltetrazoniumbromide (MTT) assay, as described previously [20]. Initially, the dose-effect relationships of each drug alone against A549 cells were subjected to the median effect plot to determine their potency which were characterised by their IC_{50} values of 67, 16, and 0.75 μM for F 11782, cisplatin and mitomycin C, respectively. Such evaluations were performed at least three times on three independent occasions, in sextuplet. Cells were then simultaneously treated with the two drugs for 48 hr and the resultant cytotoxicity evaluated. Each combination index (CI) determination results from at least three independent experiments containing a minimum of five different dilutions of the two drugs. These dilutions were established as ratios of the IC_{50} values. The combination effects of two drugs in terms of synergy, additivity or antagonism were then analysed by the median effect plot [12] using the CalcuSyn program (Biosoft), as detailed previously [20]. Thus, when $CI = 1$, the interaction is considered additive, when $CI > 1$ antagonism is indicated and when $CI < 1$ synergy is indicated.

3. Results

3.1. In vitro inhibition of NER by F 11782

As part of a routine screen using the 3D assay kit, F 11782 was evaluated for any inhibitory effects on NER. In fact, F 11782 proved a potent inhibitor of NER, characterised by a IC_{50} value of 12 μM (Table 1). Whilst such inhibition was weaker than that determined for known potent inhibitors of NER, such as aphidicolin and doxorubicin, it was considered significant since such inhibitory activity was not observed with other topoisomerases inhibitors. For example, camptothecin and etoposide [9] which, like F 11782, showed no DNA intercalating properties [11], failed to exert any effect on DNA repair activity, even at the highest concentration tested (100 μM).

3.2. In vitro inhibition of incision repair by F 11782

To determine whether one step of the NER mechanism was specifically inhibited or whether the inhibitory effects observed on overall NER results from non-specific interactions, two other assays derived from the initial 3D assay have been set up. These newer assays have taken advantage of the specific inhibition by aphidicolin of DNA-polymerases δ and ε, uniquely involved in the repair synthesis step of NER [16,17]. They have permitted measurements

Table 1
Quantitation of *in vitro* incision repair and repair synthesis inhibition by F 11782 relative to certain reference inhibitors of DNA repair

Test compound	Overall NER inhibition, IC_{50} ± SD (μM)[a]	Incision repair inhibition, IC_{50} ± SD (μM)[a]	Repair synthesis inhibition, IC_{50} ± SD (μM)[a]
F 11782	12 ± 3	1.1 ± 0.3	50 ± 5
Doxorubicin	2.4 ± 0.4[b]	4.7 ± 0.9	9.0 ± 0.7
Aphidicolin	0.8 ± 0.2[b]	>100	2.7 ± 0.4

[a] IC_{50} values correspond to the concentration (μM) of the compound necessary to reduce activity to 50% of that obtained with the solvent only. Data are the mean of five experiments (±SD).
[b] Values reproduced from Barret *et al*. [9].

of the effects of NER inhibitors on either the incision or the repair synthesis steps of NER and consequently have provided an evaluation of the specificity of NER inhibitors. Thus, the inhibition profile of F 11782 against incision repair and repair synthesis has been characterised in terms of IC_{50} values, namely 1.1 and 50 μM, respectively (Table 1). These results clearly show that F 11782 preferentially inhibits the incision repair step and that such specificity appears unusual.

3.3. Effects of F 11782 on DNA damage recognition by XPA–RPA complex

To identify which of the proteins involved in the incision step was inhibited by F 11782, DNA damage recognition by the XPA–RPA complex was investigated. These two proteins form a ternary complex with DNA damage *in vitro* [21,22] which is essential for NER [23–25]. Detection of the binding of DNA repair proteins to damaged DNA was demonstrated recently using a derivative of the 3D assay [18] and in this study, the binding of XPA and RPA to damaged DNA was investigated using the same procedure. It was shown that the presence of 100 μM F 11782 did not modify the efficacy of binding of either XPA or RPA protein to the damaged DNA (Fig. 2). Similar negative effects were found using aphidicolin (Fig. 2), etoposide and camptothecin (data not shown), as expected, since these compounds have been shown not to interfere with the incision step. Only doxorubicin inhibited the recognition of DNA damage by the XPA–RPA complex (Fig. 2). Although the reduction of XPA–RPA binding was not exactly the same when quantification was performed using either the anti-XPA or the anti-RPA antibodies, but this may be explained in terms of differences in affinities for these two antibodies.

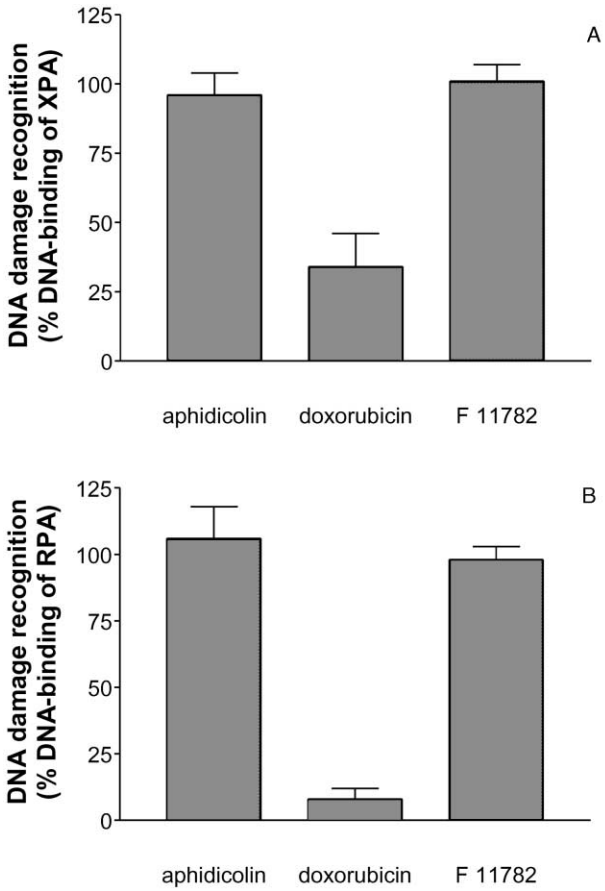

Fig. 2. DNA damage detection by the XPA–RPA complex: UV-damaged plasmids were incubated with HeLa whole-cell extract in the presence of 100 μM test drug for 30 min at 30° in the presence of the test compound. XPA–RPA complex bound to damaged DNA was then detected using anti-XPA (A) or anti-RPA antibodies (B).

3.4. Effects of F 11782 on helicase activity

The effect of the five compounds evaluated in this study were next tested on the helicase activity of AgT, which is available commercially. Inhibition of helicase activity by anthracyclines has been reported in several earlier publications against AgT, the bacterial helicase II, human helicase II and other human helicases [26–28]. Subsequently this inhibition was explained by the formation of a ternary stable complex involving anthracycline, helicase and DNA [29]. Such inhibitory activity by doxorubicin was confirmed in this study against AgT with an IC_{50} value of 0.9 μM, and so was used as a positive control. The other compound used as a reference for DNA repair inhibition, namely aphidicolin, did not inhibit the helicase activity of AgT (IC_{50} value > 100 μM). Similar inactivity against human helicase II has been reported previously [28] and is consistent with the fact that aphidicolin only inhibits the DNA repair synthesis step. F 11782 also proved inactive against the helicase activity of AgT, since no effects were detected at concentrations of ≤ 100 μM. Finally, an absence of any inhibitory activity against helicase has been demonstrated in previous studies for both etoposide and camptothecin [26–28] and was confirmed in this present study by IC_{50} values of >100 μM for both compounds.

3.5. Inhibition of NER by F 11782 in A549 cells

The DNA cross-linking agent mechlorethamine induced strong UDS by induction of DNA damage which were recognised by DNA repair (Fig. 3). However, a significant decrease in the number of nuclear grains per cell and hence in cellular DNA repair activity was obtained when F 11782 was added (Fig. 3). Using this same procedure, no inhibition was detected when etoposide, which did not inhibit NER *in vitro*, was used at concentrations up to 1 μM (data not shown). The inhibition of repair synthesis in A549 cells was detected with concentration of F 11782 ranging from 0.01 to 1 μM (Fig. 3). The use of concentrations higher than 1 μM did not permit a meaningful evaluation of UDS, since the associated cytotoxicity of F 11782 at such con-

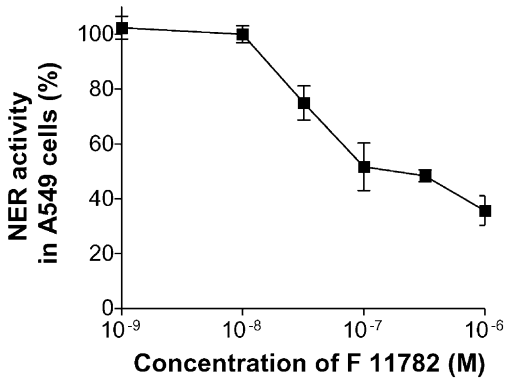

Fig. 3. Inhibition of NER in A549 cells by increasing concentrations of F 11782. A549 cells were incubated for 4 hr in the presence of the test compound plus 0.1 mM mechlorethamine to damage DNA, together with 10 μM 5-fluoro-2′-deoxyuridine and 10 mM hydroxyurea to inhibit DNA replication and labelled with 10 μCi/mL [^{3}H < /*RM* >]-thymidine, to evaluate NER activity. NER activity was not reduced significantly when F 11782 was replaced by etoposide at equicytotoxic concentrations.

centrations induced compaction of nuclei which was incompatible with grain counting.

3.6. Combination index evaluation

Each experimental point and the corresponding curve which indicates, for each fractional effect, the CI values (±1.96 SD) generated by median effect analysis are shown in Fig. 4. Most CI values were within the range of 0.2–0.6,

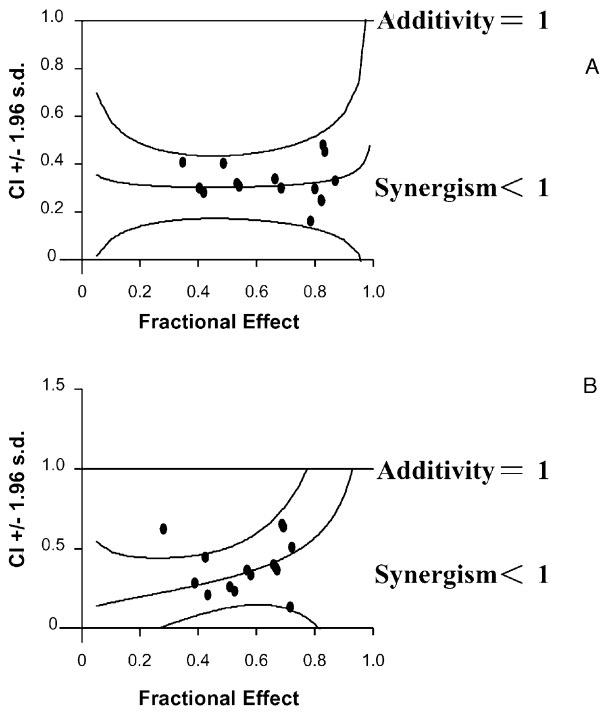

Fig. 4. Data resulting from combinations of F 11782 with either cisplatin (A) or mitomycin C (B) against A549 human lung carcinoma cells following a 48-hr of incubation and analysed by the median effect analysis program. When CI = 1, the interaction is considered additive, when CI > 1 antagonism is indicated and when CI < 1 synergy is indicated.

confirming the high extent of synergism of combinations involving F 11782 and a cross-linking agent, such as cisplatin (Fig. 4A) or mitomycin C (Fig. 4B). Interestingly, a similar high level of synergism was not observed when F 11782 was combined with a range of other anticancer drugs with modes of action other than DNA cross-linking, such as, for example, topotecan, doxorubicin, etoposide, 5-fluorouracil or vinflunine (data not shown).

4. Discussion

Screening for potential antitumour agents which inhibit NER activity has been carried out using novel *in vitro* methodologies. From such assays, F 11782 was identified as a potent inhibitor of NER. Such inhibitory activity has been only rarely identified, since as a result of screening a relatively large panel of potential and actual antitumour drugs or antibiotics, only four DNA interacting agents and aphidicolin, used as a reference, were detected as NER inhibitors [9].

F 11782 was initially selected as a potentially novel anticancer drug based on its inhibitory activities against topoisomerases I and II [11]. Any effect of F 11782 on NER resulting from such inhibitory activity is unlikely. The influence of topoisomerase II activity on DNA repair processes remains unclear, but the involvement of topoisomerases could only be indirect, i.e. via their effects on the overall topology of DNA [3]. Moreover, camptothecin and etoposide used as reference compounds for respective inhibitors of topoisomerase I and II, did not influence *in vitro* NER activity [9], confirming that inhibition of topoisomerases *per se* did not interfere directly with NER in this system.

The inhibitory activity of F 11782 against NER did not result from non-specific activity against enzymes which interfered with DNA, since F 11782 showed no inhibitory activity against several other enzymes such as DNase I, T4 polynucleotide kinase or sequenase [11]. Moreover, the testing of F 11782 on *in vitro* incision repair and repair synthesis has permitted the clear demonstration that this inhibitory activity results principally from inhibition of the initial steps of NER thus, confirming that this inhibition of NER by F 11782 was not merely non-specific. Such effects can be compared to those of reference compounds studied earlier [30]. Several spectra of activities have been identified for NER inhibitors [30]. Certain compounds, like doxorubicin, were found to inhibit both the incision repair and repair synthesis steps, without showing any specificity. Others though, like aphidicolin, preferentially inhibited repair synthesis, whilst with etoposide and camptothecin, used as negative controls, no inhibition of either step was detected at a concentration of 100 μM [30]. Thus, at present, the only NER inhibitors identified by the 3D assay are DNA-interacting agents, like doxorubicin, or the polymerase inhibitor, aphidicolin [9]. Moreover, no interaction

between DNA and F 11782 has been detected, by any of the wide range of technologies used [11]. Preferential inhibition of the incision step by F 11782, excludes any similarity with the activity of the polymerase inhibitor, aphidicolin. In this context, F 11782 appears to have an original mode of action against NER activity.

The incision step of NER can be viewed separately in terms of its three main activities, namely DNA damage recognition, the helicase and the endonuclease activities. In this study, F 11782 showed no effect on DNA damage recognition. The possibility that F 11782 affected the helicase activity of XPB and/or XPD was then investigated indirectly using AgT helicases. This model was proposed since the proteins XPB and XPD contain seven motifs conserved between two superfamilies of DNA and RNA helicases [31], providing evidence of a large degree of homology [32]. Moreover, the specificity of action of XPB and XPD may be guided, in large part, by their association with other proteins of the reparosome, rather than by their helicase activity *per se*. Nevertheless the lack of effect of F 11782 on helicase activity needs to be confirmed using purified XPB and XPD. In addition, inhibition of NER by an effect of F 11782 mediated via possible interactions between repair proteins cannot be ruled out. Nevertheless, these present results suggest that F 11782 inhibits the incision step by an effect on the endonuclease activity of the incision step. Confirmation of this though awaits data from studies based on a molecular approach using purified enzymes and such studies are under consideration. In parallel with this present study, a P388 leukaemia cell line has been selected for resistance to F 11782 [33] and, preliminary characterisation by DNA macroarray technology suggests that XPG may be underexpressed in this resistant subline. Moreover, this F 11782 resistant subline proved hypersensitive to cisplatin, a finding consistent with a reduction of NER activity. Thus, the combined results of these studies indicate that F 11782 may inhibit the endonuclease activity of XPG, whilst recent studies have shown that ERCC1-XPF plays a major role in cross-link repair [34,35].

The fact that F 11782 inhibits NER provides a rationale for using this novel anticancer drug in combination with DNA cross-linking agents like, for example, cisplatin or mitomycin C. Such a rationale can be evoked for F 11782, since results using cultured A549 cells have confirmed *in vitro* NER inhibition by F 11782, detected using the 3D assay. Concentrations of F 11782 which inhibited DNA repair in these A549 cells were slightly lower than those inducing cytotoxicity. Such results indicate that this NER inhibition may explain, at least in part, the highly synergistic cytotoxic activities identified when F 11782 was used in combination with DNA cross-linking agents like, cisplatin or mitomycin C, against these A549 cells *in vitro*. Interestingly, reflecting this same idea, fludarabine and gemcitabine when combined with cisplatin demonstrated synergistic antitumour activities, which was attributed in part to their recently identified inhibition of NER [36–39].

In conclusion, F 11782 was identified as a potent inhibitor of NER and was shown predominantly to inhibit the incision rather than the repair synthesis step. These results emphasise the unique mode of action of this novel molecule. Moreover, such an inhibition provides an explanation for the highly synergistic cytotoxicity observed against cultured A549 lung tumour cells, when F 11782 was combined with cross-linking agents, such as cisplatin or mitomycin C and provides a basis for its evaluation in clinical trials in combination with DNA cross-linking agents.

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