Optimised oligonucleotide substrates to assay XPF-ERCC1 nuclease activity for the discovery of DNA repair inhibitors
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We report the design and optimisation of novel oligonucleotide substrates for a sensitive fluorescence assay for high-throughput screening and functional studies of the DNA repair enzyme, XPF-ERCC1, with a view to accelerating inhibitor and drug discovery.

Many chemotherapy drugs kill tumour cells through the induction of DNA damage; DNA interstrand crosslinks (ICLs) are particularly toxic to cells as they prevent DNA replication and transcription. DNA cross-linking drugs, including platinum-based drugs, are used to treat a range of tumours including many hard-to-treat cancers (such as pancreatic, oesophageal and lung) for which survival rates remain low. DNA repair pathways directly combat damaging agents used in cancer therapy. Despite the urgent need to explore inhibition of XPF-ERCC1 as a therapeutic avenue, few compounds have been identified for this purpose. This is in part due to limited access to systematically optimised substrates, necessary for highly robust and sensitive screening assays.

The aim of this work was to generate and validate a highly sensitive, scalable assay for XPF-ERCC1 activity with a broad dynamic range for use in high-throughput inhibitor screens. The assay we describe utilises full-length XPF-ERCC1 and fork DNA structures which give high DNA duplex stability whilst conferring sensitivity. The position of the fluorophore and quencher relative to the junction has been varied to optimise this assay, and importantly the use of full-length XPF-ERCC1 inhibitors have also been limited to screening in silico or use truncated protein forms, leading to low hit numbers and incomplete characterisation.

Full-length XPF-ERCC1 complex was purified from insect cells using a baculovirus expression system (Fig. S1A [ESI†]). An XPF active site substitution mutant D676A (XPFD676A-ERCC1), devoid of nuclease activity, was also purified as a control for contaminating insect cell nuclease activity.

We initially characterised the activity of XPF-ERCC1 using a ‘simple fork’ structure. This was labelled at the 3′-terminus of the single-stranded arm with a fluorescent CY3, a bright and stable fluorophore (Fig. 1A). The assays employed a fixed concentration of the divalent cations previously reported to support the activity of XPF-ERCC1, (Mg\(^{2+}\) and Mn\(^{2+}\)) and increasing concentrations of XPF-ERCC1 within the range previously reported to be active on this type of substrate (10 to 70 nM), with a 60 minute incubation using a fixed, excess concentration of substrate (100 nM) (Fig. 1B).
Activity could be observed for XPF-ERCC1 at concentrations as low as 10 nM with greater activity in the Mn\(^{2+}\) buffer. As expected, XPF\(^{D676A}\)-ERCC1 (lanes marked: ND) supported no activity in either buffer when present at the highest concentration (70 nM). There have been reports of analysis of XPF-ERCC1 activities at both 30 °C and 37 °C. Here, we determined the fork substrate to be equivalently digested by 40 nM XPF-ERCC1 at both temperatures (Fig. S1B, ESI†), suggesting that activity is independent of temperature within this range.

The data presented in Fig. 1B implied that a major determinant of XPF-ERCC1 activity within our system is the divalent cation present. We consequently explored XPF-ERCC1 activity over a range of both Mg\(^{2+}\) and Mn\(^{2+}\) concentrations. XPF-ERCC1 activity is maximal at 1 mM Mn\(^{2+}\), while only weak activity is observed at concentrations up to 10 mM Mg\(^{2+}\) (Fig. 1C and Fig. S1C, ESI†), as previously reported.\(^{16}\) We therefore employed Mn\(^{2+}\), which supports greater XPF nuclease activity, at 1 mM for further optimisation experiments. Finally, we carefully mapped the major cleavage site of XPF-ERCC1 on this simple 3'-fluor-labelled fork substrate by generating a marker ladder containing 3'-fluor-labelled oligonucleotides of 2-nucleotide incremental sizes that flank the predicted incision position. This revealed that the majority (over 90%) of released products were 23-nucleotides in length, corresponding to a cleavage site 2-nucleotides 5’ to the fork junction (Fig. 1D).

Having established optimal conditions for XPF-ERCC1 analysis, we varied the position of the fluorophore within the single-stranded arm of our fork substrates and analysed the effect on activity. This was in anticipation of including a quencher moiety on the strand opposing the fluorescently labelled strand. This would permit the establishment of a real-time fluorescence incision assay, in which dsDNA cleaving by XPF-ERCC1 uncouples the fluorophore and quencher leading to increased fluorescence. This readout can subsequently be used to quantify enzyme activity and derive kinetic measurements.

In order that the fluorescent moiety can be uncoupled from the quencher incorporated elsewhere in the substrate, it is necessary to place the fluorophore on one of the substrate arms, distal to the major XPF-ERCC1 cut site, mindful that the location of the fluorophore might itself further qualitatively and quantitatively modulate the activity of XPF-ERCC1. Moreover, in order to optimally position both a fluorophore and quencher on the substrates, as became apparent (below), it might be necessary to move the fluorophore from the extreme 3'-terminus of the substrate arms. Consequently, we generated a panel of substrates with a fluorophore positioned 8-nucleotides, 4-nucleotides and 2-nucleotides 3’ to the fork junction, and determined the activity of XPF-ERCC1 on these with reference to the previously optimised substrate harbouring a terminal 3’-fluorophore. All the substrates were efficiently digested by 10 nM XPF-ERCC1, although we detected a small shift in the product size for the substrate bearing the fluorophore two nucleotides from the fork junction (Fig. 2A). Further titration of XPF-ERCC1 revealed that full cleavage of each fork substrate occurred at concentrations between 10 and 20 nM in XPF-ERCC1 (Fig. S2, ESI†), and therefore 20 nM was selected as the lowest concentration for all further analysis. By running the reaction products on a gel adjacent to the molecular weight markers we confirmed that placing the moiety two nucleotides from the junction likely moves the incision position by a single nucleotide to a position 3-nucleotides from the junction (Fig. 2B). This implies that the presence of the fluorophore so close to the junction interferes sterically with the enzyme, altering its position of incision.

Having established that XPF-ERCC1 is equally capable of digesting substrates with the fluorophore at various positions along the fork arm, with the caveat that the incision site might be altered by positioning the fluorophore close to the junction, we next designed a panel of substrates containing a Black Hole Quencher (BHQ). This has excellent spectral overlap with the Quasar 570 dye on the...
nucleotides to the 3′-terminus. However, a more complex pattern of incisions was observed for all substrates, regardless of the location of the quencher (Fig. 3C). We propose that the presence of both a fluorophore and quencher are in closer proximity than when the fluorophore is located closer to the 3′-terminus. This generated a mixture of incision products. Finally, we examined the XPF-ERCC1 cleavage characteristics on substrates where the fluorophore was placed two nucleotides to the 3′-side of the fork junction in conjunction with the same set of quencher locations used in Fig. 3A and B (substrates Fork 3.1, 3.2, 3.3 and 3.4). These structures have an advantage that the fluorophore and quencher are in closer proximity when the fluorophore is located closer to the 3′-terminus. However, a more complex pattern of incisions was observed for all substrates, regardless of the location of the quencher (Fig. 3C). We propose that the presence of both a fluorophore and quencher in the vicinity of the fork junction region, a critical feature for substrate recognition and verification by XPF-ERCC1, interferes substantially with these processes, changing the cleavage site. A summary gel provides direct comparison of the qualitative and quantitative aspects of digestion characteristics (Fig. S3, ESI†). Based on the above analyses, we did not pursue any of the DNA structures where the quencher was located in the duplex region of the substrate (Forks 2.1, 2.2, 2.3 and 2.4), as the multiple species could significantly reduce the robustness of the fluorogenic assay. In addition, none of the structures where the fluorophore is located 2-nucleotides from the junction, regardless of quencher combination, were further pursued since these also formed a complex array of products (3.1, 3.2, 3.3, 3.4).

High-throughput screens require non-gel-based assays. Development of a multi-well plate assay was necessary, where the generation of the fluorescent signal acts as a readout of XPF-ERCC1 activity. Initial assays using 100 nM DNA substrate showed reasonable signal:noise, and minimal fluorescence photobleaching. Optimisation led to the DNA concentration being raised to 200 nM, which was shown to increase the signal:noise, as well as the overall fluorescence readout. We then monitored the fluorogenic output of substrates 1.3, 1.4, 2.3 and 2.4 in a 384-well format as a function of time, with readings taken every minute for 30 min (Fig. 4A). Pleasingly all substrates produced little background fluorescence over this time course, indicating that all were stable under the reaction conditions, and that non-specific background fluorescence would not be a confounding feature in the assay. Substrates were further screened against varying concentrations of XPF-ERCC1, and this indicated that 40 nM was optimal for a robust high throughput assay (Fig. S4, ESI†).

Differing maximum fluorescence outputs were detected, where the substrate bearing the fluorescent moiety four nucleotides
characterising the functional impact of mutations in XPF and interacting proteins such as SLX4\(^24\) and XPA\(^25\) that are associated with the devastating inherited syndromes Xeroderma pigmentosum, Fanconi anaemia, Cockayne syndrome and Cerebro-Oculo-Facio-Skeletal syndrome.\(^14\)

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**Conflicts of interest**

There are no conflicts to declare.

**Notes and references**

1. P. J. McHugh, V. J. Spanswick and J. A. Hartley, *Lancet Oncol.*, 2001, 2, 483–490.
2. M. L. Dronkert and R. Kanaar, *Mutat. Res.*, 2001, 486, 217–247.
3. M. Rischel, P. Knipscheer, M. Enoiu, T. Angelov, J. Sun, J. D. Griffith, T. E. Ellenberger, O. D. Schärer and J. C. Walter, *Cell*, 2008, 134, 969–980.
4. B. Sengerova, A. T. Wang and P. J. McHugh, *Cell Cycle*, 2011, 10, 3999–4008.
5. J. Zhang and J. C. Walter, *DNA Repair*, 2014, 19, 135–142.
6. T. Helledge, E. Petermann, C. Lundin, B. Hodgson and R. A. Sharma, *Nat. Rev. Cancer*, 2008, 8, 193–204.
7. R. Plummer, *Clin. Cancer Res.*, 2010, 16, 4527–4531.
8. M. J. O’Connor, *Mol. Cell*, 2015, 60, 547–560.
9. L. Song, A. Ritchie, E. M. McNeil, W. Li and D. W. Melton, *Pigment Cell Melanoma Res.*, 2011, 24, 966–971.
10. K. Kirchner and D. W. Melton, *Anticancer Res.*, 2010, 30, 3223–3232.
11. Z. Zheng, T. Chen, X. Li, E. Haura, A. Sharma and G. Bepler, *N. Engl. J. Med.*, 2007, 356, 800–808.
12. K. A. Olussean, A. Dunant, P. Fourret, E. Brambilla, F. Andre, V. Haddad, E. Tararuchon, M. Filipits, R. Pierker, H. H. Popper, R. Stahel, L. Sabatier, J. P. Pignon, T. Tursz, T. Le Chevalier, J. C. Soria and IALT Bio Investigators, *N. Engl. J. Med.*, 2006, 355, 985–991.
13. S. Postel-Vinay and J. C. Soria, *J. Clin. Oncol.*, 2017, 35, 384–386.
14. M. Manandhar, K. S. Boulware and R. D. Wood, *Gene*, 2015, 569, 153–161.
15. A. Ciccia, N. Q. McDonald and S. C. West, *Annu. Rev. Biochem.*, 2008, 77, 259–287.
16. W. L. de Laat, E. Appeldoorn, N. G. J. Pigm. Cell Melanoma Res.
17. M. Bowles, J. Lally, A. J. Fadden, S. Mouilleron, T. Hammonds and N. O. McDonald, *Nucleic Acids Res.*, 2012, 40, e101.
18. E. M. McNeil and D. W. Melton, *Nucleic Acids Res.*, 2012, 40, 9990–10004.
19. S. Arora, J. Heyza, H. Zhang, V. Kalman-Maltese, K. Tillison, A. M. Floyd, E. M. Chaffin, G. Bepler and S. M. Patrick, *Oncotarget*, 2016, 7, 75104–75117.
20. E. M. McNeil, K. R. Astell, A. M. Ritchie, S. Shave, D. R. Houston, P. Bakrania, H. M. Jones, P. Khurana, C. Wallace, T. Chapman, M. A. Wear, M. A. Walkinshaw, B. Saxty and D. W. Melton, *DNA Repair*, 2015, 31, 19–28.
21. L. P. Jordheim, K. H. Barataek, L. Heinrich-Balard, E. Mateda, E. Cosp-Perrial, K. Boulevard, R. El Sabeh, R. Perez-Pineiro, D. S. Wishart, R. Cohen, J. Tuszynski and C. Dumontet, *Mutat. Res.* 2013, 74, 12–24.
22. U. B. Abdullah, J. F. McGonigal, S. Brohil, D. Petchelkine, A. H. El-Sagheer, T. Brown and P. J. McHugh, *EMBO J.*, 2017, 36, 2047–2060.
23. T. M. Chapman, C. Wallace, K. J. Gillen, P. Bakrania, P. Khurana, P. J. Coombs, S. Fox, E. A. Bureau, J. Brownlee, D. W. Melton and B. Saxty, *Bioorg. Med. Chem. Lett.*, 2015, 25, 4104–4108.
24. M. R. Hodkinson, J. Silhan, G. P. Crossan, J. I. Garaycoechea, S. Mukherjee, C. M. Johnson, O. D. Schärer and K. J. Patel, *Mol. Cell*, 2014, 54, 472–484.
25. B. Orelli, T. B. McClenond, O. V. Tsodikov, T. Ellenberger, L. J. Niedernhofer and O. D. Schärer, *J. Biol. Chem.*, 2010, 3705–3712.