

Supporting information for

A multi-disciplinary team-based classroom exercise for small molecule drug discovery

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XX50225: Techniques in Drug Discovery Coursework

Virtual Drug Discovery Group Exercise

Candidate Number:

Name: Adam H

Team:

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Patent Application Form

Request for grant of a patent

00001

1 Your reference:

2 Full name of applicant or of each applicant:

8a

3 Title of the invention:

4 Inventorship:

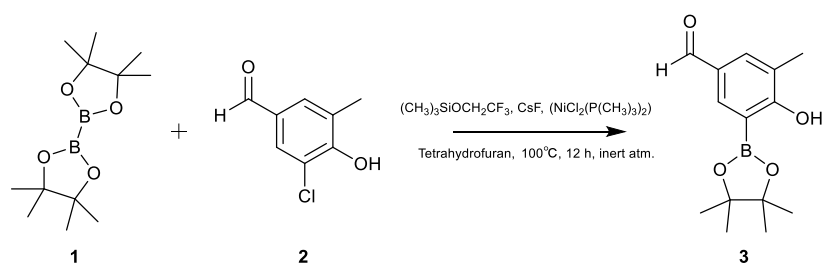
Are all the applicants named above also inventors?

YES

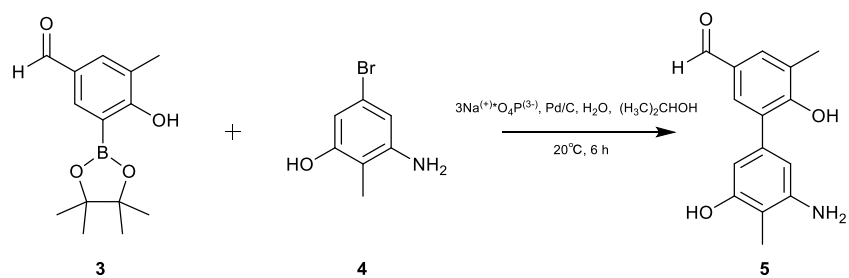
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NO

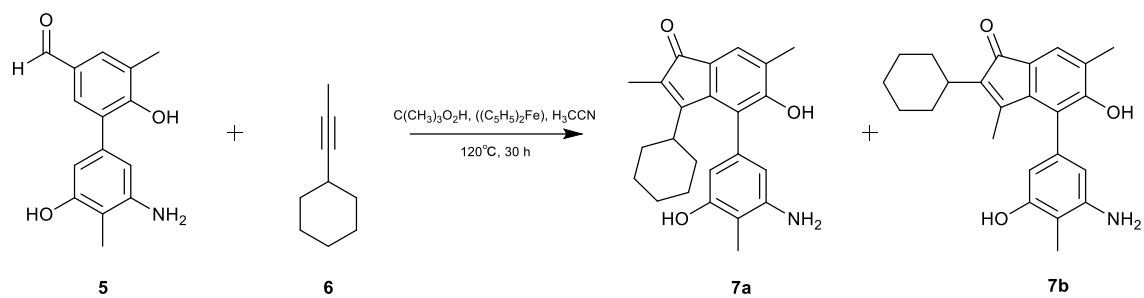
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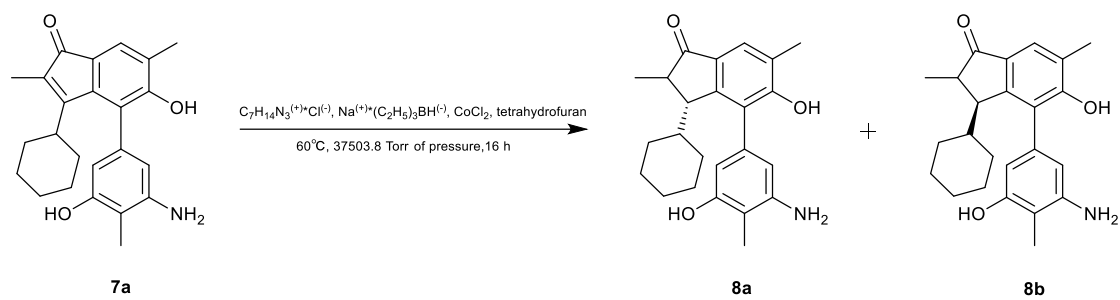
Scheme 1



Scheme 2



Scheme 3



Scheme 4

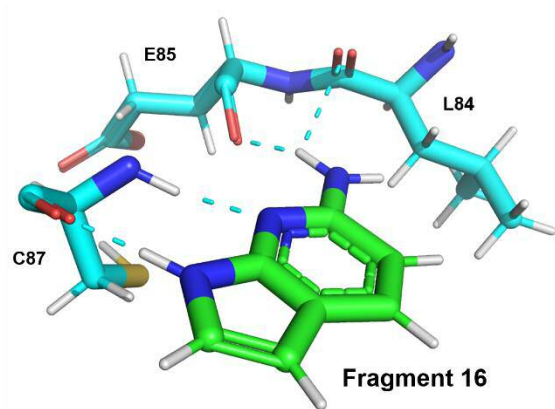


Figure 1. Fragment 16 target interaction.

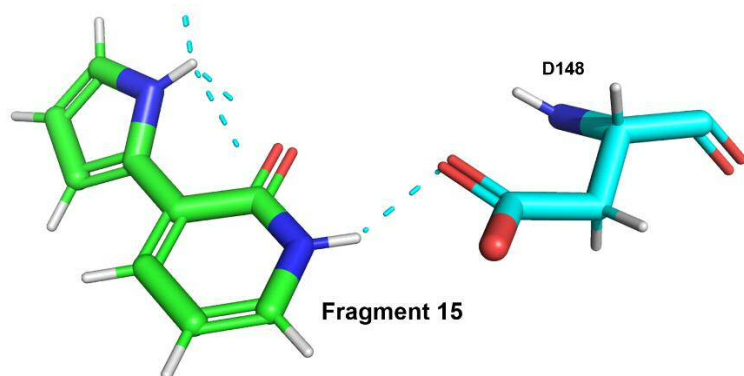


Figure 2. Fragment 15 target interaction.

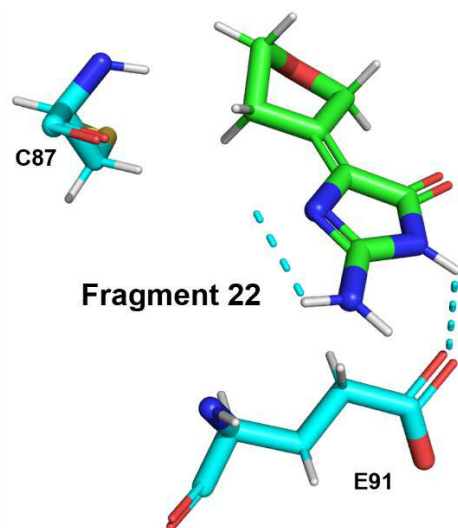


Figure 3. Fragment 22 target interaction.

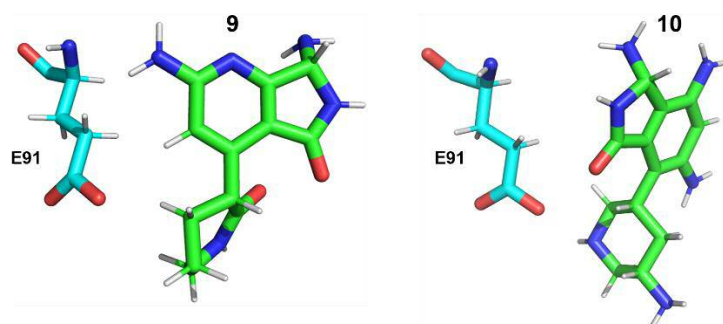


Figure 4. Compounds **9** and **10** orientation.

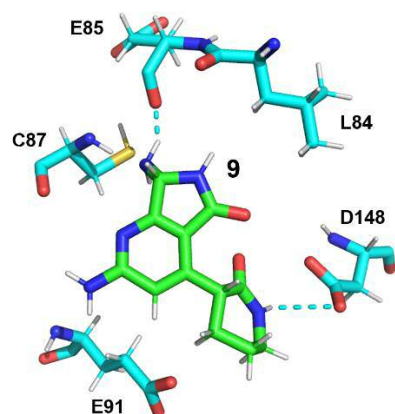


Figure 5. Compound **9** target interaction.

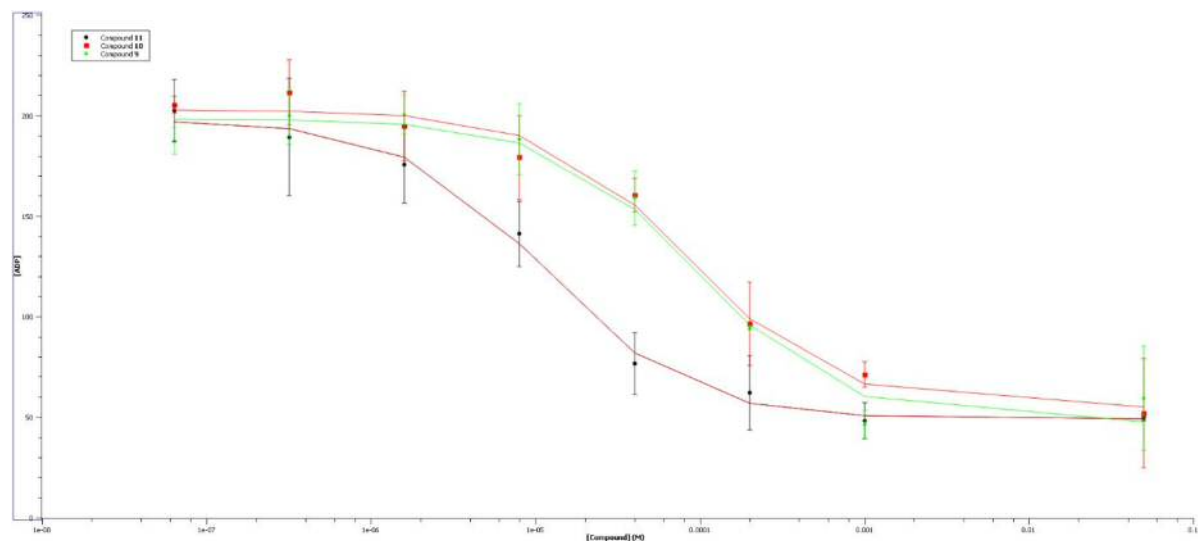


Figure 6. Compounds **9** (Yellow), **10** (red) and **11** (black) concentration response graph, y error represents standard deviation ($n = 3$).

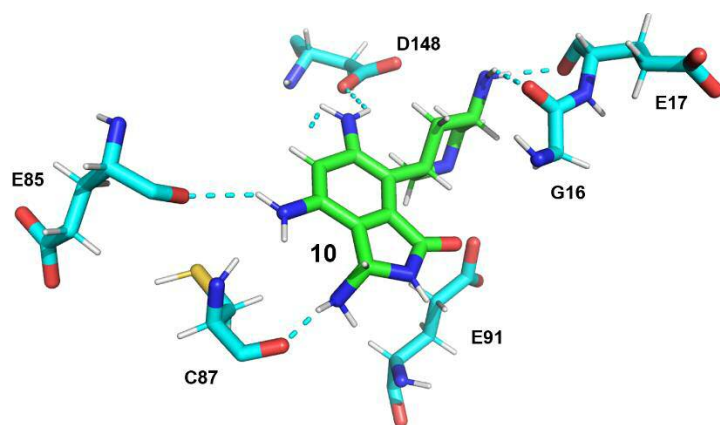


Figure 7. Compound **10** target interaction.

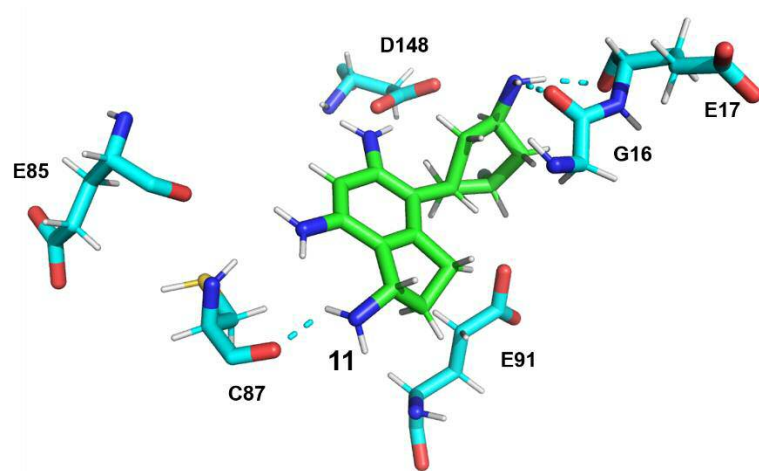


Figure 8. Compound **11** target interaction.

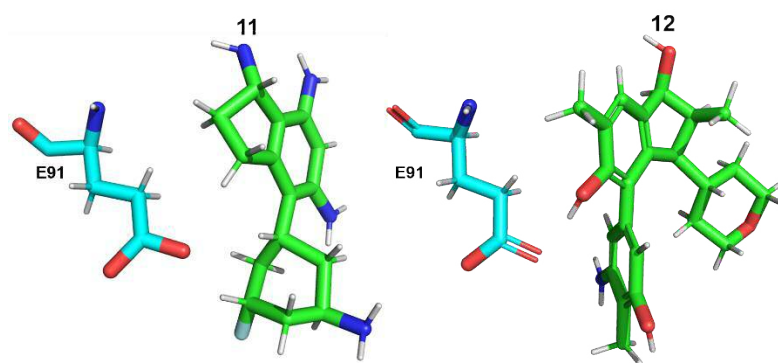


Figure 9. Compounds **11** and **12** orientation.

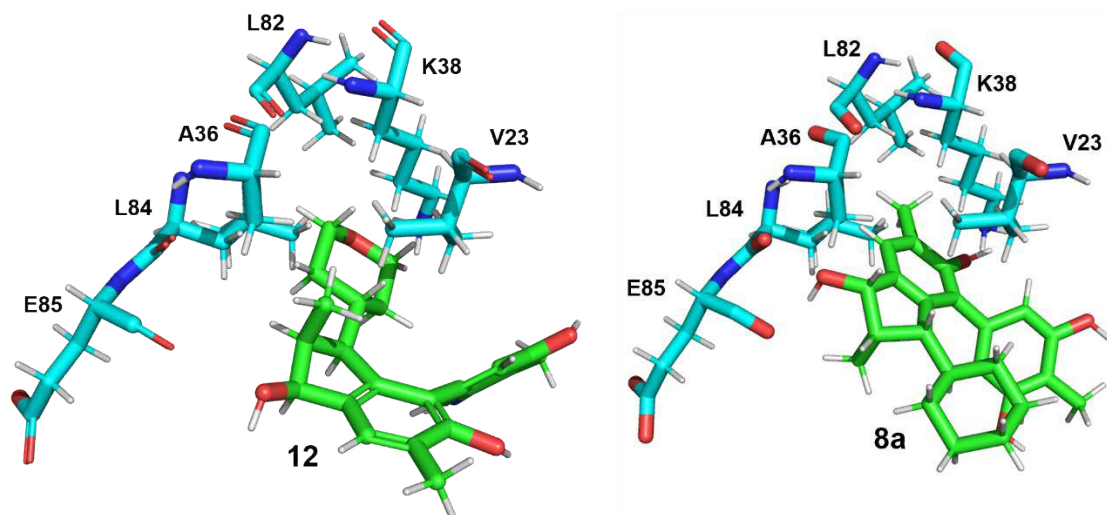


Figure 10. Compounds **12** and **8a** side pocket interactions.

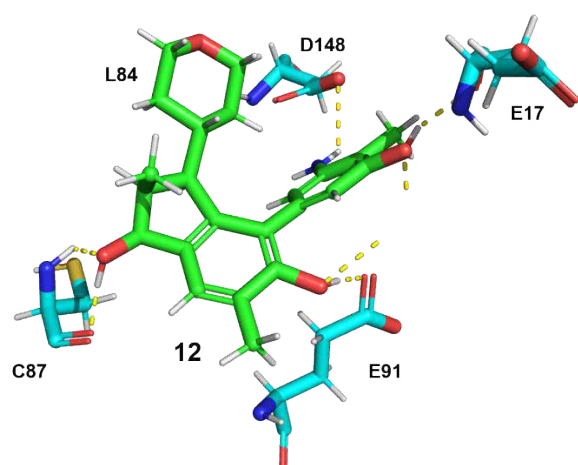


Figure 11. Compound **12** target interaction.

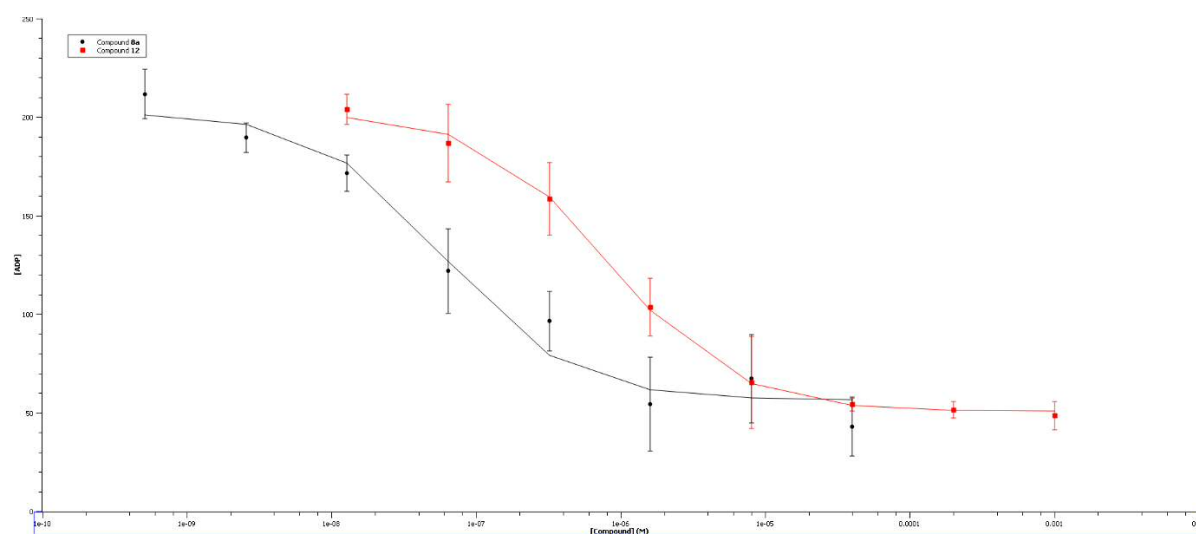


Figure 12. Compounds **12** (red) and **8a** (black) concentration response graph, y error represents standard deviation ($n = 3$).

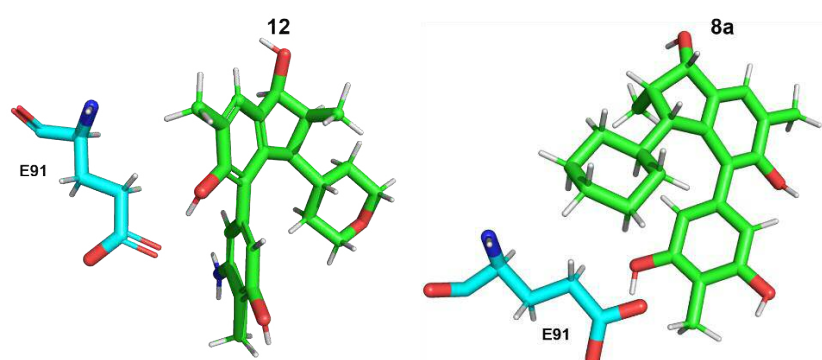


Figure 13. Compounds **12** and **8a** orientation.

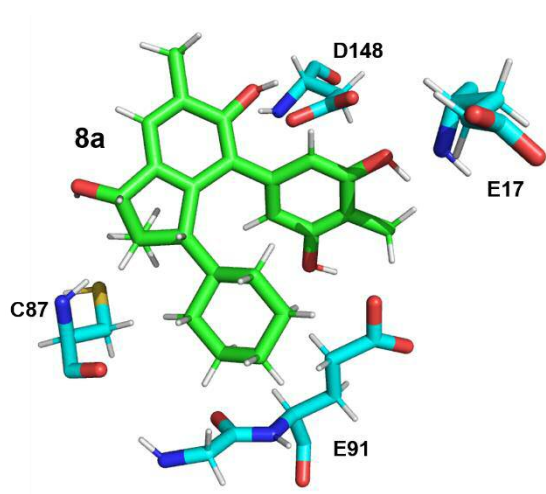


Figure 14. Compound **8a** target interaction.

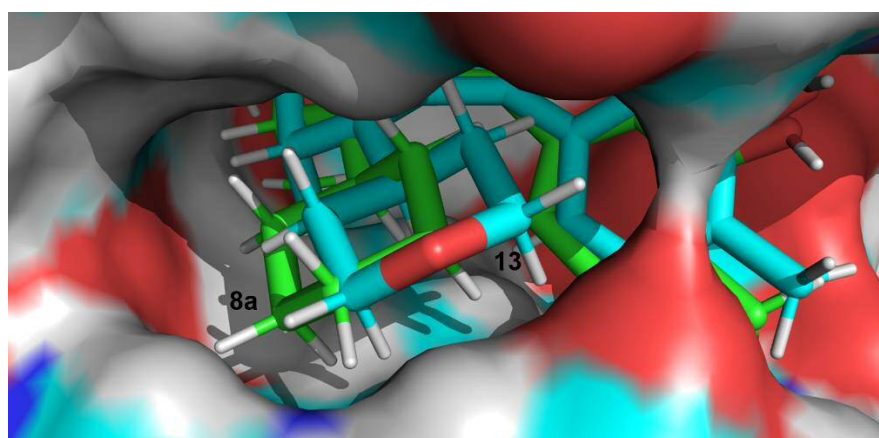


Figure 15. Compounds **13** and **8a** target occupancy.

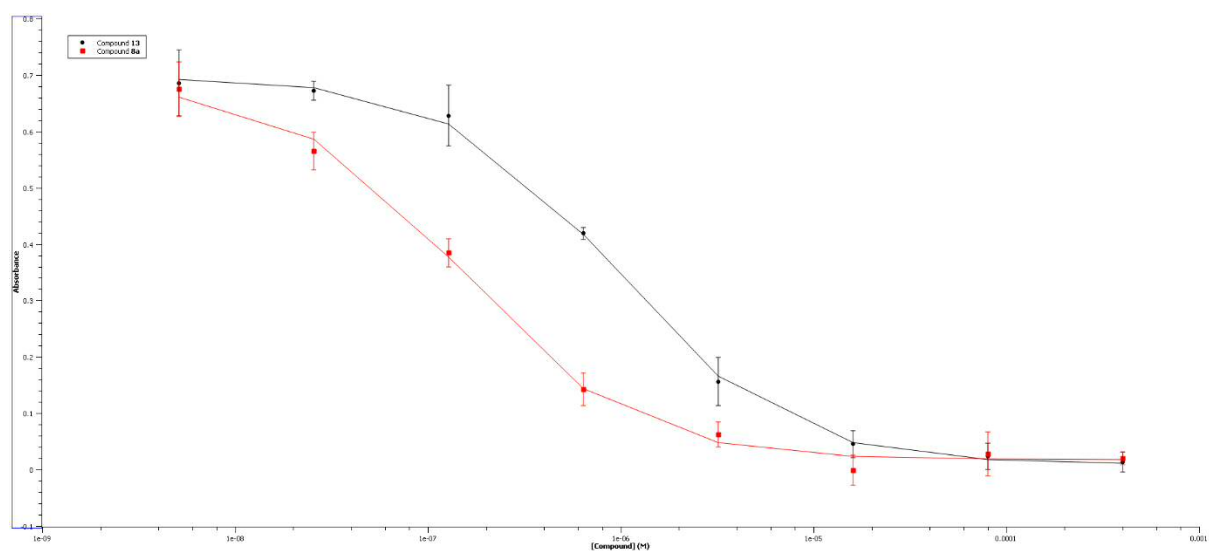


Figure 16. Compounds **13** (black) and **8a** (red) MTT assay, y error represents standard deviation ($n = 3$).

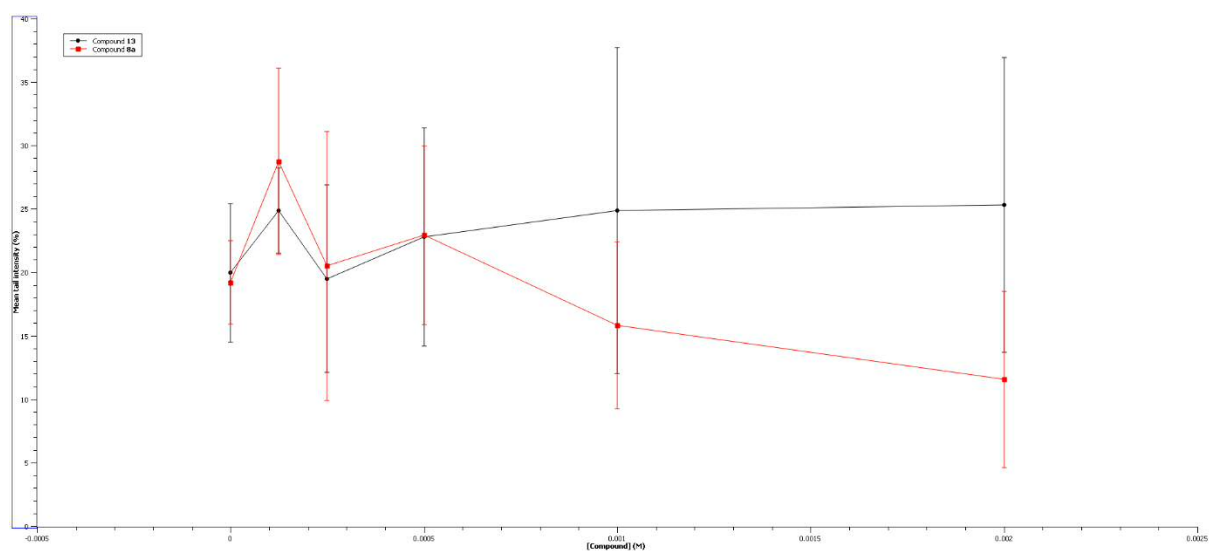


Figure 17. Compounds **12** (black) and **8a** (red) Comet assay, y error represents standard deviation ($n = 3$).

Abstract

Breast cancer is the most common cancer and, despite improvements in survival, is still a leading cause of cancer related death.

Therefore, there remains a need to explore new avenues of treatment. One approach, synthetic lethality, can be used to selectively target tumour cells carrying specific mutations which inactivate a pathway to gain a fitness advantage, at the cost of becoming reliant on alternative pathways. The inhibition of one of these other pathways can then kill the cell, without significant toxicity to normal tissue. In breast cancer, BRCA and p53 mutations are very common and leave the cells vulnerable to checkpoint inhibition, as the cells will be unable to arrest cell cycle progression despite DNA damage. Here we report the design of **8a**, a Chk1 inhibitor, that showed a potent IC₅₀ of 0.06 μ M and favourable pharmacokinetics. This novel compound could provide a new avenue for treatment in breast cancer.

Background

Breast cancer

Female breast cancer has recently become the most common cancer, and, despite the increasing survival rate, is still the fifth most common cause of cancer death (1). This is a particular issue in triple negative breast cancer, which has a five-year survival rate almost 20% lower than other breast cancer subtypes (2). Therefore, there is a need to develop new drugs for the treatment of breast cancer, especially in triple negative breast cancer patients who have an unmet need.

Synthetic lethality

One approach is synthetic lethality, which occurs when the inactivation of one pathway can be tolerated by the cell, but the inactivation of a second, complimentary pathway leads to cell death (3). Whilst the inhibition of the second pathways is lethal to tumour cells carrying the genetic event that inactivates the first, normal

cells will not have that same deficiency and so will tolerate the intervention.

Checkpoint kinase 1 (Chk1) is a series/threonine kinase that functions downstream of DNA damage (4). Upon recognition of exposed single stranded DNA, ATR phosphorylates Chk1, which in turn arrests cell cycle progression and activates DNA repair machinery (5). This offers an interesting opportunity for synthetic lethality. Chk1 inhibition will prevent cell cycle arrest and DNA damage repair, causing the cell to progress through the cell cycle despite replication stress and DNA damage (6). Alone this replication stress would be insufficient to kill the cell as tumour cells have additional mechanisms of cell cycle regulation, however if the cell is already vulnerable and is deficient in one of these other essential pathways, Chk1 inhibition could be lethal. This is particularly attractive in breast cancer, where some of the most common mutations occur in the BRCA and p53 genes (7).

The BRCA1/2 genes play an important role in DNA damage repair and, as one of the most prevalent mutation in breast cancer, show considerable clinical relevance (8). BRCA mutations lead to an increase in replication stress as cells are less able to respond to DNA damage. Therefore, the cells are more reliant on cell cycle arrest to give time for other mechanisms of repair (9). Inhibition of Chk1 will prevent checkpoint arrest and allow the cell cycle to continue despite the DNA damage, leading to cell death.

Another consequence of DNA damage is p53 activation, which then acts to arrest the cell cycle or initiate apoptosis depending on the degree of damage (10). Therefore, mutations in p53 allows cancer cells to survive and proliferate despite the accumulation of DNA damage. Whilst this introduces a replication stress, Chk1 still regulates the S and G2 checkpoints which prevents cell death (11). This reliance on Chk1 is optimal for synthetic lethality and Chk1 inhibition has shown promise in sensitising p53 deficient cells to DNA damage (12). This is particularly significant for triple negative breast

90 cancer, which lacks many of the common drug
targets but shows prevalent p53 mutations (13).
There is a significant need for new treatments
against triple negative breast cancer as it has a
significantly worse prognosis than other
95 subtypes. The prevalence of p53 however opens
the possibility of exploiting synthetic lethality as
a therapeutic approach. Against cells deficient in
p53 and BRCA, Chk1 inhibitors present an
important approach to synthetic lethality in
100 breast cancer. Here we discuss the design of a
new Chk1 inhibitor for use against human breast
cancer.

Fragment hits

The first step in inhibitor design was to screen a
105 range of fragments against Chk1. Crystal
structures were generated to assess target
interactions and the top hits were provided to
our team. These were used to inform the design
of inhibitors, which were submitted for several
110 rounds of investigation to determine their
inhibitory activity, as well as pharmacokinetic
and safety parameters.

Compound design

Fragment screen and initial lead development

115 Our investigation began with an evaluation of
the screened fragments and the interactions
they formed with Chk1. This, and the spatial
arrangement of the fragment in the active site,
informed the fragments which were chosen for
120 consolidation into a larger and more complex
structure. The reasoning for this was that, with
multiple overlapping fragments, the subsequent
molecule would show favourable interactions
that offered avenues for modification or
125 simplification where necessary.

Compound **9** incorporated fragments 15, 16 and
22, simplified to contain only the functional
groups necessary to interact with the target. One
aim during the design of compound **9** was to
130 retain the spatial orientation of the fragment
and maintain functional group interactions. To
this end, fragment 16 would become the core of
this new compound, as it could both link

fragments through its spatial orientation and
135 form multiple hydrogen bond acceptor (HBA)
and hydrogen bond donor (HBD) interactions
with Chk1 (Fig 1). One of the amine hydrogens,
part of the of the 2-aminopyridine functional
group that made up the right half of fragment
140 16, could act as a HBD to the backbone carbonyl
of either leucine or glutamic acid at positions 84
and 85, respectively. In addition, the pyridine
nitrogen acted as a HBA with the backbone
amide of the cysteine residue at position 87. The
145 five membered ring of fragment 16 would be
modified to incorporate the five membered ring
of fragment 22, which was unique in its HBD
interaction between the protonated amine of the
imidazole derivative and the side chain
150 oxygen of glutamic acid (Fig 2). In addition, the
amide of fragment 22 should still provide the
necessary HBD to interact with the backbone
carbonyl of cysteine 87, preventing the loss of a
target interaction.

155 Only three of the screened molecules extended
towards the right of the active site, meaning the
options for HBD interactions with the sidechain
oxygen of aspartate residue 148 was limited.
Therefore, to take advantage of these additional
160 interactions, one of fragments 10, 15 or 30
would have to be incorporated into the
proposed compounds to further occupy the
active site. Fragment 15 showed the best overlap
with fragment 16 and was the simplest choice,
165 requiring only the substitution of the pyrrole for
the 2-aminopyridine of fragment 16 (Fig 3).

The proposed compound had poor
pharmacokinetics, being extremely polar.
However, at this initial stage the focus was on
170 developing leads with promising interactions
and so an IC50 assay was performed to
determine which suggested lead should be taken
forwards.

Round 1, adapting to a new conformation.

175 The crystal structure revealed that compound **9**
did not bind in the expected orientation (Fig 4).
Instead, it rotated completely which led to fewer
interactions than expected. The amide predicted
to interact with cysteine at position 87, instead

180 formed a HBD interaction with the glutamic acid
residue at position 85 (Fig 5). It might be
expected that the residue would also occur, as
the change in orientation would bring the other
amide in the region of cysteine 87. However, the
185 proximity was too far and neither of the 2-
aminopyridine nitrogen formed any
interactions. The nitrogen derived from
fragment 15 was the only other interaction to
occur, forming the expected hydrogen bond
190 with aspartate 148. Despite this, compound **9**
had an IC₅₀ of 94.5 μ M \pm 27.3 (Fig 6), the best of
the proposed compounds, although still
significantly lower than desired. This was
possibly due to the fact compound **9** best fit the
195 active site and occupied multiple regions of the
binding pocket.

To improve the IC₅₀, modifications were made
to the compound to adapt it to the new
conformation. The 2-aminopyridine formed no
200 interactions, so the amine was removed, and the
pyridine nitrogen replaced with an amine, as it
was reasoned this would occupy a close enough
proximity to the cysteine backbone carbonyl at
residue 87 to form a HBD interaction. In
205 addition, another amine was introduced in the
meta position to this first amine to explore the
possibility of a HBD interaction with the
sidechain oxygen of the glutamic acid at residue
91. Finally, the cyclohexane ring was modified,
210 removing the carbonyl that formed no
interactions in favour of an amine at the meta
position. This was because, to form the HBD
interaction, the cyclohexane adopted a
conformation that brought the meta position
215 carbon in a close proximity to the roof of the
active site, where a HBD could interact with the
carbonyl backbone of glutamic acid 17.

These modifications were designed to increase
the activity and so an IC₅₀ assay was repeated.
220 The choice of assay was used as a measure of
progress, as it allowed a direct comparison to
determine the effect of the changes. Compound
10 had been designed to better interact with this
new conformation and so an increase in activity
225 was expected.

Round 2, pharmacokinetic optimisation.

Once again, the crystal structure revealed that
the modifications altered the target interaction
and led to a change in orientation (Fig 4).
230 However, due to the partial symmetry of
compound **10**, the two amines still interacted
with the cysteine and glutamic acid residues at
positions 87 and 85, just between the opposite
groups than predicted (Fig 7). The addition of the
235 amine on the cyclohexane was very successful,
forming two non-overlapping HBD interactions
to the backbone carbonyls of glutamic acid at
residue 17 and glycine at residue 16. However,
to adapt to these interactions, the cyclohexane
240 is completely orthogonal to the rest of the
molecule and so the interaction with the
sidechain oxygen of aspartate 148 was lost.
Fortunately, the change in conformation
brought the meta position phenylamine into
245 close proximity to aspartate 148 to act as a HBD
in place of the cyclohexane amine, although
depriving **10** of the predicted interaction with
glutamic acid 91. Despite the promising
interactions, compound **10** showed no
250 significant difference in IC₅₀ over **9** at 139 μ M \pm
36.9 (Fig 6).

The next step was used to alter the
pharmacokinetic profile of compound **10**
through the removal of excess functional groups
and inclusion of a fluorine to increase
255 lipophilicity. It was important to determine what
effect a more lipophilic molecule had, whether it
retained or even increased activity. The 5-
amino-2-pyrrolidinone, the five membered ring
developed from fragment 22, had been
260 unaltered since its inclusion however had also
not shown any interactions beyond the amine.
Therefore, the carbonyl and secondary amine
were removed, leaving only functional groups
265 with a confirmed interaction.

Round 3, new interactions.

Whilst the modifications did not alter
orientation, the compound lost interactions
between both the amines on the phenyl ring and
270 the target residues (Fig 8). This could potentially

be a result of the change in conformation of the cyclopentene, with the planar shape lost after the nitrogen and oxygen were removed. Despite this, compound **11** saw a marginal decrease in IC₅₀ at 99.3 μ M \pm 27.5 (Fig 6). It was clear that a significant change would have to be made to form new interactions, capable of increasing the potency, as well as further modifications to reach a favourable pharmacokinetic profile.

ACD/I-Lab identified a region of potential toxicity around the now exposed cyclopentene, therefore the first alteration involved the addition of a methyl group to mask the region of toxicity. Next, the amine group was replaced by a hydroxyl function group, as only one hydrogen was involved in HBD interactions and so the less polar OH could improve lipophilicity without compromising target interaction. Additionally, the oxygen could act as a HBA with the backbone amide of cysteine 87, although the functional groups might be too distant to interact. The phenylamine saw a similar substitution. Despite failing to form any interaction during the previous round of investigation, there is significant possibility for an interaction with the sidechain oxygen of aspartate 148. We then added a methyl group that could interact with a previously unexplored hydrophobic pocket. Due to the failure of the fluorine to meaningfully effect lipophilicity, it was removed, and a para methyl group was added. In addition, we discovered a phenyl ring was significantly more lipophilic than the cyclohexane and was likely to retain its function. The phenyl ring will possess an orthogonal conformation, driven by the two HBD interactions between the amine and the position 16 and 17 residues. This conformation was previously shown to remove the interactions between the piperidine nitrogen and the sidechain oxygen of aspartate 148, however a hydroxyl group would extend further from the ring and this different spatial arrangement may be enough, so a meta hydroxyl substitution was also evaluated. The final alteration was the largest and involved the addition of a tetrahydropyran to the cyclopentene. The oxygen will increase the total

polar surface area of the molecule whilst the carbon ring could potentially form new interactions with the hydrophobic residues at the entrance to the active site, helping the compound show a greater selectivity and potency but also forcing the molecule to sit deeper in the binding pocket. This new interaction should improve the ability of the compound to inhibit the target, so an IC₅₀ assay was repeated as the IC₅₀ was still significantly above the necessary concentration.

Round 4, final compound.

The crystal structure revealed that the orientation of compound **12** was once again rotated (Fig 9). Interestingly, the new tetrahydropyran group was embedded within the hydrophobic pocket, an interaction which may have been the driver of the change in orientation (Fig 10). This change led to a HBD interaction between the alcohol group of the phenyl ring and the sidechain oxygen of the glutamic acid residue at position 91 (Fig 11). An alternative compound proposed at this stage had a methyl group in place of the alcohol and showed negligible activity. The exact reason for this is unclear, it could be that the interactions between the hydrophobic methyl groups and oxygen of the glutamic acid side chain were unfavourable, and this influenced the orientation. The hydroxyl group of cyclopentene showed favourable interactions, forming both HBA and HBD interactions with the carbonyl and amide of the cysteine 87 backbone. Strangely, the amine of the amino-6 hydroxy-2 toluene did not interact with the glutamic acid at residue 17 and glycine at residue 16 as expected. Instead, it acted as a HBD to aspartate 148 and it was the hydroxyl group which interacted with glutamic acid 17. This was surprising as the two possible interactions of the amide would theoretically be more favourable. Despite the unexpected interactions, compound **12** showed very promising activity, with an IC₅₀ of 0.82 μ M \pm 0.07, far lower than any previous round of screening (Fig 12). One other compound this round showed a better IC₅₀ even than **12**, however it had far inferior pharmacokinetic

characteristics and the slightly lower IC₅₀ was not enough to support it going forwards. Compound **12** was chosen as the focus for the final round of investigation, it filled all the pharmacokinetic requirements and had a good IC₅₀, however its activity was still higher than ideal.

To try and increase the IC₅₀, two variants were generated. One retained the tetrahydropyran which the other substituted for a cyclohexane. In addition, both compounds substituted the amine of the amino-6 hydroxy-2 toluene for another hydroxyl group. An amine group bound to a phenyl ring represents a significant point of toxicity and as a result had to be modified to ensure the drug can be safe. The previous failure of the amine to form the two predicted HBD interactions suggests the two hydrogen are not necessary and the substitution would not affect activity. A range of pharmacokinetic and pharmacodynamic assays were run to compare the two compounds. Compound **8a** was expected to have a better activity against Chk1, however this was no guarantee of its superiority over **13** as a significant increase in potency may come at the cost of pharmacokinetics or toxicity. In addition, there is no guarantee of an increase in activity, so both compounds offered promising final compounds for this investigation.

Surprisingly, the removal of the oxygen did not improve cyclohexane binding to the side pocket, instead causing a change in orientation (Fig 13-14). It may be that polar groups are necessary for the occupancy of this binding pocket, and therefore could explain the failure of the more hydrophobic variant of compound **12** with a methyl group in place of the alcohol. The unfavourable interactions between the methyl groups and glutamic acid side chain could have driven the molecule to try and adopt a rotated conformation, but the hydrophobic groups could have had an equally unfavourable interaction with the binding pocket. In this proposed mechanism, the presence of the hydroxyl group in **8a** would have allowed it to interact favourably with the binding pocket. An alternative explanation could be that, as the

crystal structure cannot represent the dynamic nature of target-ligand interactions, the compounds can interact in either orientation (Fig 15). There is some evidence for this, as **13** was also shown in this rotated conformation, despite the only difference from **12** being an amine to hydroxyl substitution. The ability to interact with the binding pocket could also contribute to the increased potency of **8a** which showed a superior occupancy, in particular through the methyl group which extended further into the pocket (Fig 10). Interestingly, there was a visible difference between the target interactions of **8a** and **13**, with **8a**, and in particular the cyclohexane, sitting visibly lower in the binding pocket (Fig 15). This could explain the increased potency of **8a**, with a significant increase over all previously screened compounds at 0.06 $\mu\text{M} \pm 0.02$ (Fig 12). To confirm how Chk1 inhibition related to synthetic lethality, an MTT assay determined the cell viability of BRCA-negative cells treated with **8a** or **13** (Fig 16). The MTT assay is a measure of metabolic activity and can be used to find the concentration necessary to cause a 50% decrease in viable cells, therefore could be used to determine whether the compounds met the 0.5 μM IC₅₀ requirement set out in the initial report. The BRCA-negative cells are vulnerable to synthetic lethality and so both compounds showed good activity, however **5a** had a significantly higher IC₅₀ at 0.15 μM which, unlike **13** that had an IC₅₀ of 0.94 μM , met the required characteristic. This potency does come at a cost, as the total polar surface area of **8a** is only 80.92, far lower than the >90 required. In all other characteristics **8a** meets the necessary requirements, however **13** meets those requirements and the polar surface area. This means there must be a choice between the pharmacokinetics of **13** and the activity of **8a**. The significance of lipophilicity and polar surface area is clear in the plasma protein binding assay, which found only 8.2% was **8a** unbound, less than half the 16.9% of **13**. So **8a** would have a far worse bioavailability, however in terms of safety it seems a far more attractive choice, as the Comet assay may implicate **13** as a potential

460 source of genotoxicity (Fig 17). The genotoxicity
results are difficult to interpret, there is a
fluctuation in the results that suggests lower
concentrations are more genotoxic than higher
concentrations. This is unusual but important as
465 the IC50 is implicated in the range of the peak.
Therefore, it may require repeats or different
assays to confirm the results. The results do
show a strong difference between high
concentrations of the two compounds, with **13**
470 being significantly higher, however this does not
necessary mean it is a cause of genotoxicity as it
may not be significantly higher than negative
controls. Therefore, despite a worse
pharmacokinetics, **8a** was chosen as the lead
475 compound due to its greater activity and
reduced toxicity.

Experimental procedure

The two adjacent phenyl rings will be linked
through a Suzuki-Miyaura reaction. This requires
480 a halide and an organoboron, therefore the
reagents will have to be bought specially or
modified. The organoboron will be synthesised
from 4,4,4',4',5,5,5',5'-Octamethyl-2,2'-bi-1,3,2-
dioxaborolane, a complex but readily available
485 molecule that can be bought relatively cheaply
at £115 for 500 g, and 3-chloro-4-hydroxy-5-
methylbenzaldehyde which, whilst also
available, is far more expensive and may act as a
limiting factor in mass scale drug production at
490 almost US\$1200 per 5 g (*Scheme 1*). There are
two mechanisms for this reaction which differ
only in one reagent, tris(trimethylphosphine)nickel(II) chloride (14)
or dichlorobis(trimethylphosphine)nickel (15). In
495 this case, dichlorobis(trimethylphosphine)nickel
offers a far more appealing reaction as it can be
bought directly from a supplier. The proposed
reaction should not differ significantly from the
above patent and so should generate a good
500 79% yield.

The halide, 5-Bromo-3-hydroxy-2-methylaniline,
can fortunately be bought however comes with
a significant price tag of over US\$5000 for 1 g.
This may work for initial testing and clinical
505 studies however for mass production it may be

appropriate to synthesise this compound from
scratch, otherwise production costs could be
unreasonable. Fortunately, the Suzuki-Miyaura
reaction has a brilliant 98% yield and does not
510 involve expensive reagents. The expense of the
above experiments comes from the necessity of
the side chain placement, however future
development could involve the synthesis of
these starting material which should decrease
515 production costs. The Suzuki-Miyaura reaction
involves the removal of the halide and
organoboron leaving groups and cross coupling
of the products on the palladium (16) Therefore,
a significant part of the molecule can be
520 synthesised relatively easily (*Scheme 2*).

The next step involves the addition of the
cyclohexane, a reaction which will be facilitated
by the aldehyde functional group (*Scheme 3*). 1-
cyclohexylpropyne will allow provide the
525 cyclohexane to be added, as well as the alkyne
that will produce the five membered ring and
the methyl group that will provide an important
functional group. 1-cyclohexylpropyne can be
bought or synthesised relatively easily, making it
530 a suitable reagent for large scale synthesis. A
similar reaction using 1,2-Diphenylethyne
generated a good yield of 86% and it is likely to
be consistent with the proposed reaction (17).
Unfortunately, the product will generate two
535 isomers, with the cyclohexane being added to
either the desired position, or in place of the
methyl group. This is a potential issue as the
crystal structure suggests the substitution might
have a significant effect on the ability of the
540 cyclohexene to occupy its binding site. However,
the purpose of the methyl group was to limit
toxicity which will still be achieved by the
cyclohexane. Therefore, it is worth testing the
safety and efficacy of this compound in
545 preclinical studies to assess the significance of
this product and can be used to determine
whether an additional purification step is
necessary or if the compound shows activity
against the target. Regioselectivity in this
550 reaction is possible, however requires an iodine,
or bromine however this has a lower yield,
adjacent to the aldehyde (18). Despite some

effort, it was not possible to selectively generate a starting compound with all the necessary functional groups. An iodine can be added to the 3-chloro-4-hydroxy-5-methylbenzaldehyde, first through the addition of an amine then its conversion into an iodine, however there was no way to selectively add it to only one of the two available positions and this would have resulted in significant off target production. Alternatively, phenyl aldehyde with chloride and iodide substitutions can be bought, however the methyl and hydroxyl functional groups cannot reliably be added to the correct sites. Further work may be able to achieve this and improve the yield of the synthesis.

The final step involved the reduction of cyclopentene, to remove the planar conformation and allow the functional groups to interact with the target (*Scheme 4*). There are numerous mechanisms for this and the most appropriate is unclear. The chosen method for scheme 4 was the most complex and so the most costly, however it had a very good 90% yield (19). Alternatively, a method using only hydrogen, platinum(IV) oxide and ethyl acetate would prove far cheaper but had a yield of only 62% (20). The simplest used only hydrogen and platinum(IV) oxide but did not list a yield. Therefore, a choice must be made between cost and yield. In this case it may be yield is more important, as the stereocentres generated during reduction will generate optical isomers. It is unclear if the R enantiomer will have the same activity as the desired S enantiomer. The crystal structure suggests the same interaction may not be possible so may need to be investigated for activity.

The consistent high yield of each step is promising; however, the initial cost of starting material and off target isomers are potential limitations to synthesis. This can be managed; dedicated production of the starting materials will decrease cost and further optimisation of the synthesis may facilitate regioselectivity and a greater yield. Additionally, activity and toxicological assessments of the isomers are necessary to reveal what further steps, if any,

should be taken to optimise the synthesis pathway.

Assays

Crystal structures were generated for each proposed inhibitor to determine target interactions and active site occupancy. Chk1 crystals were soaked in a 1 mM solution of the compound and imaged at a resolution of 2.3 to 2.7 Å. The ability of these compounds to inhibit the target was measured using an ADP glo assay, which uses the amount of ATP converted to ADP over a 1 h period to act as a measure of activity (21). The assay was repeated in triplicate for each compound at an increasing concentration and the average and standard deviation of the results were used to find the IC₅₀. SciDAVis was used to plot the data, which was then fit using the equation:

$$\frac{1}{y} = \frac{1}{y_{\text{max}}} + \frac{1}{y_{\text{max}}} \left(\frac{x}{x + \text{IC}_{50}} \right)^n$$

This allowed the IC₅₀ of each compound to be calculated and compared, to determine which proposed molecule should progress. To confirm how IC₅₀ translated to efficacy, an MTT assay (Abcam) was performed against a BRCA-negative breast cancer cell line to measure cell viability after treatment with the compounds (22). Viable cells convert MTT to formazan which acts as a measure of metabolic activity. Because formazan generates a purple colouration, the intensity of the colour is related to how many cells are metabolically active and therefore viable. Inhibition of Chk1 in BRCA-negative breast cancer cells will be synthetically lethal and so can provide a measure of the anti-cancer cytotoxicity of the inhibitors. Once a suitable compound was chosen, a range of pharmacokinetic assays were run to determine the safety and bioavailability of the drug. For safety, genotoxicity was measured using a Comet assay, performed by the external CRO (contract research organisation) ADMEexpress Ltd. TK6 cells were treated with the compound, 1% DMSO (negative control) or Etoposide

(positive control) for a 3 h exposure time prior to staining and visualisation (23). For the pharmacokinetics, plasma protein binding (PPB) provides a measure of the free drug in a system and is therefore an important part of drug bioavailability. A common technique to measure PPB involves equilibrium dialysis, which measures the ability of a compound to cross from a protein containing, to a protein free compartment separated by a semi-permeable membrane (24).

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