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DMXAA (Vadimezan, ASA404) is a multi-kinase inhibitor targeting VEGFR2 in particular

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A B S T R A C T

The flavone acetic acid derivative DMXAA [5,6-dimethylXAA (xanthenone-4-acetic acid), Vadimezan, ASA404] is a drug that displayed vascular-disrupting activity and induced haemorrhagic necrosis and tumour regression in pre-clinical animal models. Both immune-mediated and non-immune-mediated effects contributed to the tumour regression. The vascular disruption was less in human tumours, with immune-mediated effects being less prominent, but nonetheless DMXAA showed promising effects in Phase II clinical trials in non-small-cell lung cancer. However, these effects were not replicated in Phase III clinical trials. It has been difficult to understand the differences between the pre-clinical findings and the later clinical trials as the molecular targets for the agent have never been clearly established. To investigate the mechanism of action, we sought to determine whether DMXAA might target protein kinases. We found that, at concentrations achieved in blood during clinical trials, DMXAA has inhibitory effects against several kinases, with most potent effects being on members of the VEGFR (vascular endothelial growth factor receptor) tyrosine kinase family. Some analogues of DMXAA were even more effective inhibitors of these kinases, in particular 2-MeXAA (2-methylXAA) and 6-MeXAA (6-methylXAA). The inhibitory effects were greatest against VEGFR2 and, consistent with this, we found that DMXAA, 2-MeXAA and 6-MeXAA were able to block angiogenesis in zebrafish embryos and also inhibit VEGFR2 signalling in HUVECs (human umbilical vein endothelial cells). Taken together, these results indicate that at least part of the effects of DMXAA are due to it acting as a multi-kinase inhibitor and that the anti-VEGFR activity in particular may contribute to the non-immune-mediated effects of DMXAA on the vasculature.

INTRODUCTION

DMXAA [5,6-dimethylXAA (xanthenone-4-acetic acid), ASA404, Vadimezan] is an analogue of flavone acetic acid that has antitumour activity [1]. In pre-clinical mouse tumour models it was demonstrated that administration of DMXAA rapidly leads to disruption of the existing vasculature in the tumour and consequent haemorrhagic

Key words: angiogenesis, antivascular, 5,6-dimethylxanthenone-4-acetic acid (DMXAA), kinase inhibition, screening, vascular endothelial growth factor receptor (VEGFR).

Abbreviations: CK2, casein kinase 2; DLAV, dorsal longitudinal anastomotic vessel; ERK, extracellular-signal-regulated kinase; FBS, fetal bovine serum; GFP, green fluorescent protein; hpf, h post-fertilization; HIPK2, homeodomain-interacting protein kinase 2; HUVEC, human umbilical vein endothelial cell; ISV, intersegmental vessel; PDE, phosphodiesterase; TNFα, tumour necrosis factor α; TrK, tropomyosin-receptor kinase; VEGF, vascular endothelial growth; VEGFR, VEGF receptor; XAA, xanthenone 4-acetic acid; DMXAA, 5,6-dimethylXAA; MeXAA, methyl-XAA.

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necrosis of the tumour [2–8]. This was consistent with the finding that a single dose of DMXAA induced a prolonged reduction in the growth of xenografted tumours in animal models. The ability to disrupt the vasculature in these pre-clinical models has been attributed to a rapid induction of cytokines, particularly TNFα (tumour necrosis factor α) [1]. This mechanism seems to differ from other vascular disruptors such as combretastatin [9] or ZD6126 [10], which elicit their effects by directly binding to tubulin.

Despite the fact that the molecular targets for the drug remained unknown [11], the promising pre-clinical results led to DMXAA being selected for clinical development [12–14]. Results of Phase I trials showed some restriction of tumour blood flow within 24 h of treatment, although this was not as dramatic as seen in pre-clinical models [15]. Unlike the animal models, there was also very little evidence for the rapid death of blood vessels or for increases in TNFα levels in human tumours [15,16]. No difference in antitumour activity, cytokine induction or toxicity was observed between two parallel Phase I trials, one dosed weekly [13] and the other dosed every 3 weeks [17]. Therefore the drug proceeded to Phase II clinical trials, dosed every 21 days in combination with chemotherapeutic agents [18–20]. These trials indicated the drug had small benefits in the treatment of non-small-cell lung cancer and prostate cancer [18–20]. However, a subsequent Phase III clinical trial was not able to reproduce this response and clinical development was halted [21]. The reasons for this are not clear, but it is unlikely that these problems can be resolved until appropriate biomarkers are developed to monitor efficacy or a patient population can be identified that would particularly benefit from DMXAA treatment [11]. However, this would require an understanding of the molecular targets of DMXAA.

To date the only molecular targets of DMXAA identified are members of the PDE (phosphodiesterase) family, which are partially inhibited at the concentrations achieved in clinical trials [22]. The inhibition of PDE6 explained the visual disturbances observed in clinical trials [22], but the inhibition of the members of the PDE family is unlikely to explain the antivascular effects of DMXAA [23,24].

In the present study, we have tested whether DMXAA might target protein kinases. Our enzyme screens show that, at pharmacologically relevant concentrations, DMXAA inhibits several protein kinases, including HIPK2 (homeodomain-interacting protein kinase 2), CK2 (casein kinase 2), Haspin, Aurora kinases, PIM kinases, c-FMS and TrKs (tropomyosin-receptor kinases), but the most potent inhibition was of VEGFR [VEGF (vascular endothelial growth factor) receptor] tyrosine kinases. We go on demonstrate that a range of DMXAA analogues have similar properties and to show that these compounds functionally inhibit VEGFR function in cells. Taken together, these findings provide evidence to help understand the molecular targets and mechanisms of action of DMXAA and related molecules, and suggest future directions for developing this drug.

MATERIALS AND METHODS

Chemical synthesis

Synthesis of XAA, DMXAA, α-MeXAA (methylXAA), XPA (xanthenone propionic acid) and 5-phenylXAA was performed as described previously [25–28].

Enzyme screens

Enzyme screens were performed by two different service providers: the National Centre for Protein Kinase Profiling (Dundee, U.K.) using the method detailed previously [29], and the European Screening Centre (Invitrogen). The latter Centre used both the Z′-LYTE assay and the LanthaScreen® Eu Kinase Binding Assay [30]. All assays were performed using the apparent K_m for ATP. The European Screening Centre also performed inhibitor IC_{50} determinations.

Cell culture experiments

Early passage (<8) HUVECs (human umbilical vein endothelial cells) were cultured in medium 200 supplemented with LSGS (low-serum growth supplement) (Invitrogen), according to the supplier's instructions. HUVECs (4×10^5 cells/well) were subcultured into six-well plates pre-coated with 1 % gelatin (Sigma) and allowed to grow to approximately 80 % confluence before starvation for 16 h in medium 200/0.5 % FBS (fetal bovine serum). HUVECs were then pre-incubated for 1 h with medium 200/0.1 % FBS in the presence/absence of inhibitors at the stated concentrations, before stimulation for 10 min with 50 ng/ml human $VEGF_{165}$ (Symansis). Cells were lysed and the lysates were analysed by Western blotting using antibodies against phosphorylated ERK (extracellular-signal-regulated kinase) 1/2 (Thr^{202}/Tyr^{204}) or phosphorylated VEGFR2 (Tyr^{951}) (Cell Signalling Technology). AV-951 (Tivozanib, KRN-951; Symansis) was used as a control inhibitor at a concentration of 50 nM.

Zebrafish experiments

Friend leukaemia integration 1a transgenic zebrafish (*fli1a:EGFP*), which express GFP (green fluorescent protein) in the developing vasculature [31], were used in our experiments. We used 15–20 embryos per experimental group, and each experiment was carried out in two independent replicates. Embryos were maintained in E3 solution at 28 °C. A 4 hpf (h post-fertilization), zebrafish embryos were placed in 600 μl of E3 solution supplemented with penicillin/streptomycin (Invitrogen) and either AV951 or DMXAA/analogues

or vehicle (DMSO) and maintained in the dark. At 24 hpf, 0.003 % 1-phenyl-2-thiourea was added to maintain the optical transparency of the zebrafish embryos. At 48 hpf, zebrafish embryos were manually dechorinated, anaesthetized with 0.01 % tricaine (3-aminobenzoic acid ethyl ester) and mounted in 3 % methylcellulose for imaging on either a NikonD-Eclipse C1 confocal microscope or a Nikon SMZ1500 stereomicroscope equipped with a DS-U2/L2 camera. ISVs (intersegmental vessels) were counted manually and appraised as 'complete' when they had extended to the level of the DLAV (dorsal longitudinal anastomotic vessel). For simplicity, ISVs were only counted on one side of each embryo.

Molecular modelling

Modelling was performed as described recently [32]. The images were generated using PyMOL version 1.4 (http://www.pymol.org). The ATP-binding site surface was generated from the VEGFR2 structure (PDB code 2OH4) [33].

RESULTS

To identify kinases that might be targeted by DMXAA we first performed screens at 50 μM DMXXA as this is within the range of concentrations at which DMXAA gives effects in cultured cells [2,34–37]. The full results of our first screen against a panel of kinases are shown in Supplementary Table S1 (at http://www.clinsci.org/cs/122/cs1220449add.htm) with those compounds showing a greater than 50 % inhibition being summarized in Table 1. To confirm and extend these results, we performed a second screen on a larger panel of kinases (see Supplementary Table S2 at http://www.clinsci.org/cs/122/cs1220449add.htm) with those compounds showing a greater than 50 % inhibition also summarized in Table 1. These results demonstrate that DMXAA has the potential to inhibit a select range of kinases at the concentrations that are observed after therapeutic dosing [22,38].

To understand more about the structure–activity relationship of these inhibitory effects, we screened 12 DMXAA analogues (Figure 1) against a range of kinases (Table 2 and Supplementary Table S3 at http://www.clinsci.org/cs/122/cs1220449add.htm). Broadly, the compounds displayed a similar target profile, but several of these compounds were consistently more potent than DMXAA against many of the targets, most notably XPA, 2-MeXAA and 6-MeXAA.

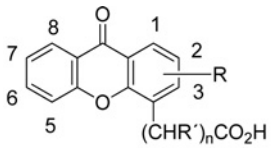
The greatest degree of inhibition in these screens was of VEGFRs, which were strongly inhibited by 50 μM DMXAA and were also inhibited by most of the analogues (Table 1). Given the role these receptors play in angiogenesis, we determined the IC_{50} of these compounds against VEGFR1 and VEGFR2 (Table 3). These results show that a number of the compounds are able to inhibit VEGFR2 in the low-micromolar range, particularly 2-MeXAA and 6-MeXAA, although they were approximately 10-fold less potent as inhibitors of VEGFR1.

Table 1 Summary of kinases showing greater than 50 % inhibition by 50 μM DMXAA

The first eight kinase hits result from screening against 102 kinases in the National Centre for Protein Kinase Profiling screen (Dundee), and the remaining kinase hits result from screening against 267 kinases in the European Screening Centre screen (Invitrogen). JAK3, Janus kinase 3; FGFR4, fibroblast growth factor 4; IRAK1, interleukin-1-receptor-associated kinase 1; NTRK1 etc., neurotrophic tyrosine kinase receptor type 1 etc; NUAK1, sucrose-non-fermenting kinase-1-like kinase 1.

Kinase	Activity remaining at 50 μM DMXAA (%)
Aurora B	48 ± 8
NUAK1	41 ± 2
CK2	29 ± 6
PIM1	25 ± 1
PIM3	12 ± 3
HIPK2	35 ± 3
TrkA	32 ± 4
VEGFR1	19 ± 2
AURKA (Aurora A)	33 ± 1
CSF1R (FMS)	34 ± 1
FGFR4	35 ± 1
FLT3 D835Y	44 ± 0
FLT4 (VEGFR3)	20 ± 2
IRAK1	41 ± 0
JAK3	48 ± 0
KDR (VEGFR2)	15 ± 0
NTRK1 (TrkA)	39 ± 3
NTRK2 (TrkB)	23 ± 2
NTRK3 (TrkC)	22 ± 1
GSG2 (Haspin)	47 ± 7

These findings suggest that these compounds could have anti-angiogenic effects, so to test this we used an embryonic zebrafish model in which blood vessels were labelled with GFP. Representative results are shown in Figure 2(A) and a quantitative analysis of all embryos is shown in Figure 2(B). In these studies, 25 μM 1-MeXAA and 5-phenyl-XAA were toxic. However, 25 μM DMXAA was not toxic and had some anti-angiogenic effect. 2-MeXAA and 6-MeXAA were also non-toxic and were very effective inhibitors of angiogenesis, and, like the control compound AV-951, caused complete inhibition of ISV formation. Overall, the anti-angiogenic effects of the different drugs correlated very well with the degree to which they inhibited VEGFR1 and VEGFR2 (Figure 2B and Table 3). These studies clearly demonstrate that DMXAA and some



XAA	R = H, R′ = H, n = 1
DMXAA	R = 5,6-dimethyl, R′ = H, n = 1
1-Me XAA	R = 1-methyl, R′ = H, n = 1
2-Me XAA	R = 2-methyl, R′ = H, n = 1
3-Me XAA	R = 3-methyl, R′ = H, n = 1
5-Me XAA	R = 5-methyl, R′ = H, n = 1
6-Me XAA	R = 6-methyl, R′ = H, n = 1
7-Me XAA	R = 7-methyl, R′ = H, n = 1
8-Me XAA	R = 8-methyl, R′ = H, n = 1
α-Me XAA	R = H, R′ = methyl, n = 1
XPA XAA	R = H, R′ = H, n = 2
5-Cl XAA	R = 5-chloro, R′ = H, n = 1
5-Ph XAA	R = 5-phenyl, R′ = H, n = 1

Figure 1 Structure of DMXAA and its analogues tested

of its analogues have the ability to block embryonic angiogenesis in this zebrafish model.

To further directly test whether these compounds were blocking VEGFRs in mammalian cells, we examined the effects of the drugs on $VEGF_{165}$-induced signalling in HUVECs (Figure 3). VEGFRs were immunoprecipitated from $VEGF_{165}$-treated HUVECs and then analysed by Western blotting using a VEGFR2-specific phosphotyrosine antibody (Figure 3A). In these experiments, the VEGFR2 inhibitor AV951 [39], 2-MeXAA and 6-MeXAA blocked receptor phosphorylation. Further evidence that these drugs were inhibiting VEGFR signalling was provided by the finding that DMXAA and its analogues were able to block signalling pathways activated by $VEGF_{165}$. VEGF potently stimulates ERK signalling in these cells and the small-molecule VEGFR inhibitor AV-951 blocked this, as expected, but the effect was also blocked by DMXAA, 2-MeXAA and 6-MeXAA (Figures 3A and 3B). It is notable that, in these same experiments, the total level of VEGFR2 extracted in the lysis buffer was reduced by $VEGF_{165}$ treatment (Figure 3B), which is consistent with ligand-induced receptor internalization and degradation [40]. This was reversed by AV951, 2-MeXAA and 6-MeXAA, which provides further evidence that these compounds can directly inhibit VEGFR2 signalling in mammalian cells. We next investigated the potency of these effects and found that by 30 μM DMXAA had begun to significantly reduce the VEGF-induced activation of ERK in cells, despite the fact that some serum (and hence binding proteins) was present in the cell culture medium (Figure 3C). This is consistent with the potency with which DMXAA inhibits VEGFR2 *in vitro*. 2-MeXAA and 6-MeXAA were even more potent inhibitors of ERK activation (Figure 3C), consistent with their greater potency against the VEGFRs *in vitro*.

DISCUSSION

The findings of the present study demonstrate that DMXAA and its analogues inhibit a range of kinases in the low-to-mid-micromolar concentrations. For most drugs this would not be relevant to their therapeutic effects, but levels of free DMXAA found in the circulation are relatively high at therapeutic dosing levels in humans. The C_{max} following doses used in clinical trials (1200 mg/m^2) have been reported to be up to 20 μM (reviewed in [41]) and free DMXAA levels of 240 μM were achieved at only a four times higher dose (4800 mg/m^2) in pharmacology studies [38]. Together, this suggests that DMXAA and some of its analogues could act as multi-kinase inhibitors at the dosing levels used in humans.

The important question is how these inhibitory effects on kinases might relate to the therapeutic effects of DMXAA and its ability to disrupt the

Table 2 DMXAA analogues which inhibit targets by more than 50% in the initial screen from the National Centre for Protein Kinase Profiling Centre

Cl, chloro; Ph, phenyl.

		Activity remaining at 50 μM of the drug (%)											
Kinase	Drug . . .	XAA	1-MeXAA	2-MeXAA	3-MeXAA	5-MeXAA	6-MeXAA	7-MeXAA	8-MeXAA	α-MeXAA	XPA	5-ClXAA	5-PhXAA
Aurora B		26 ± 3	44 ± 0	14 ± 1	20 ± 1	30 ± 2	12 ± 1	16 ± 2	38 ± 2	21 ± 7	26 ± 0	52 ± 5	36 ± 1
NUAK1		24 ± 1	34 ± 4	17 ± 1	26 ± 2	18 ± 1	21 ± 1	12 ± 1	66 ± 17	9 ± 2	39 ± 2	30 ± 1	16 ± 1
CK2		35 ± 1	15 ± 0	34 ± 3	49 ± 0	37 ± 1	20 ± 1	53 ± 6	32 ± 1	40 ± 0	3 ± 0	41 ± 2	35 ± 5
PIM1		46 ± 3	34 ± 0	33 ± 4	25 ± 2	15 ± 0	24 ± 1	32 ± 3	29 ± 4	21 ± 1	9 ± 0	31 ± 5	7 ± 1
PIM3		17 ± 0	20 ± 0	17 ± 1	14 ± 1	11 ± 0	13 ± 3	10 ± 1	9 ± 0	10 ± 0	8 ± 0	13 ± 1	7 ± 1
HIPK2		13 ± 1	17 ± 1	22 ± 1	18 ± 0	10 ± 0	11 ± 1	19 ± 0	14 ± 1	10 ± 0	4 ± 0	12 ± 1	10 ± 0
TrkA		63 ± 5	95 ± 18	23 ± 3	31 ± 5	37 ± 4	26 ± 4	34 ± 1	77 ± 10	40 ± 1	29 ± 1	49 ± 5	14 ± 2
VEGFR1		18 ± 2	67 ± 6	7 ± 3	13 ± 0	29 ± 3	9 ± 1	24 ± 2	33 ± 3	19 ± 1	15 ± 1	28 ± 4	19 ± 2

Table 3 IC_{50} **values for the effects of the DMXAA analogues on VEGFR1 and VEGFR2 from the screen at the European Screening Centre**

Cl, chlorinyl; Ph, phenyl.

	Drug IC_{50} (μM)												
Kinase	XAA	DMXAA	1-MeXAA	2-MeXAA	3-MeXAA	5-MeXAA	6-MeXAA	7-MeXAA	8-MeXAA	α-MeXAA	XPA	5-ClXAA	5-PhXAA
VEGFR1	172	119	2707	13	167	173	49	800	171	336	137	112	185
VEGFR2	16	11	80	1	16	19	9	56	61	27	25	19	9

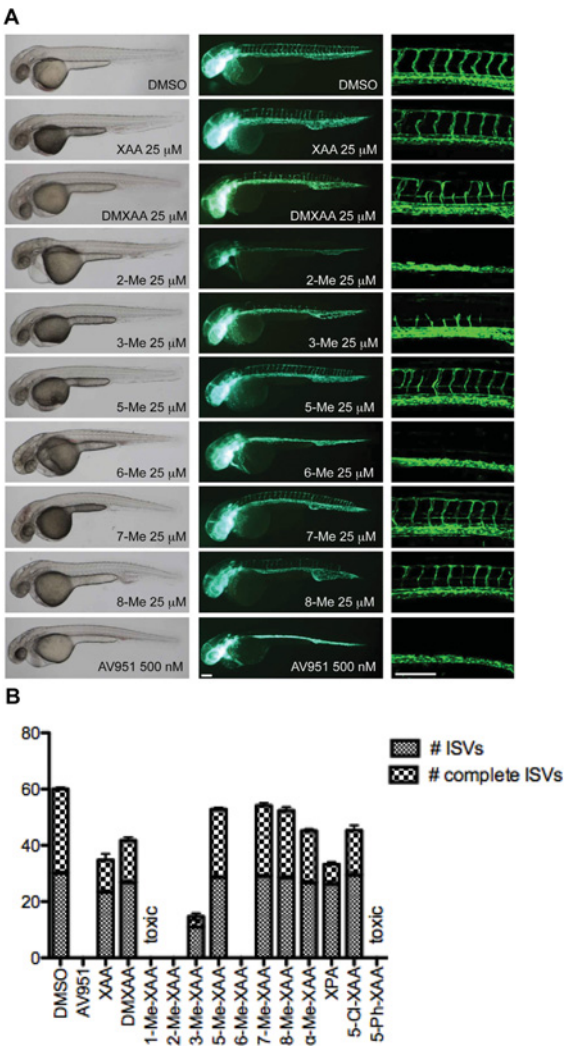


Figure 2 Effect of DMXAA and its analogues on angiogenesis in zebrafish

Friend leukaemia integration 1a transgenic zebrafish (*fli1a:EGFP*) embryos were incubated with the indicated drug concentration and analysed as described in the Materials and methods section. (A) Representative results for the controls, XAA, DMXAA and the methyl (Me) analogues of XAA are shown. Scale bar, 200 μm. (B) Quantification of all of the compounds tested; # ISV, total number of ISVs counted; # complete ISV, number of ISVs which extend to the DLAV.

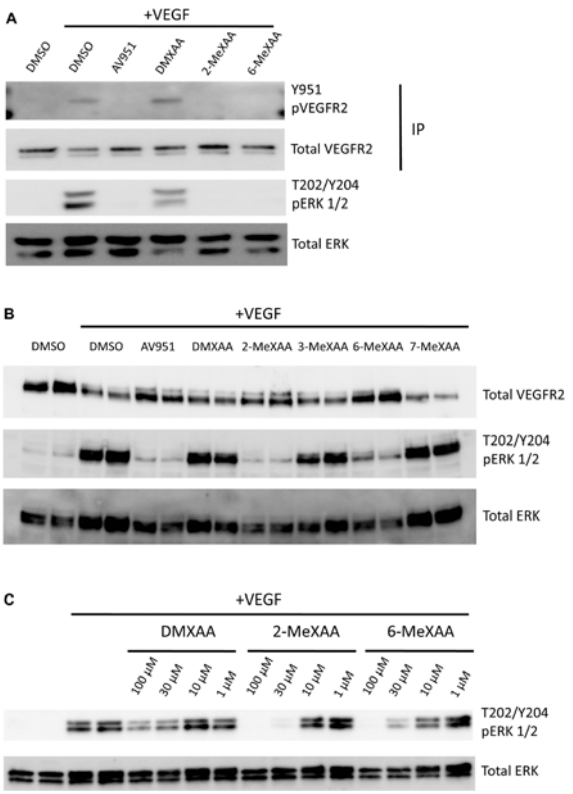


Figure 3 Effect of DMXAA and its analogues on VEGFR signalling in HUVECs

HUVECs were serum-starved overnight in medium 200 with 0.5 % FBS. Cells were then incubated fresh medium with 0.1 % FBS. (A) Cells were treated with 50 nM AV951, or 100 μM of DMXAA, 2-MeXAA or 6-MeXAA for 60 min before the addition of 50 ng/ml $VEGF_{165}$ for a further 10 min. Cells were lysed and the lysates were immunoprecipitated (IP) with an anti-VEGFR2-specific antibody and then Western blots were performed with an antibody specific for the tyrosine-phosphorylated VEGFR2. (B) The indicated DMXAA analogue (100 μM) was added for 60 min before the addition of 50 ng/ml $VEGF_{165}$ for a further 10 min. Cells were lysed and the resulting lysates were analysed by Western blotting with the indicated antibody. Results are representative of at least three independent experiments. (C) The indicated concentrations of DMXAA, 2-MeXAA or 6-MeXAA were added 60 min before the stimulation with 50 ng/ml $VEGF_{165}$ for 10 min. Cells were lysed and the lysates were analysed by Western blotting with the indicated antibodies. Results are representative of at least three independent experiments.

vasculature. None of the inhibitory effects on the kinases that we have observed are likely to explain the dramatic immune-mediated antivascular effects of DMXAA observed in some mouse tumour models [2,4,5,7,8,42]. In fact, the results with the DMXAA analogues support this, for example the inhibition of VEGFR2 is not sufficient to induce the vascular disruption, as 2-MeXAA and 6-MeXAA were even more potent inhibitors of VEGFR2 signalling, but both of these have were less effective than DMXAA in disrupting the vasculature in colon-38 xenograft models [27,43]. Further studies will be required to determine the molecular targets of DMXAA responsible for the immune-modulated vascular-disrupting effects seen in animal models. However, DMXAA also has non-immune-mediated effects and a number of the kinases inhibited by DMXAA, and its analogues have the potential to contribute to such antitumour activity. Kinases targeted by DMXAA that have been implicated in cancer include the serine/threonine kinases CK2 [44,45], Haspin [46], Aurora kinase [47,48] and PIM kinases [49]. They also include a number of receptor tyrosine kinases, including c-FMS [50], VEGFRs [51] and TrKs [52]. The activity against VEGFRs was of particular interest given the role these play in angiogenesis [51]. In the present study, we have focussed on VEGFR2 as this is the most important VEGFR controlling angiogenesis [51], and DMXAA was a more potent inhibitor of this than other kinases in our screen. Our studies provide evidence for inhibition of VEGFR2 signalling in HUVECs by DMXAA and its analogues. This indicates that DMXAA will attenuate VEGF signalling *in vivo*. This is supported by the finding that DMXAA and its analogues have anti-angiogenic effects in zebrafish, the potency of which correlates reasonably well with the potency with which these compounds inhibit VEGFR2. Several of the other kinases inhibited by DMXAA also have roles in blood vessel formation, including CK2 [44,45], Haspin [46], PIM-1 [53] and various isoforms of VEGFR [51]. The results in the present study have focussed only on VEGFR2 and provide strong evidence that the anti-VEGFR2 activity of DMXAA can contribute to anti-angiogenic activity. Further studies will be required to determine whether the inhibition of the other kinases might also contribute to the anti-angiogenic activity observed.

Because of the dramatic vascular-disrupting effects of DMXAA in animal models [2,4,5,7,8,42], subsequent studies have mostly focussed on its potential to act as a vascular-disrupting agent. The rapid disruption of blood vessels leading to haemorrhagic necrosis of tumours seen in mouse models is linked with a rapid induction of high levels of cytokines, particularly TNFα [54]. The effects of DMXAA on the vasculature in human tumours are not as dramatic as those seen in mouse xenograft models and this may be due to the fact that DMXAA does not induce such large increases in tumour TNFα [15,16] or other cytokines [12] in humans. The reason for this is not clear, but may explain why the clinical efficacy was lower in humans when DMXAA was used as a single agent. However, although often overlooked, DMXAA also had anti-angiogenic effects in pre-clinical models [55]. Compounds specifically designed as VEGFR inhibitors have similar anti-angiogenic effects in both pre-clinical models [56–58] and in human tumours [58,59]. This suggests that the anti-VEGFR activity of DMXAA has the potential to contribute to the therapeutic antitumour effects of DMXAA. However, this would require continual suppression of the VEGFR and so would require constant presence of drug at micromolar concentrations in blood. This would not have been achieved in the Phase II and Phase III clinical trials as dosing was only once every 3 weeks [41]. The pharmacology suggests that once or twice daily dosing would be required to achieve a level of drug exposure to allow effective inhibition of angiogenesis [41]. This dosing regime has not yet been tested in humans.

Our findings that, in addition to its vascular-disrupting activity, DMXAA inhibits VEGFR2 may have other implications for efficacy. The literature suggests that the combinations of these two effects can synergize in blocking tumour growth. In one such study, the anti-vascular effects of TNFα are potentiated by the VEGFR inhibitor ZD6474 (Vandetinib) in animal models [60]. In another study, the effects of a vascular-disrupting agent targeting microtubules and ZD6474 were also found to synergize [10]. This could help explain the dramatic effects of DMXAA on the vasculature in animals. Such combinations have not yet been tried in humans.

The present findings also provide some direction for the future development of this drug family, in particular they suggest that further investigations of analogues of DMXAA such as 2- and 6-MeXAA are warranted. To gain some insight into how DMXAA might inhibit the VEGFR2 kinase activity, the compound series was modelled into the VEGFR2 kinase domain (Figure 4). Binding modes were predicted that indicated a possible hydrogen bond between the central carbonyl group of the DMXAA analogues and the backbone amide of Cys^{919} in the inter-lobe linker region of the kinase domain, an interaction seen in ligand-bound VEGFR2 protein structures used in the present study. This is consistent with the results presented in Table 1, which illustrate a negative effect of substitutions adjacent to the carbonyl group at positions 1 and 8. These may sterically hinder the essential ligand linker region interaction. The best binding mode predicted for the most active analogue (2-MeXAA) was found in the active site of PDB code 2XIR, and indicated that the carboxylate group is directed toward the mouth of the ATP-binding site, whereas the 2-Me group is in close proximity to the surface created by Leu^{840} and Phe^{918}. In this orientation the 5 and 6 positions of DMXAA (in PDB code 2OH4) are orientated toward

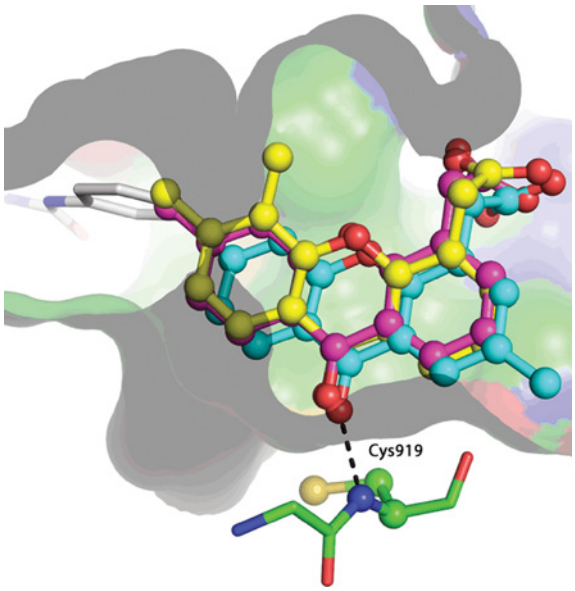


Figure 4 Models of DMXAA and its analogues in the VEGFR2 structure

Models of DMXAA (yellow ball and stick), 2-MeXAA (cyan ball and stick) and 6-MeXAA (magenta ball and stick) bound in the ATP-binding site of the VEGFR2 kinase domain. A possible interaction with the backbone amide of Cys^{919} (green ball and stick) is shown as a broken line. The ATP-binding site surface was generated from the VEGFR2 structure (PDB code 2OH4) in which a DMXAA-binding mode similar to that best ranked for 2-MeXAA was predicted. The carboxylate group is orientated toward solvent, whereas the 6 position is orientated toward the rear of the active site and a pocket occupied by other VEGFR kinase inhibitors. A fragment of the benimidazole inhibitor present in the VEGFR2 structure (PDB code 2OH4) is shown to indicate the pocket location (shown by white sticks).

the back of the pocket, with the 6 position directed toward a site occupied by aromatic groups in other VEGFR inhibitors [61–63], as is shown in Figure 4. This suggests that addition of bulk at the 6 position would be tolerated and that such compounds may be more selective and potent inhibitors of VEGFR2.

In summary, the results of the present study have identified new potential molecular targets for DMXAA which will help in understanding how this drug works *in vivo* and how it might be optimized for clinical use.

AUTHOR CONTRIBUTION

Christina Buchanan, Jen-Hsing Shih, Jonathan Astin, Gordon Rewcastle, Jack Flanagan, Philip Crosier and Peter Shepherd all participated in planning the experiments. Christina Buchanan, Jen-Hsing Shih, Jonathan Astin, Gordon Rewcastle and Jack Flanagan all performed the experiments. Christina Buchanan, Jonathan Astin, Philip Crosier and Peter Shepherd interpreted the results. Christina Buchanan, Jack Flanagan and Peter Shepherd wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

DMXAA (Vadimezan, ASA404) is a multi-kinase inhibitor targeting VEGFR2 in particular

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See following pages for Supplementary Tables 1–3.

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Table S1 Screening against 50 μM DMXAA for kinases in the National Centre for Protein Kinase Profiling screen

JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MKK1, MAPK kinase 1; MAPKAPK2, MAPK-activated protein kinase 2; RSK, p90 ribosomal S6 kinase; PDK1, phosphoinositide-dependent kinase 1; PKA, protein kinase A; PKC, protein kinase C; PKB, protein kinase B; SGK, serum- and glucocorticoid-induced protein kinase; S6K1, p70 ribosomal S6 kinase 1; ROCK2, Rho-dependent protein kinase 2; PKD1, protein kinase D1; MSK, mitogen- and stress-activated kinase; PRAK, p38-regulated activated kinase; CaMKKβ, CaMK kinase β; CaMK1, calmodulin-dependent kinase 1; PHK, phosphorylase kinase; PAK, p21-activated protein kinase; MST, mammalian homologue Ste20-like kinase; GSK, glycogen synthase kinase; CSK, C-terminal Src kinase; BTK, Bruton's tyrosine kinase; JAK2, Janus kinase 2; SYK, spleen tyrosine kinase; EPH, ephrin; FGFR1, fibroblast growth factor receptor 1; IR, insulin receptor; IRR, insulin-related receptor; IGF1R, insulin-like growth factor-1 receptor; CHK1 checkpoint kinase 1; PRK, PKC-related kinase; MNK, MAPK-interacting kinase; smMLCK, smooth muscle myosin light-chain kinase; MEKK, MAPK/ERK kinase kinase; MLK, mixed-lineage kinase; TAK, transforming growth factor-β-activated kinase; RIPK1, receptor-interacting serine/threonine protein kinase1.

Kinase	Activity remaining at 50 μM DMXAA (%)
MKK1	120 $\pm$ 26
ERK1	145 $\pm$ 6
ERK2	116 $\pm$ 19
JNK1	84 $\pm$ 4
JNK2	104 $\pm$ 13
JNK3	87 $\pm$ 6
p38α MAPK	108 $\pm$ 4
p38β MAPK	110 $\pm$ 0
p38γ MAPK	121 $\pm$ 18
p38δ MAPK	101 $\pm$ 4
ERK8	67 $\pm$ 3
RSK1	113 $\pm$ 2
RSK2	114 $\pm$ 15
PDK1	103 $\pm$ 1
PKBα	109 $\pm$ 5
PKBβ	125 $\pm$ 15
SGK1	126 $\pm$ 9
S6K1	110 $\pm$ 13
PKA	102 $\pm$ 2
ROCK2	98 $\pm$ 10
PRK2	105 $\pm$ 10
PKCα	115 $\pm$ 4
PKCζ	105 $\pm$ 3
PKD1	103 $\pm$ 10
MSK1	104 $\pm$ 15
MNK1	94 $\pm$ 18
MNK2	84 $\pm$ 0
MAPKAPK2	106 $\pm$ 1
MAPKAPK3	89 $\pm$ 7
PRAK	94 $\pm$ 11
CAMKKβ	53 $\pm$ 5
CAMK1	132 $\pm$ 24
SmMLCK	62 $\pm$ 8
PHK	68 $\pm$ 2
PAK4	65 $\pm$ 4
PAK5	93 $\pm$ 6
PAK6	97 $\pm$ 4
MST2	89 $\pm$ 3
MST4	82 $\pm$ 2
GCK	52 $\pm$ 3
MINK1	75 $\pm$ 2
MEKK1	103 $\pm$ 11
MLK1	68 $\pm$ 16
MLK3	82 $\pm$ 2
TAK1	75 $\pm$ 7
IRAK4	52 $\pm$ 4
RIPK2	88 $\pm$ 6
TTK	83 $\pm$ 5
Src	79 $\pm$ 11
Lck	112 $\pm$ 11
CSK	117 $\pm$ 17
YES1	99 $\pm$ 0
BTK	56 $\pm$ 5
JAK2	111 $\pm$ 5
SYK	85 $\pm$ 16
EPHA2	110 $\pm$ 3
EPHA4	104 $\pm$ 5
EPHB3	111 $\pm$ 10
EPHB4	95 $\pm$ 2
FGFR1	73 $\pm$ 6
HER4	98 $\pm$ 2
IGF1R	88 $\pm$ 5
IR	82 $\pm$ 1
IRR	57 $\pm$ 5
TrkA	32 $\pm$ 4
VEGFR	19 $\pm$ 2
CHK1	109 $\pm$ 3

Table S2 Screening against 50 μM DMXAA for kinases in the European Screening Centre screen

PKB, protein kinase B; AMPK, AMP-activated protein kinase; BSRK, brain-specific kinase; CaMKK, Ca^{2+}/calmodulin-dependent protein kinase; BTK, Bruton's tyrosine kinase; CDC, cell division cycle; CDK, cyclin-dependent kinase; CHK, checkpoint kinase; IKK, IκB kinase; CLK, CDC2p/CDC28p-like kinase; CSK, C-terminal Src kinase; DAPK, death-associated protein kinase; DCK, DC kinase; DNA-PK, DNA-activated protein kinase; DYRK, dual-specificity tyrosine-phosphorylated and -regulated kinase; EGFR, epidermal growth factor receptor; EPH, ephrin; FGFR, fibroblast growth factor receptor; EEF2K, eukaryotic elongation factor 2 kinase; mTOR, mammalian target of rapamycin; GRK, G-protein-coupled-receptor kinase; GSK, glycogen synthase kinase; JAK, Janus kinase; IRR, insulin-related receptor; IGF1R, insulin-like growth factor-1 receptor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; JNK, c-Jun N-terminal kinase; MAPKAPK, MAPK-activated protein kinase; MARK, microtubule-associated protein-regulating kinase; IRAK, interleukin-1-receptor-associated kinase; MELK, maternal embryonic leucine zipper kinase; NEK, never in mitosis in *Aspergillus nidulans*-related kinase; PAK, p21-activated kinase; PDGFR, platelet-derived growth factor receptor; PDK, phosphoinositide-dependent kinase; PHKG, phosphorylase kinase γ; PI4K, phosphoinositide 4-kinase; PKA, protein kinase A; PKC, protein kinase C; FAK, focal adhesion kinase; ROCK, Rho-dependent protein kinase; RSK, p90 ribosomal S6 kinase; PKD, protein kinase D; PKG, protein kinase G; MSK, mitogen- and stress-activated kinase; S6K, S6 kinase; SGK, serum- and glucocorticoid-induced protein kinase; TBK, TNF-receptor-associated factor-associated nuclear factor-κB activator-binding kinase 1; ZAP70, ζ-chain (T-cell receptor)-associated protein kinase of 70 kDa.

Kinase	Activity remaining at 50 μM DMXAA (%)
ABL1	61 ± 1
ABL1 E255K	77 ± 1
ABL1 G250E	83 ± 1
ABL1 T315I	76 ± 0
ABL1 Y253F	56 ± 1
ABL2 (Arg)	83 ± 1
ACVR1B (ALK4)	97 ± 2
ADRBK1 (GRK2)	102 ± 3
ADRBK2 (GRK3)	106 ± 1
AKT1 (PKBα)	95 ± 2
AKT2 (PKBβ)	105 ± 1
AKT3 (PKBγ)	95 ± 2
ALK	91 ± 0
AMPK A1/B1/G1	79 ± 0
AMPK A2/B1/G1	100 ± 1
AURKA (Aurora A)	33 ± 1
AURKB (Aurora B)	85 ± 4
AURKC (Aurora C)	86 ± 2
AXL	73 ± 1
BLK	84 ± 3
BMX	86 ± 2
BRAF	95 ± 3
BRAF V599E	108 ± 7
BRSK1 (SAD1)	88 ± 4
BTK	85 ± 0
CAMK1	91 ± 0
CAMK1D	99 ± 3
CAMK2A	86 ± 2
CAMK2B	80 ± 3
CAMK2D	68 ± 1
CAMK4	75 ± 4
CDC42 BPA (MRCKA)	80 ± 2
CDC42 BPB (MRCKB)	87 ± 2
CDK1/cyclin B	95 ± 3
CDK2/cyclin A	99 ± 3
CDK5/p25	92 ± 1
CDK5/p35	98 ± 1
CDK7/cyclin H/MNAT1	97 ± 1
CDK9/cyclin T1	66 ± 2
CHEK1 (CHK1)	97 ± 3
CHEK2 (CHK2)	84 ± 2
CHUK (IKKα)	102 ± 4
CLK1	99 ± 1
CLK2	95 ± 0
CLK3	79 ± 3
CSF1R (FMS)	34 ± 1
CSK	106 ± 1
CSNK1A1	109 ± 4
CSNK1D	99 ± 0
CSNK1E	94 ± 1
CSNK1G1	105 ± 3
CSNK1G2	106 ± 2
CSNK1G3	109 ± 2
CSNK2A1	90 ± 1
CSNK2A2	66 ± 3
DAPK1	51 ± 5
DAPK3 (ZIPK)	80 ± 0
DCAMKL2 (DCK2)	99 ± 1
DNA-PK	89 ± 0
DYRK1A	79 ± 3
DYRK1B	95 ± 3
DYRK3	94 ± 1
DYRK4	92 ± 1
EEF2K	111 ± 1
EGFR (ErbB1)	93 ± 6
EGFR (ErbB1) L858R	93 ± 4
EGFR (ErbB1) L861Q	93 ± 3
EGFR (ErbB1) T790M	81 ± 0
EGFR (ErbB1) T790M L858R	80 ± 3
EPHA1	91 ± 2
EPHA2	84 ± 3
EPHA3	95 ± 1
EPHA4	67 ± 1
EPHA5	81 ± 0
EPHA8	95 ± 1
EPHB1	93 ± 0

Table S2 Continued

Kinase	Activity remaining at 50 μM DMXAA (%)
EPHB2	91 ± 1
EPHB3	86 ± 0
EPHB4	91 ± 2
ERBB2 (HER2)	92 ± 1
ERBB4 (HER4)	91 ± 7
FER	88 ± 3
FES (FPS)	94 ± 1
FGFR1	68 ± 3
FGFR2	70 ± 0
FGFR3	71 ± 1
FGFR3 K650E	65 ± 0
FGFR4	35 ± 1
FGR	83 ± 1
FLT1 (VEGFR1)	54 ± 0
FLT3	52 ± 1
FLT3 D835Y	44 ± 0
FLT4 (VEGFR3)	20 ± 2
FRAP1 (mTOR)	103 ± 0
FRK (PTK5)	100 ± 5
FYN	88 ± 1
GRK4	99 ± 2
GRK5	102 ± 1
GRK6	90 ± 1
GRK7	91 ± 1
GSG2 (Haspin)	47 ± 7
GSK3A (GSK3α)	86 ± 2
GSK3B (GSK3β)	89 ± 2
HCK	87 ± 1
HIPK1 (Myak)	95 ± 0
HIPK2	72 ± 1
HIPK3 (YAK1)	98 ± 1
HIPK4	74 ± 4
IGF1R	104 ± 0
IKBKB (IKKβ)	98 ± 2
IKBKE (IKKε)	113 ± 2
INSR	109 ± 3
INSRR (IRR)	86 ± 2
IRAK1	41 ± 0
IRAK4	78 ± 1
ITK	105 ± 4
JAK1	78 ± 2
JAK2	88 ± 3
JAK2 JH1 JH2	97 ± 5
JAK2 JH1 JH2 V617F	90 ± 1
JAK3	48 ± 0
KDR (VEGFR2)	15 ± 0
KIT	57 ± 3
KIT T670I	85 ± 0
LCK	97 ± 0
LTK (TYK1)	84 ± 1

Table S2 Continued

Kinase	Activity remaining at 50 μM DMXAA (%)
LYN A	90 ± 2
LYN B	90 ± 2
MAP2K1 (MEK1)	75 ± 3
MAP2K2 (MEK2)	76 ± 2
MAP2K6 (MKK6)	103 ± 0
MAP3K8 (COT)	104 ± 5
MAP3K9 (MLK1)	99 ± 1
MAP4K2 (GCK)	77 ± 4
MAP4K4 (HGK)	78 ± 3
MAP4K5 (KHS1)	79 ± 4
MAPK1 (ERK2)	90 ± 8
MAPK10 (JNK3)	83 ± 1
MAPK11 (p38β)	102 ± 2
MAPK12 (p38γ)	92 ± 4
MAPK13 (p38δ)	96 ± 3
MAPK14 (p38α)	92 ± 2
MAPK14 Direct	97 ± 2
MAPK3 (ERK1)	96 ± 4
MAPK8 (JNK1)	71 ± 3
MAPK9 (JNK2)	64 ± 1
MAPKAPK2	111 ± 0
MAPKAPK3	94 ± 3
MAPKAPK5 (PRAK)	100 ± 1
MARK1 (MARK)	109 ± 3
MARK2	115 ± 5
MARK3	86 ± 2
MARK4	82 ± 2
MATK (HYL)	86 ± 5
MELK	63 ± 2
MERTK (cMER)	92 ± 2
MET (cMet)	83 ± 5
MET M1250T	93 ± 1
MINK1	66 ± 4
MKNK1 (MNK1)	99 ± 3
MST1R (RON)	94 ± 1
MST4	84 ± 3
MUSK	85 ± 0
MYLK2 (skMLCK)	67 ± 0
NEK1	87 ± 1
NEK2	103 ± 1
NEK4	65 ± 1
NEK6	89 ± 4
NEK7	115 ± 3
NEK9	86 ± 0
NTRK1 (TRKA)	39 ± 3
NTRK2 (TRKB)	23 ± 2
NTRK3 (TRKC)	22 ± 1
NUAK1 (ARK5)	64 ± 6
PAK1	79 ± 5
PAK2 (PAK65)	89 ± 2

Table S2 Continued

Kinase	Activity remaining at 50 μM DMXAA (%)
PAK3	84 ± 7
PAK4	88 ± 2
PAK6	108 ± 6
PAK7 (KIAA1264)	95 ± 3
PASK	83 ± 4
PDGFRA	81 ± 3
PDGFRA D842V	93 ± 1
PDGFRA T674I	67 ± 4
PDGFRA V561D	77 ± 1
PDGFRB	95 ± 3
PDK1	109 ± 5
PDK1 Direct	143 ± 7
PHKG1	88 ± 3
PHKG2	88 ± 3
PI4KA (PI4Kα)	100 ± 14
PI4KB (PI4Kβ)	68 ± 8
PIK3C2A	108 ± 2
PIK3C2B	81 ± 2
PIK3C3 (hVPS34)	72 ± 1
PIK3CA/PIK3R1	80 ± 3
PIK3CD/PIK3R1	72 ± 4
PIK3CG	120 ± 2
PIM1	62 ± 3
PIM2	81 ± 1
PKN1 (PRK1)	132 ± 3
PLK1	90 ± 3
PLK2	92 ± 0
PLK3	101 ± 0
PRKACA (PKA)	92 ± 2
PRKCA (PKCα)	85 ± 2
PRKCB1 (PKCβI)	81 ± 1
PRKCB2 (PKCβII)	98 ± 1
PRKCD (PKCδ)	78 ± 6
PRKCE (PKCε)	112 ± 2
PRKCG (PKCγ)	77 ± 3
PRKCH (PKCη)	85 ± 6
PRKCI (PKCι)	83 ± 7
PRKCN (PKD3)	105 ± 3
PRKCQ (PKCθ)	104 ± 3
PRKCZ (PKCζ)	107 ± 3
PRKD1 (PKCμ)	87 ± 1
PRKD2 (PKD2)	88 ± 4
PRKG1	97 ± 8
PRKG2 (PKG2)	88 ± 5
PRKX	88 ± 3
PTK2 (FAK)	91 ± 1
PTK2B (FAK2)	88 ± 3
PTK6 (Brk)	103 ± 2
RAF1 (cRAF) Y340D Y341D	74 ± 5
RET	57 ± 2
RET V804L	57 ± 0

Table S2 Continued

Kinase	Activity remaining at 50 μM DMXAA (%)
RET Y791F	62 ± 0
ROCK1	94 ± 4
ROCK2	91 ± 8
ROS1	100 ± 2
RPS6KA1 (RSK1)	88 ± 1
RPS6KA2 (RSK3)	84 ± 4
RPS6KA3 (RSK2)	85 ± 1
RPS6KA4 (MSK2)	100 ± 3
RPS6KA5 (MSK1)	85 ± 6
RPS6KA6 (RSK4)	88 ± 4
RPS6KB1 (p70S6K)	95 ± 3
SGK (SGK1)	89 ± 6
SGK2	84 ± 6
SGKL (SGK3)	92 ± 4
SNF1LK2	94 ± 4
SPHK1	93 ± 7
SPHK2	106 ± 2
SRC	92 ± 0
SRC N1	89 ± 1
SRMS (Srm)	65 ± 2
SRPK1	86 ± 6
SRPK2	102 ± 2
STK22B (TSSK2)	103 ± 1
STK22D (TSSK1)	91 ± 1
STK23 (MSSK1)	84 ± 4
STK24 (MST3)	66 ± 2
STK25 (YSK1)	69 ± 4
STK3 (MST2)	93 ± 0
STK4 (MST1)	89 ± 5
SYK	70 ± 2
TAOK2 (TAO1)	101 ± 1
TBK1	75 ± 2
TEK (Tie2)	92 ± 2
TXK	89 ± 0
TYK2	81 ± 5
TYRO3 (RSE)	95 ± 0
YES1	88 ± 1
ZAP70	95 ± 0

Table S3 Screening of the kinases against the DMXAA analogues at 50 μM

See Tables S1 and S2 for abbreviations. 5-ClXAA, 5-chloroXAA, 5-PhXAA, 5-phenylXAA.

		Activity remaining at 50 μM (%)											
Kinase	Drug . . .	XAA	1-MeXAA	2-MeXAA	3-MeXAA	5-MeXAA	6-MeXAA	7-MeXAA	8-MeXAA	α-MeXAA	XPA	5-ClXAA	5-PhXAA
MKK1		79 ± 3	93 ± 8	69 ± 6	87 ± 9	61 ± 1	66 ± 10	67 ± 2	74 ± 11	48 ± 1	47 ± 2	75 ± 0	44 ± 4
ERK1		114 ± 3	118 ± 3	127 ± 2	129 ± 15	130 ± 3	124 ± 19	117 ± 3	124 ± 8	128 ± 3	131 ± 7	136 ± 2	125 ± 4
ERK2		99 ± 8	94 ± 5	97 ± 7	79 ± 10	80 ± 11	92 ± 2	106 ± 11	90 ± 12	109 ± 12	97 ± 6	101 ± 7	93 ± 6
JNK1		86 ± 4	67 ± 4	79 ± 2	61 ± 2	65 ± 5	51 ± 5	86 ± 4	68 ± 3	83 ± 19	49 ± 3	76 ± 5	56 ± 4
JNK2		94 ± 2	60 ± 7	77 ± 7	62 ± 2	86 ± 0	65 ± 3	95 ± 8	71 ± 1	98 ± 11	80 ± 2	100 ± 6	74 ± 4
JNK3		70 ± 2	46 ± 4	67 ± 6	56 ± 13	60 ± 6	45 ± 3	86 ± 9	55 ± 8	60 ± 4	45 ± 6	63 ± 7	39 ± 8
p38α MAPK		99 ± 3	110 ± 9	104 ± 2	94 ± 9	101 ± 19	98 ± 27	100 ± 18	86 ± 10	94 ± 5	71 ± 24	107 ± 23	93 ± 10
p38β MAPK		98 ± 17	111 ± 15	102 ± 27	86 ± 11	81 ± 11	102 ± 32	103 ± 12	98 ± 22	99 ± 2	84 ± 10	102 ± 16	112 ± 5
p38γ MAPK		107 ± 12	104 ± 4	112 ± 9	98 ± 4	86 ± 4	95 ± 12	114 ± 6	103 ± 6	109 ± 11	38 ± 0	89 ± 4	80 ± 3
p38δ MAPK		95 ± 4	99 ± 11	99 ± 4	101 ± 1	99 ± 2	94 ± 5	106 ± 7	98 ± 0	95 ± 14	94 ± 5	96 ± 9	99 ± 4
ERK8		90 ± 6	58 ± 1	98 ± 1	91 ± 3	69 ± 3	74 ± 6	92 ± 12	68 ± 7	102 ± 9	33 ± 7	83 ± 11	40 ± 3
RSK1		78 ± 2	82 ± 3	57 ± 5	74 ± 1	65 ± 8	69 ± 2	70 ± 7	91 ± 9	55 ± 4	71 ± 1	74 ± 4	39 ± 6
RSK2		83 ± 1	104 ± 10	72 ± 9	71 ± 12	71 ± 2	82 ± 12	87 ± 5	95 ± 2	85 ± 4	84 ± 6	83 ± 6	64 ± 8
PDK1		97 ± 15	89 ± 12	60 ± 7	70 ± 9	72 ± 2	75 ± 6	62 ± 3	68 ± 6	90 ± 3	80 ± 5	83 ± 4	85 ± 8
PKBα		89 ± 7	72 ± 3	76 ± 7	26 ± 2	60 ± 5	47 ± 5	45 ± 9	68 ± 6	54 ± 0	87 ± 3	61 ± 3	80 ± 7
PKBβ		119 ± 5	108 ± 11	116 ± 8	104 ± 1	83 ± 3	105 ± 12	108 ± 10	95 ± 10	99 ± 5	90 ± 8	94 ± 3	72 ± 2
SGK1		109 ± 11	99 ± 14	90 ± 6	89 ± 3	66 ± 3	79 ± 11	99 ± 6	93 ± 21	90 ± 10	64 ± 11	75 ± 8	86 ± 10
S6K1		96 ± 7	107 ± 14	84 ± 1	79 ± 9	72 ± 6	91 ± 5	92 ± 12	97 ± 12	93 ± 8	68 ± 4	82 ± 19	79 ± 3
PKA		98 ± 1	91 ± 3	94 ± 8	88 ± 1	83 ± 2	84 ± 1	98 ± 5	79 ± 3	114 ± 5	83 ± 1	94 ± 3	78 ± 4
ROCK2		90 ± 7	81 ± 5	68 ± 5	71 ± 5	83 ± 16	73 ± 2	83 ± 6	79 ± 0	67 ± 5	88 ± 2	84 ± 12	71 ± 0
PRK2		87 ± 3	108 ± 2	106 ± 6	93 ± 3	77 ± 5	82 ± 0	88 ± 3	82 ± 2	74 ± 1	97 ± 2	84 ± 1	80 ± 3
PKCα		92 ± 1	80 ± 2	91 ± 5	85 ± 1	87 ± 4	98 ± 3	97 ± 1	88 ± 2	83 ± 2	71 ± 1	94 ± 2	63 ± 1
PKCζ		103 ± 6	99 ± 0	105 ± 1	106 ± 7	119 ± 5	107 ± 5	109 ± 13	101 ± 7	125 ± 7	106 ± 2	115 ± 6	103 ± 0
PKD1		60 ± 1	57 ± 4	65 ± 14	64 ± 6	44 ± 8	65 ± 5	70 ± 9	60 ± 10	55 ± 3	75 ± 9	65 ± 1	36 ± 0
MSK1		67 ± 8	88 ± 10	71 ± 7	79 ± 10	77 ± 11	71 ± 8	83 ± 13	92 ± 27	67 ± 2	65 ± 2	84 ± 6	85 ± 13
MNK1		82 ± 15	77 ± 5	79 ± 6	68 ± 12	78 ± 3	66 ± 4	71 ± 1	65 ± 3	80 ± 13	77 ± 9	79 ± 18	72 ± 2
MNK2		81 ± 11	79 ± 9	44 ± 23	44 ± 4	74 ± 7	38 ± 6	60 ± 11	77 ± 1	107 ± 32	70 ± 1	95 ± 15	74 ± 1
MAPKAPK2		92 ± 6	101 ± 1	105 ± 8	109 ± 17	99 ± 13	108 ± 13	99 ± 10	95 ± 4	106 ± 8	100 ± 9	110 ± 23	88 ± 2
MAPKAPK3		94 ± 4	86 ± 11	86 ± 8	89 ± 2	76 ± 3	81 ± 4	105 ± 15	73 ± 2	110 ± 3	85 ± 16	69 ± 4	60 ± 17
PRAK		88 ± 11	98 ± 5	102 ± 7	85 ± 1	86 ± 4	97 ± 10	44 ± 0	81 ± 4	82 ± 9	71 ± 5	101 ± 7	67 ± 7
CAMKKβ		41 ± 2	62 ± 2	47 ± 0	53 ± 2	44 ± 3	45 ± 0	29 ± 0	29 ± 0	33 ± 3	27 ± 4	41 ± 1	32 ± 6
CAMK1		58 ± 3	41 ± 2	47 ± 5	48 ± 2	53 ± 2	56 ± 1	46 ± 3	30 ± 8	68 ± 1	62 ± 2	80 ± 5	68 ± 9
SmMLCK		31 ± 0	36 ± 2	25 ± 4	37 ± 3	20 ± 1	29 ± 2	30 ± 6	25 ± 2	18 ± 1	39 ± 5	18 ± 1	15 ± 0
PHK		34 ± 2	84 ± 2	44 ± 2	69 ± 1	67 ± 8	44 ± 5	51 ± 9	92 ± 7	32 ± 0	30 ± 13	85 ± 0	57 ± 2
DAPK1		71 ± 16	74 ± 12	95 ± 5	93 ± 5	41 ± 5	58 ± 4	70 ± 2	50 ± 5	94 ± 11	65 ± 2	42 ± 7	10 ± 2
CHK1		115 ± 11	130 ± 15	110 ± 24	99 ± 7	125 ± 27	98 ± 19	95 ± 2	102 ± 9	152 ± 38	109 ± 1	111 ± 12	117 ± 6
CHK2		25 ± 5	42 ± 2	21 ± 1	47 ± 3	48 ± 12	45 ± 6	31 ± 1	61 ± 15	39 ± 4	36 ± 0	90 ± 8	64 ± 4
GSK3β		92 ± 2	88 ± 1	89 ± 10	82 ± 3	74 ± 0	81 ± 7	98 ± 6	75 ± 8	82 ± 1	44 ± 1	80 ± 4	49 ± 0
CDK2/cyclin A		95 ± 0	94 ± 3	104 ± 13	94 ± 5	84 ± 9	92 ± 1	97 ± 8	84 ± 2	100 ± 15	77 ± 1	88 ± 1	67 ± 6
PLK1		105 ± 4	102 ± 5	122 ± 1	123 ± 8	113 ± 9	114 ± 9	122 ± 16	123 ± 15	94 ± 11	96 ± 5	107 ± 1	93 ± 7
Aurora A		71 ± 1	67 ± 11	44 ± 1	80 ± 15	59 ± 4	34 ± 3	63 ± 7	54 ± 14	45 ± 4	28 ± 3	75 ± 3	48 ± 2
Aurora B		26 ± 3	44 ± 0	14 ± 1	20 ± 1	30 ± 2	12 ± 1	16 ± 2	38 ± 2	21 ± 7	26 ± 0	52 ± 5	36 ± 1
LKB1		84 ± 10	88 ± 6	89 ± 3	84 ± 2	51 ± 1	93 ± 2	83 ± 1	80 ± 8	58 ± 4	57 ± 3	59 ± 6	61 ± 3
AMPK		84 ± 6	87 ± 6	71 ± 7	82 ± 6	68 ± 11	85 ± 1	76 ± 1	83 ± 5	74 ± 2	18 ± 4	79 ± 2	45 ± 1
MARK1		89 ± 1	100 ± 1	106 ± 5	110 ± 3	102 ± 3	103 ± 11	108 ± 3	98 ± 8	106 ± 2	90 ± 3	103 ± 1	86 ± 3
MARK2		93 ± 3	88 ± 7	83 ± 3	73 ± 11	79 ± 11	82 ± 3	90 ± 2	81 ± 2	96 ± 2	72 ± 4	92 ± 15	77 ± 9
MARK3		103 ± 11	85 ± 4	82 ± 8	79 ± 4	77 ± 3	81 ± 3	92 ± 3	80 ± 4	72 ± 3	62 ± 3	83 ± 16	64 ± 2
MARK4		80 ± 2	84 ± 15	74 ± 6	75 ± 5	78 ± 5	80 ± 14	83 ± 6	87 ± 2	66 ± 1	82 ± 4	92 ± 8	78 ± 1

Table S3 Continued

Kinase	Drug . . .	Activity remaining at 50 μM (%)											
		XAA	1-MeXAA	2-MeXAA	3-MeXAA	5-MeXAA	6-MeXAA	7-MeXAA	8-MeXAA	α-MeXAA	XPA	5-ClXAA	5-PhXAA
BRSK1		89 ± 3	86 ± 5	62 ± 4	64 ± 2	80 ± 4	71 ± 19	65 ± 1	78 ± 11	78 ± 9	71 ± 5	96 ± 4	63 ± 2
BRSK2		76 ± 4	84 ± 5	64 ± 3	68 ± 0	101 ± 0	72 ± 3	64 ± 1	83 ± 5	66 ± 11	80 ± 9	102 ± 9	86 ± 2
MELK		67 ± 1	84 ± 0	45 ± 6	56 ± 4	47 ± 2	61 ± 11	50 ± 2	82 ± 13	57 ± 4	51 ± 4	54 ± 5	52 ± 2
NUAK1		24 ± 1	34 ± 4	17 ± 1	26 ± 2	18 ± 1	21 ± 1	12 ± 1	66 ± 17	9 ± 2	39 ± 2	30 ± 1	16 ± 1
CK1		66 ± 9	69 ± 1	77 ± 6	84 ± 6	46 ± 4	75 ± 6	84 ± 1	49 ± 6	81 ± 9	39 ± 6	49 ± 0	95 ± 4
CK2		35 ± 1	15 ± 0	34 ± 3	49 ± 0	37 ± 1	20 ± 1	53 ± 6	32 ± 1	40 ± 0	3 ± 0	41 ± 2	35 ± 5
DYRK1A		44 ± 10	39 ± 2	35 ± 1	27 ± 2	30 ± 3	26 ± 1	28 ± 2	30 ± 1	25 ± 2	17 ± 0	36 ± 3	39 ± 4
DYRK2		51 ± 0	54 ± 4	56 ± 1	56 ± 3	51 ± 2	25 ± 0	70 ± 5	56 ± 1	54 ± 2	53 ± 1	51 ± 3	52 ± 2
DYRK3		59 ± 1	68 ± 3	85 ± 5	65 ± 0	69 ± 1	47 ± 2	71 ± 1	74 ± 1	44 ± 0	69 ± 1	83 ± 1	60 ± 2
NEK2α		100 ± 0	99 ± 5	104 ± 16	96 ± 11	43 ± 4	88 ± 2	85 ± 4	111 ± 12	111 ± 2	95 ± 3	85 ± 14	83 ± 1
NEK6		102 ± 3	88 ± 11	115 ± 4	110 ± 9	91 ± 11	92 ± 1	115 ± 6	95 ± 0	114 ± 15	99 ± 4	109 ± 16	74 ± 2
IKKβ		99 ± 2	100 ± 10	95 ± 2	93 ± 14	81 ± 6	96 ± 4	104 ± 20	82 ± 6	92 ± 6	68 ± 11	92 ± 16	67 ± 15
IKKε		74 ± 6	77 ± 5	49 ± 7	77 ± 9	73 ± 4	71 ± 1	70 ± 1	78 ± 6	86 ± 18	62 ± 7	80 ± 3	69 ± 4
TBK1		72 ± 5	75 ± 7	39 ± 1	77 ± 8	79 ± 0	66 ± 2	52 ± 1	78 ± 7	83 ± 5	41 ± 1	93 ± 12	63 ± 6
PIM1		46 ± 3	34 ± 0	33 ± 4	25 ± 2	15 ± 0	24 ± 1	32 ± 3	29 ± 4	21 ± 1	9 ± 0	31 ± 5	7 ± 1
PIM2		86 ± 3	73 ± 2	81 ± 8	101 ± 9	51 ± 6	65 ± 2	58 ± 7	59 ± 4	62 ± 5	23 ± 1	64 ± 5	37 ± 0
PIM3		17 ± 0	20 ± 0	17 ± 1	14 ± 1	11 ± 0	13 ± 3	10 ± 1	9 ± 0	10 ± 0	8 ± 0	13 ± 1	7 ± 1
SRPK1		103 ± 2	81 ± 1	77 ± 9	84 ± 2	92 ± 4	90 ± 10	87 ± 3	89 ± 12	98 ± 2	59 ± 3	93 ± 6	67 ± 0
EF2K		95 ± 4	99 ± 14	84 ± 14	84 ± 12	75 ± 10	80 ± 10	86 ± 6	72 ± 12	98 ± 5	95 ± 3	85 ± 7	74 ± 1
HIPK1		94 ± 6	76 ± 10	90 ± 4	71 ± 6	72 ± 9	93 ± 6	106 ± 15	65 ± 4	95 ± 1	32 ± 1	67 ± 2	74 ± 4
HIPK2		13 ± 1	17 ± 1	22 ± 1	18 ± 0	10 ± 0	11 ± 1	19 ± 0	14 ± 1	10 ± 0	4 ± 0	12 ± 1	10 ± 0
HIPK3		107 ± 6	78 ± 3	96 ± 8	93 ± 1	83 ± 6	91 ± 4	109 ± 3	79 ± 9	102 ± 23	36 ± 2	79 ± 3	87 ± 8
CLK2		71 ± 4	62 ± 3	55 ± 8	74 ± 2	42 ± 7	71 ± 6	60 ± 14	66 ± 11	51 ± 1	40 ± 15	51 ± 8	27 ± 4
PAK2		75 ± 3	65 ± 1	77 ± 3	80 ± 7	100 ± 4	86 ± 11	82 ± 1	64 ± 11	80 ± 3	96 ± 0	98 ± 0	79 ± 3
PAK4		94 ± 10	71 ± 4	64 ± 8	81 ± 11	128 ± 23	46 ± 6	82 ± 0	70 ± 16	66 ± 12	53 ± 1	71 ± 3	39 ± 0
PAK5		78 ± 4	64 ± 3	64 ± 5	58 ± 4	108 ± 2	70 ± 3	76 ± 15	57 ± 1	92 ± 13	73 ± 8	84 ± 3	46 ± 5
PAK6		92 ± 9	96 ± 2	91 ± 5	90 ± 1	117 ± 11	89 ± 1	93 ± 7	86 ± 4	102 ± 16	91 ± 16	102 ± 5	89 ± 11
MST2		66 ± 8	95 ± 4	21 ± 0	52 ± 0	38 ± 4	59 ± 1	28 ± 1	96 ± 9	21 ± 1	29 ± 5	54 ± 2	34 ± 5
MST4		100 ± 2	105 ± 3	106 ± 2	98 ± 5	83 ± 12	105 ± 5	93 ± 3	92 ± 3	96 ± 2	87 ± 1	84 ± 5	89 ± 1
GCK		21 ± 0	55 ± 5	9 ± 0	29 ± 3	28 ± 1	23 ± 2	18 ± 3	53 ± 3	8 ± 1	22 ± 2	38 ± 7	22 ± 2
MINK1		44 ± 7	66 ± 2	65 ± 64	50 ± 7	32 ± 2	41 ± 1	13 ± 1	57 ± 7	27 ± 4	37 ± 5	46 ± 0	30 ± 1
MEKK1		54 ± 2	72 ± 1	33 ± 5	56 ± 6	67 ± 25	58 ± 5	28 ± 1	52 ± 1	39 ± 2	53 ± 12	58 ± 2	35 ± 2
MLK1		68 ± 6	92 ± 3	44 ± 5	49 ± 1	43 ± 1	62 ± 5	63 ± 7	75 ± 0	73 ± 20	48 ± 1	74 ± 7	65 ± 32
MLK3		62 ± 9	52 ± 4	21 ± 0	59 ± 0	55 ± 2	54 ± 1	34 ± 2	58 ± 9	45 ± 9	58 ± 3	68 ± 8	44 ± 2
TAK1		70 ± 6	100 ± 9	36 ± 5	66 ± 6	36 ± 3	75 ± 4	48 ± 6	82 ± 12	47 ± 1	44 ± 5	61 ± 8	41 ± 3
IRAK4		17 ± 0	48 ± 4	20 ± 2	31 ± 4	35 ± 3	20 ± 4	23 ± 1	40 ± 0	20 ± 2	43 ± 4	48 ± 6	28 ± 3
RIPK2		84 ± 4	93 ± 3	56 ± 3	82 ± 5	100 ± 2	85 ± 2	74 ± 8	100 ± 1	68 ± 12	65 ± 5	95 ± 6	83 ± 3
TTK		81 ± 7	83 ± 4	75 ± 6	75 ± 3	71 ± 8	75 ± 8	85 ± 14	70 ± 10	91 ± 5	63 ± 1	75 ± 3	64 ± 5
Src		96 ± 1	103 ± 3	57 ± 3	83 ± 6	98 ± 14	70 ± 2	74 ± 7	95 ± 27	55 ± 8	83 ± 9	110 ± 8	70 ± 1
Lck		104 ± 10	109 ± 15	51 ± 4	90 ± 1	81 ± 32	80 ± 9	89 ± 7	113 ± 11	70 ± 7	79 ± 9	64 ± 20	56 ± 2
CSK		108 ± 10	96 ± 12	88 ± 5	76 ± 4	83 ± 1	74 ± 4	82 ± 4	78 ± 1	104 ± 2	68 ± 11	102 ± 6	71 ± 1
YES1		87 ± 13	78 ± 4	65 ± 7	79 ± 1	88 ± 3	83 ± 4	77 ± 0	76 ± 13	95 ± 4	82 ± 5	94 ± 5	76 ± 6
BTK		46 ± 5	41 ± 5	8 ± 1	31 ± 5	17 ± 1	20 ± 1	18 ± 1	41 ± 2	14 ± 1	11 ± 1	40 ± 8	12 ± 1
JAK2		79 ± 7	81 ± 4	58 ± 7	83 ± 2	65 ± 5	84 ± 0	72 ± 5	86 ± 3	60 ± 9	39 ± 2	78 ± 6	17 ± 1
SYK		95 ± 1	90 ± 2	70 ± 4	75 ± 1	77 ± 1	86 ± 0	88 ± 7	76 ± 16	75 ± 2	72 ± 2	95 ± 2	72 ± 2
EPHA2		98 ± 9	89 ± 5	64 ± 6	87 ± 10	93 ± 5	97 ± 0	74 ± 2	101 ± 3	88 ± 8	75 ± 12	94 ± 8	89 ± 16
EPHA4		98 ± 8	101 ± 0	46 ± 0	77 ± 0	97 ± 2	83 ± 6	64 ± 2	95 ± 3	72 ± 4	70 ± 12	98 ± 3	82 ± 2
EPHB2		121 ± 3	108 ± 12	63 ± 7	79 ± 12	94 ± 4	104 ± 5	66 ± 4	96 ± 18	97 ± 9	99 ± 1	93 ± 3	85 ± 18
EPHB3		104 ± 2	116 ± 12	74 ± 1	89 ± 2	84 ± 6	76 ± 8	83 ± 4	86 ± 7	78 ± 7	64 ± 8	82 ± 1	81 ± 5

Table S3 Continued

Kinase	Drug . . .	Activity remaining at 50 μM (%) XAA	1-MeXAA	2-MeXAA	3-MeXAA	5-MeXAA	6-MeXAA	7-MeXAA	8-MeXAA	α-MeXAA	XPA	5-ClXAA	5-PhXAA
EPHB4		103 ± 11	107 ± 3	106 ± 12	109 ± 3	110 ± 4	107 ± 6	104 ± 5	108 ± 6	106 ± 9	102 ± 16	106 ± 23	92 ± 5
FGFR1		60 ± 1	63 ± 2	23 ± 1	59 ± 3	56 ± 0	18 ± 3	68 ± 1	67 ± 1	66 ± 5	40 ± 8	64 ± 7	28 ± 2
HER4		89 ± 16	74 ± 2	58 ± 6	93 ± 8	79 ± 9	93 ± 14	86 ± 11	87 ± 2	69 ± 16	64 ± 4	88 ± 7	77 ± 2
IGF1R		71 ± 12	87 ± 4	55 ± 8	88 ± 11	60 ± 12	101 ± 10	87 ± 4	105 ± 7	40 ± 2	81 ± 7	71 ± 10	42 ± 14
IR		92 ± 7	91 ± 1	31 ± 2	77 ± 5	82 ± 1	106 ± 13	92 ± 2	114 ± 1	41 ± 7	110 ± 7	82 ± 5	25 ± 0
IRR		61 ± 1	52 ± 0	69 ± 1	66 ± 3	40 ± 6	50 ± 8	65 ± 1	45 ± 2	56 ± 2	36 ± 6	47 ± 3	40 ± 2
TrkA		63 ± 5	95 ± 18	23 ± 3	31 ± 5	37 ± 4	26 ± 4	34 ± 1	77 ± 10	40 ± 1	29 ± 1	49 ± 5	14 ± 2
VEGFR11		18 ± 2	67 ± 6	7 ± 3	13 ± 0	29 ± 3	9 ± 1	24 ± 2	33 ± 3	19 ± 1	15 ± 1	28 ± 4	19 ± 2

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