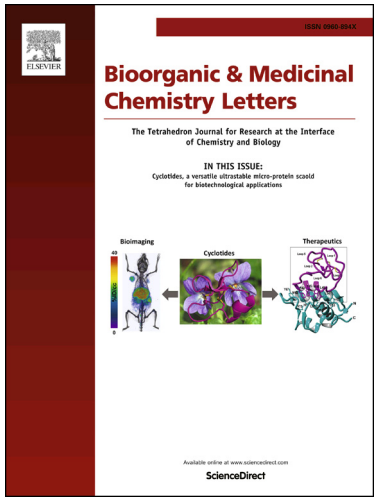

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Design and Synthesis of Triple Inhibitors of Janus Kinase (JAK), Histone Deacetylase (HDAC) and Heat Shock Protein 90 (HSP90)

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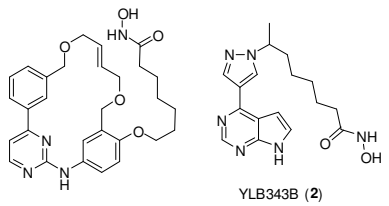
ABSTRACT

Inhibition of multiple signaling pathways in a cancer cell with a single molecule could result in better therapies that are simpler to administer. Efficacy may be achieved with reduced potency against individual targets if there is synergy through multiple pathway inhibition. To achieve this, it is necessary to be able to build multi-component ligands by joining together key pharmacophores in a way which maintains sufficient activity against the individual pathways. In this work, designed triple inhibiting ligands are explored aiming to block three completely different target types: a kinase (JAK2), an epigenetic target (HDAC) and a chaperone (HSP90). Although these enzymes have totally different functions they are related through inter-dependent pathways in the developing cancer cell. Synthesis of several complex multi-inhibiting ligands are presented along with initial enzyme inhibition data against 3 biological target classes of interest. A lead compound, **47**, was discovered which had low micromolar activity for all 3 targets. Further development of these complex trispecific designed multiple ligands could result in a 'transient drug', an alternative combination therapy for treating cancer mediated via a single molecule.

Combination drug treatment is the mainstay of modern cancer therapy.[1] However even with the welcome progress made with immune-oncology therapeutics,[2] there remains a high unmet need in treatment of many cancers and especially drug resistant disease.[3] Combination therapies are typically given as two or more separate drugs which need to be scheduled to ensure maximal efficacy with the lowest possible toxicity. However this is not always successful and drug-drug interactions can be a particular problem.[4] New ways of delivering combination therapy are therefore urgently needed. The new concept of the transient drug, where less potency at the target is balanced with multiple target inhibition creating a synergistic effect which is efficacious but without overt toxicity, could be one such new approach.[5] To approach such a challenge molecules able to inhibit multiple targets will need to be designed and synthesized. The choice of target is crucial and must include targets important in disease pathways, or known to be important when combined with inhibition of certain other pathways.[6] In this regard, we have been interested in inhibition of kinase signaling pathways, epigenetic targets and chaperone proteins.

Our previous work has led to dual inhibitors of JAK (Janus) kinases and HDACs (histone deacetylases), EY3238 (**1**) and YLB343B (**2**), with low nanomolar potency against members of both target families.[7, 8] In this present work, we aimed to stretch the possibilities of small molecule chemistry to assess the feasibility of preparing single molecules which could have potential to be inhibitors of 3 different pathways by inhibition of 3 different types of enzyme target.



EY3238 (**1**)
JAK2 IC_{50} = 1.4 nM
HDAC6 IC_{50} = 2.1 nM

YLB343B (**2**)
JAK1 IC_{50} = 11 nM
HDAC1 IC_{50} = 6 nM
HDAC6 IC_{50} = 1 nM

Figure 1. Structures of the JAK2-HDAC dual inhibitors EY3238 (**1**) and YLB343B (**2**).

We aimed to build upon our previous work in JAK-HDAC dual inhibitors by adding on a third activity against the chaperone Heat Shock Protein 90 (HSP90). HSP90 is massively upregulated in developing cancer cells due to its importance in ensuring the correct folding of a wide array of client proteins many of which are oncoproteins.[9] HSP90 promotes activation of the oncogenic inflammatory mediator STAT-3.[10] JAK kinases are direct activators of STATs[11] hence inhibition of HSP90 and JAK could be synergistic. Inhibition of HSP90 has been shown to be effective in cells with an activated JAK-STAT pathway.[12] HDACs, in particular HDAC6, are known to be important in modulating HSP90 complexes.[13, 14] Hence exploring a triple inhibitor of all three enzymes could have value in treating cancer with JAK-STAT and/or HSP90 dependency. We selected the marketed drugs ruxolitinib (**3**),[15] vorinostat (**4**)[16] and the published HSP90 inhibitor BEP800 (**5**)[17] as the base molecules for initial exploratory work towards a triple inhibitor of JAK-HDAC-HSP90.

Analysis of published crystal structures of **4**[18] and **5**[19] indicates clearly which regions of the structures are solvent exposed. There are no published crystal structures of **3** in complex with JAK kinases but structures of similar molecules such as tofacitnib are available.[20] These structures indicate that there are two regions of the structure of **3** which are solvent exposed, or with a direct path out to solvent: the nitrile group and the central carbon of the pyrimidine. These were therefore considered as viable attachment points for either an HDAC or HSP90 binding moiety. Compound **4** has a solvent exposed terminal phenyl group which we have established as an attachment point for a JAK inhibiting moiety. HSP90 binds small molecules in its ATP site which is a deep pocket but the structure of molecules related to **5** indicates two solvent exposed regions: the amide alkyl group and the meta position of the phenyl.[19] Using this information our initial designs focused on using a simplified core based on **3** with attachment of a HSP90 binding moiety, based on **5**, on the pyrimidine ring and an HDAC binding moiety, based on **4**, on the pyrazole (compare with **2**[8]) (Figure 2a). Alternatively we planned to explore the HDAC binding group attached to the pyrimidine and the HSP90 binding group attached to the pyrazole (Figure 2b).

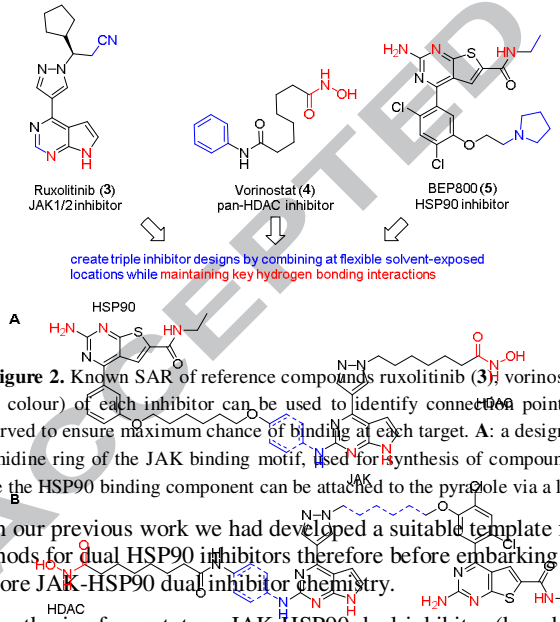


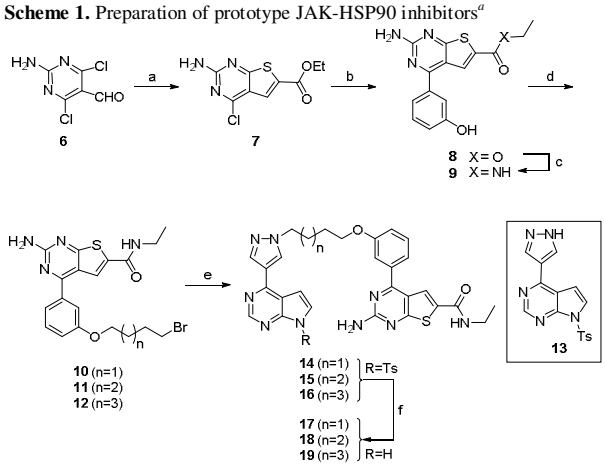
Figure 2. Known SAR of reference compounds ruxolitinib (**3**), vorinostat (**4**) and BEP800 (**5**) enables design of multiple ligands. Solvent exposed areas (blue colour) of each inhibitor can be used to identify connection points for combination compounds. Key hydrogen bonding groups (red colour) are preserved to ensure maximum chance of binding at each target. **A**: a design based on the compact structure of **2**[8] with attachment of the HSP90 binder to the pyrimidine ring of the JAK binding motif, used for synthesis of compound **39** ; B: HDAC binder is attached through the pyrimidine of the JAK inhibitor while the HSP90 binding component can be attached to the pyrazole via a linker, used for synthesis of compounds **17**-**19**, **26**, **27**, **46**, **47**.

In our previous work we had developed a suitable template for JAK-HDAC dual inhibitors based on **3**[8] but we had not developed methods for dual HSP90 inhibitors therefore before embarking on the challenging synthesis of a triple inhibitor we decided to firstly explore JAK-HSP90 dual inhibitor chemistry.

Synthesis of a prototype JAK-HSP90 dual inhibitor (based on the designs of Figure 2a), started by building the HSP90 portion from aldehyde **6** through a SNAr reaction using 2-mercaptoethylacetate and cyclisation of the intermediate to form thienyl pyrimidine **7** in good yield (Scheme 1). Suzuki coupling with hydroxyphenyl boronic acid gave **8** followed by ethyl amide formation gave phenol **9** in moderate overall yield. Installation of linkers with 4, 5 and 6 carbons was achieved with the respective dibromo alkanes giving bromides **10**-**12**. The previously reported[8] tosyl-protected JAK binding fragment, **13**, could then be attached via N-alkylation giving **14**-**16**. Finally deprotection with sodium methoxide gave the desired products **17**-**19**.

These three prototype JAK-HSP90 inhibitors (**17-19**) were tested in a JAK2 ATPase kinase assay using radio-labelled ATP and in an HSP90 fluorescence polarisation assay using a geldanamycin-based probe. Although **18** and **19** had JAK2 IC_{50}s of around 1 µM unfortunately **17** was not active against JAK2 and none of the compounds registered any activity against HSP90 at concentrations up to 10 µM. We reasoned that this could be due to a weak HSP90 pharmacophore. In published studies leading to **5**, halogen substituents on the phenyl ring played an important role in achieving good potency. Hence we prepared two analogues, **26** and **27**, with a 2,4-dichlorophenyl in the HSP90 portion using similar chemistry with the expectation that this change would enhance HSP90 potency (Scheme 2). Following enzyme assay testing we were pleased to observe that both **26** and **27** inhibited HSP90 with IC_{50}s of 0.4 and 0.11 µM, respectively (Table 1).

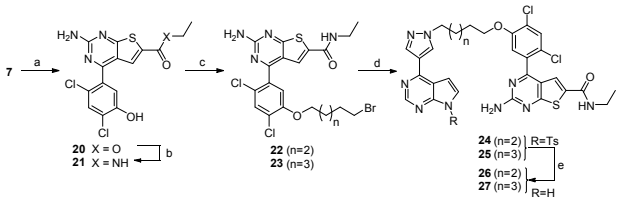
Scheme 1. Preparation of prototype JAK-HSP90 inhibitors[a]



[a]Reagents and Conditions: (a) $HSCH_2CO_2Et$, K_2CO_3, MeCN, reflux, O/N, 70%; (b) 3-hydroxyphenyl boronic acid, $Pd(PPh_3)_4$, K_3PO_4, dioxane, 100 °C, O/N, 68%; (c) $EtNH_2$, EtOH, MW, 130 °C, 3 h, 30%; (d) 1,4-dibromobutane (n=1), 1,4-dibromopentane (n=2), 1,4-dibromohexane (n=3), DMF, RT, 18 h, 46%; (e) **13**, DMF, K_2CO_3, RT, 96% (n=1); (f) NaOMe, MeOH, THF, 70 °C, 3 h, 62-76%.

Having validated the connection strategy for the BEP800-based HSP90 fragment, we then progressed towards synthesis of the first triple inhibitors (Schemes 3 and 4). Pyrazole boronate ester **28** was protected with ethyl vinyl ether and coupled to dichloropyrrolopyrimidine **30** in good yield (Scheme 3). This building block offers a functional group handle via the chloro attached to the pyrimidine which is later used to install the HSP90 binding fragment.

Scheme 2. Preparation of 2,4-dichlorophenyl products[a]



[a]Reagents and Conditions: (a) 2,4-dichloro-5-hydroxyphenyl boronic acid, $Pd(PPh_3)_4$, K_3PO_4, dioxane, 100 °C, O/N, 57%; (b) $EtNH_2$, EtOH, MW, 130 °C, 3 h, 36%; (c) 1,4-dibromopentane (n=2), 1,4-dibromohexane (n=3), DMF, RT, 18 h, 59-68%; (d) **13**, DMF, K_2CO_3, RT; (e) NaOMe, MeOH, THF, 70 °C, 3 h, 34-44% (over 2 steps).

Table 1. JAK2 and HSP90 enzyme data for JAK-HSP90 dual inhibitors

Compound	IC_{50} (μM)[a]	
	JAK2	HSP90
Gd	-	0.016[b]
1	1.4[c]	
17	>10	>10
18	1.09	>10
19	1.42	>10
26	3.45	0.40
27	1.71	0.11

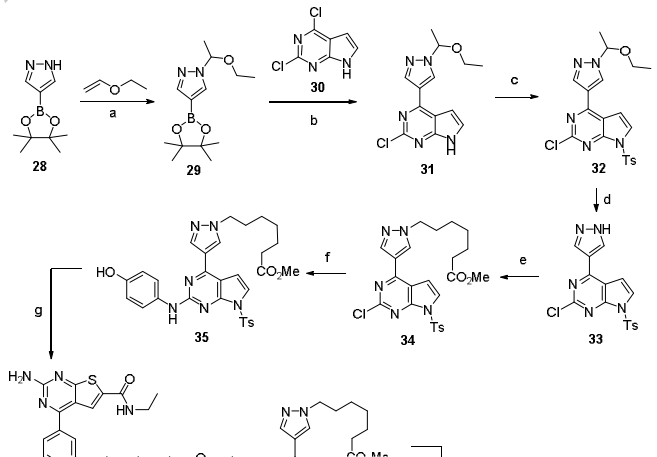
[a] 10 dose IC_{50} determination.[7] [b] Reported value (http://www.reactionbiology.com). [c] reported value from same assay[7]

Protection of the pyrrole nitrogen was necessary to enable control of the subsequent reaction and was achieved with a tosyl group giving **32**. Removal of the ethoxy ethyl protecting group under acidic conditions revealed the pyrazole **33** which could then be alkylated with the linker using methyl bromoheptanoate furnishing ester **34**. Aminophenol displacement of the chloro to give **35** was followed by alkylation with the HSP90 fragment **23** (see Scheme 1) using potassium carbonate in DMF. Having all the carbons of the target compound in place all that remained was to install the hydroxamate, via THP-protected hydroxylamine, and then deprotect the tosyl and THP groups (**37**, **38**) to give the first triple target, **39**.

Following the design of Figure 2b, a similar strategy was used to synthesise **46** (Scheme 4). In this case, the carboxylic acid **47** was also isolated probably from some incomplete hydroxamate formation. This acid was considered worthy of biological testing given that acids have been described as HDAC inhibitors, albeit weaker than hydroxamates. The acid was synthesized directly from **43** via saponification.

The three target compounds were tested against HDAC1, HDAC6, JAK2 and HSP90 (Table 2). Surprisingly, **39** was very potent against HDAC6 with an IC_{50} of 8 nM. This is not surprising since it is based on compounds very similar to **2** with the preferred 6-carbon linker connecting the hydroxamate to the heterocycle.[8] However it was considerably less active against HDAC1 and no significant activity was detected against either JAK2 or HSP90 at the top concentration tested (10 µM). It is generally the case with dual inhibitors that HDAC1 potency is more challenging to maintain due to the size and shape of the binding site at the immediate surface next to the HDAC substrate pocket.[7, 8] This indicates that attaching the HSP90 binder to the pyrimidine of the JAK binding core is not a suitable combination. Hence the proven strategy explored with the prototype JAK-HSP90 inhibitors (Schemes1 and 2, Table 1) was expected to provide better results (Table 2). Indeed, **46** did have activity for JAK2 and HDAC6 but unfortunately not for HSP90. However, the carboxylic acid **47** did fully inhibit HSP90 with an IC_{50} of 20.2 µM as well as JAK2 (3.76 µM) and HDAC6 (6.3 µM).

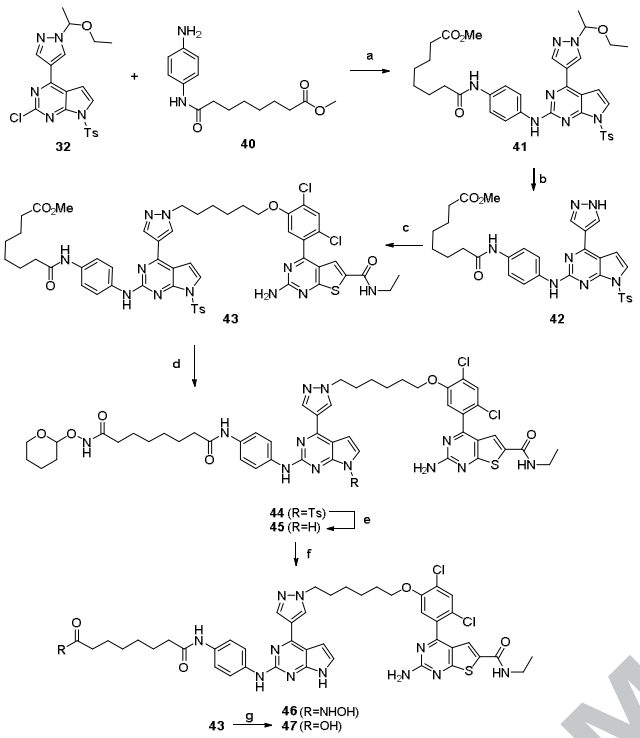
Scheme 3. Preparation of JAK-HSP90-HDAC triple inhibitor based on design shown in figure 2a[a]



[a]Reagents and Conditions: (a) toluene, 35°C,13 h; (b) $Pd(PPh_3)_4$, Na_2CO_3, dioxane/H_2O, 100°C, overnight, 70%; (c) TsCl, Et_3N, DMAP, DCM, rt, 3 h, 68%; (d) HCl (aq.), THF, rt, ON; (e) methyl-7-bromoheptanoate, K_2CO_3, DMF, RT, ON, 86%; (f) *p*-nitrophenol, $Pd_2(dba)_3$, X-phos, K_2CO_3, tBuOH, 100°C, 24 h, 43%; (g) **23**, K_2CO_3, DMF, rt, ON, 65%; (h) (1) $THPONH_2$, LiOH, THF, H_2O; (2) HATU, TEA, DMSO, rt, ON, 74%; (i) MeONa, MeOH, THF, 75°C, 5 h 70 %; (j) HCl, dioxane, rt, 18h, 46%.

This broad micromolar activity demonstrated with **47** shows that it is possible to inhibit three different biological targets with a single small molecule. Although the inhibition is in the micromolar range , if there is synergy between the inhibition of these targets, this could be the first step towards a so-called 'transient' drug, i.e. a molecule which inhibits a group of specific targets weakly, but strongly enough to elicit desired biological effects through the combination of its inhibition profile without causing overt toxicity.[5] Further work will be required to optimize these initial structures and to evaluate cell permeable trispecific inhibitors for the optimal balance of target potency for best effects in cancer cells and tumors.

Scheme 4. Preparation of JAK-HSP90-HDAC triple inhibitor based on design shown in figure 2b[a]



[a]Reagents and Conditions: (a) $Pd_2(dba)_3$, XPhos, K_2CO_3, tBuOH, 100°C, overnight, 77%; (b) HCl (aq), THF, rt, 24h; (c) **23**, K_2CO_3, DMF, 60°C, 24 h; (d) (1) $THPONH_2$, LiOH, THF, H_2O; (2) HATU, TEA, DMSO, RT, ON; (e) MeONa, MeOH, THF, 70°C, 5 h; (f) HCl (aq.), 1,4-dioxane rt, 18h; (g) NaOMe, THF, MeOH, 75°C, 5 h, 59%.

Table 2. Enzyme inhibition for designed triple inhibitors

Compound	IC_{50} (μM)[a]			
	HDAC1[b]	HDAC6[b]	JAK2[c]	HSP90α[d]
39	2.47	0.008	>100	>100
46	>100	2.82	14.1	>100
47	>100	6.30	3.76	20.2

[a] 10 dose IC_{50} determinations.[7] [b] Vorinostat used as a positive control (HDAC1 IC_{50} = 306 nM; HDAC6 IC_{50} = 20 nM). [c] Ruxolitinib used as a positive control (IC_{50} <1nM). [d] Geldanamycin used as a positive control (IC_{50} = 27 nM).

In conclusion, we have shown in this work that it is possible to develop small molecules which have specific inhibition of three different enzyme classes, all of which are strongly implicated in oncogenesis. The relatively weak inhibition exhibited by the triple inhibitor **47** may be the first step towards a transient drug.[5] Further work will be required to understand the scope of these designs and to develop cell permeable inhibitors.

Acknowledgments

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Supplementary Material

Supplementary material describing methods of synthesis and characterization of compounds is available.

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Graphical Abstract

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