Josa-Cullere et al 2023 Phenotypic screening identifies a trisubstituted imidazo[1,2-a]pyridine series that induces differentiation in multiple AML cell lines

1. Introduction

Acute myeloid leukaemia (AML) is a type of blood cancer characterised by a block in the differentiation of myeloid cells. This block causes an accumulation of immature cells in the bone marrow and leads to a disruption of regular haematopoiesis [1]. AML is more prevalent in elderly people [2,3] – older than 65 years– and is a highly heterogeneous disease where patients are often categorised into different subtypes [4,5].

The current standard of care is focused on cytotoxic chemotherapy, usually with the anti-metabolite cytarabine in combination with anthracycline-derived antibiotics such as daunorubicin; these are often poorly tolerated and do not provide sustained efficacy [6]. The leukaemic cells are often not completely eradicated, resulting in high rates of tumour resistance and relapse in patients. Newer therapies targeted to specific genetic profiles, signalling pathways or epigenetic regulators are under investigation and at different stages of clinical development [7,8]. In spite of a better safety profile, these targeted agents address limited patient populations, and their full utility remains to be seen [9–12]. An emerging alternative strategy to cytotoxic therapy aims to promote differentiation of the leukaemic blasts [13–15] which are arrested at an immature stage. The first successful example of this approach was the use of all-trans retinoic acid (ATRA) to treat patients with acute promyelocytic leukaemia (APL), a subset of 10% of AML patients [16]. The prognosis for APL was previously very poor, but since the introduction of treatment with the combination of ATRA and arsenic trioxide, it now has an 85% 5-year survival rate [16,17]. This was a breakthrough for differentiation therapy and since then many other examples have been reported [14]. Recent examples of FDA-approved drugs that induce differentiation in AML are inhibitors of mutated IDH enzyme, enasidenib [18] and ivosidenib [19], and FLT3 kinase, gilteritinib [20] and midostaurin [21]. However, since these agents have been developed for specific targets that are effective only in patient populations with particular genetic mutations – IDH1/2, found in 20% of patients [22], and FLT3, found in 25% of patients, there remains both a large unaddressed patient population, as well as a continued need for more broad acting therapeutic options [23,24]. We recently reported the development of a phenotypic screen which utilised the pan-myeloid differentiation marker CD11b as a marker of compound activity against a range of AML cell lines [25]. Herein, using the same strategy we describe the identification of a trisubstituted imidazo[1,2a] pyridine hit series and optimisation of activity and metabolic stability towards the discovery of OXS007464, a potent small molecule differentiator of various AML cells with anti-tumour efficacy when tested in vivo.

2. Materials and methods

Experimental procedures can be found in the Supplementary Information file.

3. Results and discussion

3.1. Hit identification: a phenotypic assay identifies OXS000651, which differentiates a range of AML cell lines in vitro A phenotypic pilot screen to detect differentiation of AML cells was performed utilising the cell-surface marker CD11b, which is overexpressed

in differentiated myeloid cells [25]. A panel of three genetically diverse cell lines (HL-60, THP-1 and OCI-AML3) was treated with a commercial library of 1000 structurally diverse compounds at 10 μM for 4 days. Upregulation of CD11b in the live cell population was then quantified by flow cytometry. Those compounds upregulating CD11b higher than 10%, as compared to the isotype control were considered as “actives”, and validated further towards hit confirmation. One particular example, trisubstituted imidazo[1,2-a]pyridine OXS000651 (1) (Fig. 1A) displayed the highest CD11b upregulation in our panel of AML cell lines, increasing the expression up to 78% in HL-60 cells at 10 μM (Fig. 1B). To complete the hit confirmation process, resynthesis of OXS000651 was undertaken using the route outlined in Scheme 1 and the compound retested. The synthetic strategy is inspired by the synthesis of a related family of trisubstituted imidazo[1,2-a]pyridines [26], however in this case initial attempts to introduce the formamide at the beginning of the synthesis resulted in low solubility of the intermediates, and poor isolated yields. Alternatively, it was found that N-protection with the Boc group significantly enhanced solubility, which improved chromatographic purification and product isolation. Thus, after N-Boc protection of the commercially available 6-bromo-8-aminoimidazo[1,2-a]pyridine 1, Suzuki-Miyaura reaction at the 6-position afforded 4. Regioselective bromination and subsequent cross-coupling provided 6. The N-Boc-to-formamide exchange in the last step was performed under refluxing formic acid to afford OXS000651 in 5 steps and 21% overall yield. Hit confirmation of the authentic solid sample of OXS000651 was completed with evaluation in the 3 AML cell lines. At 1 μM, consistently high CD11b expression of 14–58% was observed (Fig. S1). At 10 μM, lower CD11b+cell numbers and higher variability was observed in some cell lines, probably due to some toxic effects at this higher dose, leading to low total cell numbers. Pleasingly, concentration-dependent treatments confirmed its relatively potent EC50 (34–210 nM) in each of the 3 different cell lines (Fig. 1C and S1B,C). Next, the effect of the confirmed hit on AML cell numbers and viability after treatment was evaluated. Treatment of 6 AML cell lines (HL-60, THP-1, OCI-AML3, KG-1, ME-1 and Kasumi-1) with OXS000651 led to a significant decrease of cell number with little effect on viability (Fig. 1D and S2). A compound inducing cells to move along the differentiation pathway would indeed be expected to cause a decrease on the proliferation ability of the leukaemic blasts whilst not causing direct cell death, as also observed with our positive control phorbol-12-myristate-13-acetate (PMA), a well studied differentiation-inducing chemical [28]. Finally, morphological assessment of the cells after treatment showed size variation, nuclear segmentation as well as wider and lighter cytoplasm, and presence of granules and vacuoles. These cellular characteristics were consistent with myeloid differentiation (Fig. 1E and S3-S6).
While OXS000651 effectively differentiated AML cells in vitro, an initial assessment of its physicochemical and selected in vitro ADME properties revealed the compound had poor metabolic stability and relatively low solubility (Table 1). Further optimisation of the compound was therefore needed prior to its progression to in vivo studies. Despite possessing Lipinski-like calculated physicochemical properties (Fig. 1A) [29], and a suitable lipophilic ligand efficiency (LLE =-logEC50-clogP) of 4.4 for a hit compound [30], three potential metabolic soft-spots were identified. Firstly, the N-formyl moiety at position 8 was readily cleaved by a cofactor-independent mechanism (plausibly esterase or amidase enzymes), when incubated with mouse S9 (mS9) fraction without NADPH and UGDPA (Table 1). In addition, the methoxy-containing phenyl rings at

position 3 and 6 could potentially be demethylated by cytochrome P450 (CYP) enzymes, and this would lead to the formation of a quinone toxicophore [31]. Our hit-to-lead optimisation effort was therefore aimed at improving the metabolic stability of OXS000651 at position 8, and modify the 3- and 6-aryl substituents whilst retaining its activity. Biological activity of the analogues was evaluated using the CD11b readout in HL-60 cells, which is the cell line that showed the highest and more robust shift of fluorescence of the CD11b+population.

3.2. Hit-to-lead optimisation

3.2.1. Replacement of the 8-formamide group increases metabolic stability. We first focused on replacing the formamide moiety at position 8, ensuring an overall cLogP <4 to retain a LLE >3. Complete removal of the formamide or the formyl unit, exemplified by 7 and 8 respectively, was accompanied by a significant reduction in potency. However, these analogues confirmed that the formamide was a metabolic lability by a cofactor independent mechanism (Table 1). Due to the apparent key role played by the formamide for activity, the amide bond at position 8 was conserved. Attempts to gain metabolic stability by methylation (9), cyclisation to both β- and γ-lactams (10, 11), and homologation to acetamide 12 led to a decrease in activity without completely resolving the cofactor independent liability (Table 1). Amide bioisosteres [32] were next investigated, including carbamate 15, methyl amine 13, sulfonamide 16, sulfonimidamide 18, sulfone 19, sulfoximine [33] 20, and the reversed amide 21 (Table 1). Despite a notable drop in potency compared to OXS000651, compound 16 bearing a methyl sulfonamide at position 8 appeared to have the best balance of metabolic stability and solubility, and the second highest LLE of 3.1. Sulfonamide 16 therefore became the basis for the next phase of optimisation, with a focus on restoring potency to a level comparable with the initial hit OXS000651. The synthetic route towards the above analogues made use of the common intermediate 5 (Scheme 1). The 8-NHBoc group was either readily deprotonated to react with electrophiles, or deprotected under acidic conditions to yield the free amine, which could be subsequently coupled to afford the other analogues. For the reversed amide 21, the cyano precursor 24 was prepared using an analogous sequence to the amine derivatives (Scheme 2), and subsequently hydrolysed. Sulfone 19 and sulfoximine 20 were prepared from the fluoro precursor 25 via SNAr with sodium methanethiolate, followed by oxidation [34].

3.2.2. Core replacement enables further exploration of the C-8 position, and identifies alternative cores which retain activity. Since only thiolates reacted on 8-fluoroimidazo[1,2-a]pyridine 25 in an SNAr fashion, this limited the exploration of a wider chemical space at the C-8 position. To enable a broader SAR examination, a closely related core was investigated. 8-Bromoimidazo[1,2-a]pyrazine core 26 reacted with a diverse set of amines via SNAr, which followed by double Suzuki cross-coupling allowed the incorporation of a range of potential alternative formamide bioisosteres (Scheme 3A) such as morpholine 33, 2-oxa-6-azaspiro[3.3]heptane 34 [35], 3-aminooxetane 35 [36,37], and 1,2,4-triazole [38] 36. Replacing the imidazo[1,2-a]pyridine core with the imidazo[1,2-a]pyrazine notably lowered the lipophilicity while retaining similar activity, as exemplified by the pairs OXS000651/30, 13/31, and 14/32 (Tables 1 and 2 and Fig. 2). However, this core swap did not provide any significant biological advantage, and these imidazo [1,2-a]pyrazine analogues were particularly insoluble, which limited our interest in this core substitute. Prompted by the acceptable metabolic and solubility profile of disubstituted imidazo[1,2-a]pyridine 7 but relatively

high lipophilicity and LLE<2 (Table 1), the less lipophilic imidazo[1,2-b]pyrimidine, imidazo[4,5-c]pyridine, and triazolo[4,3-a] pyridine cores were also probed (Scheme 3B-D and Table 2). While the imidazo[1,2-b]pyrimidine 37 and the triazolo[4,3-a]pyridine 38 were inactive, imidazo[4,5-c]pyridine 40 and triazolo[4,3-a]pyridine 39 were one order of magnitude more potent than the corresponding imidazo[1,2-a]pyridine 7. The optimisation of the imidazo[4,5-c]pyridine series, which presented a better overall profile than the triazolo[4,3-a] pyridine series, was reported in previous work [39].
3.2.3. Optimisation of the 3-substituent improves biological activity. Substitution of the catechol methyl ether at position 3 of the aryl group was investigated in parallel to SAR at C-8 (Table 3). It was of interest to identify a substituent that increased metabolic stability and biological potency, to enable incorporation of the 8-sulfonamide with sufficient levels of activity. Starting from the previously described intermediate 5 (Scheme 1), variation at position 3 was achieved by successive cross-coupling and formylation reactions through an equivalent route to that used for the synthesis of OXS000651 (Scheme 1). Complete removal of the phenyl group (44) or deletion of both methoxy groups (45) was detrimental and resulted in a loss of biological activity. The presence of only one methoxy moiety in either the meta (47) or para (46) position was sufficient to retain an encouraging level of potency, with the latter exhibiting comparable potency to the corresponding dimethoxy (OXS000651). Interestingly, the ortho-methoxy analogue 48 was devoid of activity. To modify the electronic properties of the ring and reduce susceptibility to possible demethylation, electron-withdrawing fluorine (49) or nitrile (50) were added adjacent to the methoxy group, and pleasingly, no significant effect on intrinsic potency was observed. Incorporation of nitrogen atoms in this position next to the methoxy group (51, 52) led to a 20- and 100-fold loss of activity respectively. Replacement of the para-methoxy with an OCHF2 (53) [39] similarly led to a drop in potency. Other catechol methyl ether isosteres were also synthesised, the 1,3-benzodioxole 54 and dimethylindazoles [40] 55–57. Of these, only 57 was marginally more active than OXS000651, but significantly less soluble. The combination of the catechol methyl ether bioisosteres and the attractive potency of the para monomethoxy led to the design of the benzofuran 58, 2-methylbenzoxazole 59 and monomethyl indazole 60. These variations greatly improved the intrinsic potency, with 60 having an EC50 of 5 nM in the CD11b assay. This analogue also possessed the highest LLE amongst the C-3 analogues and was used for subsequent SAR modifications. Even more encouraging was that combination of the methylindazole at C-6 and the sulfonamide at C-8 (66, Table 4) led to a compound with the same levels of potency compared to the original hit OXS000651, but with a higher LLE and without the liabilities of the formamide and 1,2-dimethoxybenzene groups. 3.2.4. Optimisation of the 6-substituent improves solubility. To facilitate investigation of the SAR at the C-6 position, the synthetic route was modified to allow for late-stage incorporation of the 6- aromatic group by introducing an 8-iodo substituent before the two subsequent cross-coupling reactions (Scheme 4). At position 6, varying the regiochemistry of the methoxy substituent, ortho-66, meta-67 and para-68, was found to favour the ortho position (Table 4). Indeed, sulfonamide 66 appeared particularly attractive. We have previously described [39] that the ortho-substitution seemed to induce a twist between the phenyl ring at the 6 position and the core, which positively affects potency as well as solubility. The ortho-chloro and ortho-methyl analogues were also similarly active, but the chloro

and methoxy were less soluble than ortho-methyl (70 and 72 vs OXS007464 71). Addition of a substituent at the para position seemed to also marginally improve solubility and offered the added benefit of protecting the para position of the aromatic ring from potential metabolic oxidation. Analogues with a para-fluoro (70–72) retained the same levels of potency, while the para-cyano (74 and 75) led to a 10-fold loss of activity. Despite slightly increasing the lipophilicity, these compounds displayed an acceptable LLE>4.5. An attempt to improve solubility by increasing the fraction of sp3 carbon (Fsp3) [41] character of the series with the 2-methylcyclohexene 76 did not lead to any improvements. Overall, substitution of the formamide group of the original hit OXS000651 by a sulfonamide led to a significant improvement in the metabolic stability of the compounds. Optimisation of the C-3 and C-6 substituents to 2-methyl-4-fluorophenyl and 2-methyl indazole recovered biological potency to an acceptable level, and enabled further improvement of overall properties. This resulted in the discovery of OXS007464, which based on its encouraging profile, was selected for progression into more detailed studies. Assessment of the in vitro ADME properties of OXS007464 indicated that it had reasonable stability in mouse hepatocytes (mHep), high Caco-2 permeability with no apparent active efflux, and high plasma protein binding (Table 5). 3.3. OXS007464 differentiates genetically distinct AML cells in vitro The in vitro potency of OXS007464 in our wider panel of AML cell lines was evaluated. Gratifyingly, despite the incorporated structural modifications, the differentiation profile of OXS007464 in all cell lines appeared to be maintained. The compound increased CD11b expression of HL-60, THP-1 and OCI-AML3 cells with EC50 values of 36, 69 and 25 nM respectively (Fig. 3A). This suggests a mechanism independent of mutation status or patient subtype, which is an important distinction from most existing differentiating agents. After a 4-day treatment at 123nM, OXS007464 significantly decreased live cell numbers in all 6 cell lines (Fig. 3B, Fig. S7). Viability was lower than that observed with 10 μM of OXS000651, which could either be due to an early block in cell proliferation, producing cell death of the differentiated cells after 4 days, or an additional mechanism also causing direct cytotoxicity. Cell morphology analysis (Fig. 3C, Figs. S8–S13) also confirmed that OXS007464 induced differentiation of all 6 AML cell lines (HL-60, THP-1, OCI-AML3, KG-1, ME-1 and Kasumi-1), as indicated by variations in cell size and shape, nuclear segmentation, nuclear:cycloplasmatic ratio and presence of granules and vacuoles. Despite the modest solubility of OXS007464, its overall in vitro profile justified evaluation of its in vivo pharmacokinetic properties. 3.4. Pharmacokinetic studies inform the dose used for in vivo efficacy testing. Pharmacokinetic evaluation in mice was undertaken to investigate compound exposure and to assist in determining the dose level and dosing frequency required for in vivo studies. In order to maximise the likelihood of seeing efficacy in a xenograft study, our initial goal was to maintain compound levels in blood at 10 x EC50 value for over 8 h. Assessment of the pharmacokinetic properties of OXS007464 was undertaken using male CD-1 mice. The compound was administered at a single dose, both intravenous (iv) at 0.25 mg/kg and per os (po) at 3 mg/kg, and the compound concentration in blood was measured over 24 h Fig. 4A). OXS007464 showed a promising profile, with low clearance (16 mL/min/kg), moderate volume of distribution (3.1 L/kg) and high bioavailability (100%). After po administration at this relatively low dose, Cmax was noted as being 22 times higher than the in vitro cellular EC50 (36 nM ≡16 ng/mL), and compound levels were maintained above the EC50 for 18 h. To investigate the effect of

repeat dosing, OXS007464 was administered once daily for 4 days at 20 mg/kg, which was expected to provide sustained levels above the EC50, and compound blood levels were measured on days 1 and 4 (Fig. 4B). Similar results in terms of AUC and Cmax in both days suggested no accumulation over time (Fig. 4E). However, exposure from 0 to 8 h after the first dose (AUC0–8h =4461ng/mL·h) at 20 mg/kg was approximately the same as the exposure from 3 mg/kg (3062 ng/mL·h), suggesting non-linear PK for this compound between these two doses. A possible explanation is that bioavailability could be limited by incomplete absorption, due to the low aqueous solubility of the compound (21 μM). The plateau in exposure observed between 2 and 8 h would be consistent with this explanation. We noted during pre-formulation that a fully solubilised solution of this compound was not possible, hence administration of a fine suspension was necessary. Alternative dosing vehicles suitable for low solubility compounds were not investigated but could be considered in the future. OXS007464 was also administered once daily at a lower dose of 1mg/kg for 4 days. In this case, exposure was approximately a third of the 3 mg/kg dosing, indicating linear PK at these dose levels (Fig. 4C), further supporting the hypothesis that exposure was limited by compound solubility. The data from the 3 mg/kg study was fitted to a single compartment PK model (Fig. S14, see supplementary information for details), which was used to predict exposure levels at 3 mg/kg twice daily dosing and at10 mg/kg once daily (Fig. 4D). A dose of 3 mg/kg twice daily seemed appropriate to sustain constant levels above the EC50, and even above 10 x EC50 in some timepoints. In subsequent tolerance studies, male CD-1 mice were administered OXS007464 po at this dose regimen for 7 days and showed no adverse effects. These pharmacokinetic studies identified 3 mg/kg twice daily as a suitable dosing route and frequency, which enabled progression of the compound into an in vivo efficacy model. The results of this study are reported separately in our recent paper, which also discusses the mechanism of action of these compounds as inhibitors of beta-tubulin polymerisation [25].

4. Conclusions

A phenotypic screen was performed based on CD11b expression to identify compounds able to differentiate AML cells from a range of genetic subtypes. The confirmed hit OXS000651 was highly potent in the CD11b assay but had poor solubility and low metabolic stability. SAR studies revealed that replacement of the formamide group with a sulfonamide significantly decreased metabolic clearance, albeit with a significant drop in biological activity. Further optimisation of the C-3 and C-6 aromatic substituents delivered OXS007464, which had an encouraging in vitro activity profile. OXS007464 was able to differentiate six AML cell lines, as proven by CD11b overexpression, a block in proliferation, and consistent changes in cell morphology. Although solubility was still not optimal, significant improvements in the metabolic profile of the series were made. Pharmacokinetic studies provided results consistent with the in vitro ADME predictions; OXS007464 showed high bioavailability and low clearance, with a dose of 3 mg/kg bid predicted to be sufficient to maintain constant blood levels significantly above EC50. In a subsequent in vivo subcutaneous xenograft study published elsewhere [25], OXS007464 was shown to decrease tumour volume to levels comparable to known literature standard molecules. However, chronic dosing revealed adverse effects preventing further progression of this compound. Further optimisation will be necessary to progress this compound series further. Overall, this work supports the value of phenotypic screens to identify small molecules with broad ranging activity

mechanisms, and in particular with differentiation effects in a wide range of AML cell types.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: A. J.R., T.A.M., S.G.D. and P.V. are founders and minor shareholders of OxStem Oncology, a subsidiary company of OxStem Ltd. A.J.R. is a paid consultant for OxStem Ltd. G.M.W. is a paid consultant for and minor shareholder of OxStem Ltd. T.A.M. is currently a paid consultant for and minor shareholder of Dark Blue Therapeutics Ltd.

Data availability

Data will be made available on request.

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