Discovery and Optimization of Allosteric Inhibitors of Mutant Isocitrato Dehydrogenase 1 (R132H IDH1) Displaying Activity in Human Acute Myeloid Leukemia Cells

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Supporting Information

ABSTRACT: A collaborative high throughput screen of 1.35 million compounds against mutant (R132H) isocitrato dehydrogenase IDH1 led to the identification of a novel series of inhibitors. Elucidation of the bound ligand crystal structure showed that the inhibitors exhibited a novel binding mode in a previously identified allosteric site of IDH1 (R132H). This information guided the optimization of the series yielding submicromolar enzyme inhibitors with promising cellular activity. Encouragingly, one compound from this series was found to induce myeloid differentiation in primary human IDH1 R132H AML cells in vitro.

INTRODUCTION

The enzyme isocitrato dehydrogenase 1 (IDH1) is a nicotinamide adenine dinucleotide phosphate (NADP⁺)-dependent dehydrogenase which catalyzes the conversion of isocitrato to α-ketoglutarato (α-KG). Mutations have been found in both IDH1 (R132H) and IDH2 (R140Q, R172K, R172M) in several cancer types, including up to 70% of low grade and secondary glioma cases and up to 10% of acute myeloid leukemia cases. 1−4 IDH1 has been found to be frequently mutated in its active site at residue arginine 132 to histidine (R132H). This mutation leads to a loss of the normal enzymatic activity of IDH1 and the acquisition of a neomorphic activity, the conversion of α-KG to 2-hydroxyglutarato (2-HG). 5−7 2-HG is considered to be an “oncometabolite” which accumulates to high levels and competitively inhibits α-ketoglutarato-dependent enzymes, such as histone demethylases and TET2, which mediates DNA demethylation. There is an increasing body of research to investigate the effect of inhibition of IDH1 R132H on the cancer disease state. 8−13

A number of inhibitors of IDH1 R132H have been reported 14−24 (Figure 1). The crystal structures of IDH1 R132H containing isocitrato (ICT) have provided some understanding of the change of function of the enzyme. 25 Of the reported IDH1 R132H inhibitors, a number have associated crystallographic data, and these may be separated into two distinct classes—those that occupy the active site of the protein, 16,21,22 such as 2 and 4 (Figure 1), and those that occupy a remote allosteric site which then renders the protein inactive through conformational change, such as 3 and 6. 20,23

As part of the AstraZeneca (AZ) Open Innovation initiative, the Cancer Research UK Manchester Institute (CRUK-MI) and AZ entered into a collaboration to screen IDH1 (R132H) against the AZ compound collection. A high-throughput screen (HTS) of approximately 1.35 million compounds was undertaken against the R132H mutant form of IDH1 in order to identify novel chemical matter for development as inhibitors.

The results of HTS screening (see Figure 2 for screening cascade) are described below. The primary HTS performed well, with a mean Z’ of 0.58, and generated an initial hit rate of 2.1% using a cut off of 50% inhibition at 30 μM compound concentration in the IDH1 R132H biochemical assay.

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Compounds were then retested at a lower concentration (10 μM), resulting in 61.3% of hits at 30 μM reconforming (17126 compounds). False positives (e.g., those that interfered with the assay detection system) were identified using an orthogonal mass spectrometry assay, leaving 50.5% confirmed as active.

Prior to IC50 determination, confirmed actives were subjected to cluster analysis and virtual chemical filtering (to remove potential reactive and assay interference compounds), resulting in the selection of 5976 compounds alongside 2748 "near neighbors" for 10 point dose response determination. In order to further improve the quality of the hit output, compounds were tested in a ratio assay, i.e. testing at 1× and 10× enzyme concentration to identify any additional assay interference compounds. Compounds showing a greater than 0.5 log10 shift in IC50 were removed from further testing, with the majority of these showing a decrease in potency under the 10× standard enzyme concentration conditions, potentially due to redox cycling or colloidal aggregation.

After these measures, further clustering by substructural analysis and Tanimoto similarity gave 585 compounds of interest, with IC50 values ranging from 0.3 to 30 μM. These compounds were tested against wild-type IDH1 in a biochemical assay to determine whether the compounds were selective for the mutant isoform. None of the compounds tested showed significant activity against wild-type IDH1.

In parallel, compounds were also examined in a surface plasmon resonance (SPR) assay to confirm direct binding to the IDH R132H enzyme. Compounds that bound with a 1:1 stoichiometry were prioritized for further exploration (data not shown).

The overall result from the described screening cascade and triaging process was that compounds belonging to three distinct chemical series were identified for further evaluation. Previously we had found that the IDH1 (R132H) biochemical assay was sensitive to a number of metal contaminants including palladium, potentially giving rise to false positives. Several other hits, which had used palladium in a late stage of their synthesis, were resynthesized with particular care taken to eliminate any potential contamination of the products with residual palladium by the use of a palladium scavenger (Quadrapure MPA) and purification of reaction products by both normal and reverse phase chromatography. However, one hit series did not use palladium in the synthetic route, and examples were resynthesized in-house without these additional precautions.

On testing the in-house prepared compounds in the IDH1 R132H biochemical assay, only this palladium-free series reconfirmed strongly, suggesting that contamination of the original HTS samples (potentially by palladium) was responsible for the false positive response. The results for the one remaining series are shown in Table 1.

Compounds from this chemical series have previously been reported by AZ as inhibitors of 11β-hydroxysteroid dehydrogenase Type 1 (11β-HSD1), offering us the opportunity to...
Table 1. Activity of Original and Resynthesized Hits

| Compound | Structure | Biochemical IC₅₀ (µM)¹ | Library sample | Resynthesized |
|----------|-----------|------------------------|---------------|--------------|
| 8        | ![Structure](image1.png) | 10.1 (0.66) | 7.6 (3.7) | |
| 9        | ![Structure](image2.png) | 2.3 (0.20) | 2.2 (0.62) | |
| 10       | ![Structure](image3.png) | 3.0 (n/a) | 1.6 (0.04) | |
| 11       | ![Structure](image4.png) | 1.63 (0.07) | 1.4 (0.64) | |

¹IC₅₀’s are mean values of a minimum of two replicates. Standard deviations are listed in parentheses.

As an aid to guide optimization, attempts were made early in the project to determine the binding mode of the initial hits using X-ray crystallography. The crystal structure of compound 9 complexed with IDH1 (R132H) homodimer in the presence of NADPH (PDB submission code 5LS8) was obtained at 3.0 Å resolution (Figure 4). Although several loop regions proved to be highly disordered at this resolution, the crystal structure revealed that the unit cell contained a symmetrical dimer of (R132H) IDH1 in an “open” conformation, with electron density for compound 9 and the NADP cofactor found in both protomer subunits.

The inhibitor occupies a binding site at the dimer interface in a similar region to that described previously for other allosteric inhibitors. This binding site is distinct from the substrate and cofactor binding sites, with the closest distance between 9 and NADPH being approximately 14 Å. Notably, the packing of the two protomers differs significantly between our crystal structure and the structure of (R132H) IDH bound to α-ketoglutarate (PDB 3INM), with considerable disruption of the helical dimer interface, in particular with the helix Asn271-Tyr285 being completely disordered.

The binding interactions around 9 are mainly lipophilic in nature, with the only direct protein hydrogen bonds arising from an ionic interaction between the carboxylic acid of compound 9 and Arg119. The central pyridine ring is z-stacked with the side chain of Tyr285. The pyridyl 2-cyclohexylthio group occupies a discrete lipophilic pocket bounded by the residues Trp124 and Trp 267 of chain A and Val255 and Met259 of chains A and B. The -NH- of the pyridine 3-carboxamide potentially forms a hydrogen bond with the 2-pyridyl sulfur atom, which may serve to constrain the ligand conformation. While the side chain of R132H is located close to the substrate binding site in the α-ketoglutarate-bound crystal structure 3INM (where it presumably modulates the neomorphic catalytic activity), in our structure it has rotated approximately 5 Å away from the substrate site and toward compound 9. Although the 3-carboxamide carbonyl group of 9 points toward R132H, it appears too distant for direct hydrogen bonding. However, the recruitment of R132H to form part of the binding cavity, away from the catalytic site, offers some explanation as to the loss of neomorphic activity in the presence of these compounds. The pendant hydroxy adamantyl group is situated in an area which is largely unresolved (the residues Tyr272–Val281 are missing in the electron density), giving only partial structural information. The adamantyl moiety appears loosely bound, although it makes several lipophilic contacts with Ile251, Ile251B, Val255B, and Trp267A. The hydroxyl group is positioned close to the side chain of Asn271 but does not appear to make any direct hydrogen bonds.

Examination of the X-ray crystal structure suggested a number of avenues for the rapid exploration of the SAR (Figure 5). Thus, for Region 1 (R1), the potential for hydrogen bonding between the pyridyl carbonyl and the protein could be explored, alongside attempts to increase potency by finding additional contacts in the large pocket currently occupied by the hydroxy adamantyl group. The crystal structure shows that the pocket occupied by the -S-cyclohexyl moiety, Region 2 (R2), is a discrete lipophilic pocket and is likely to respond to the precise nature and shape of any “small” substituents. The effects of changes to the linkage could also be explored. Region 3 (R3) is dominated by the charged interaction between the carboxylic acid and Arg119. However, the linkage between the carboxylic acid and the pyridyl ring offers scope for exploration. It was imagined that modification of each of the areas in the molecule in turn would give an understanding of the component SAR, which may allow a rationale for the design of molecules with acceptable in vitro and in vivo DMPK properties, alongside improvements in selectivity against 11β-HSD1.

### CHEMISTRY

A series of compounds allowing simple substitution at the 2-position of the central pyridine (R2) were prepared with the 3-carboxamide function held as cyclohexyl and the 6-carboxylic acid function maintained as (S)-2-(piperidin-3-yl)acetic acid using the methods shown in Scheme 1.

2,6-Dichloronicotinoyl chloride was condensed with cyclohexylamine in dichloromethane (DCM) in the presence of diisopropylethylamine (DIPEA) to give 2,6-dichloro-N-cyclohexynicotinamide 13 as a key intermediate. Displacement of the 2-chloro group was accomplished using the appropriate alcohols and either sodium hydride in DMF or potassium tert-butoxide in THF to give the 2-O-substituted 6-chloro-N-cyclohexyl nicotinamides 14a–az. The regiochemistry of this reaction was confirmed for R = -O-isopentyl by removal of the remaining chlorine atom by hydrogenation and examination of the ¹H NMR aryl region for characteristic coupling patterns. The 6-chloro group was then displaced by ethyl (S)-2-(piperidin-3-yl)acetate using potassium carbonate in butyronitrile at 110 °C to give the intermediate esters 15a–az, which
were hydrolyzed to the acid using aqueous sodium hydroxide in methanol to give the carboxylic acids 16a−az. The final ester hydrolysis step was frequently carried out on unpurified isolated esters, allowing expedient production of final compounds.

Compounds designed to explore the SAR around the 3-carboxamide group, R1, were prepared either by the sequential route shown in Scheme 1 (although steps were “telescoped” by taking crude isolated products into the next reaction and using preparative HPLC to isolate the final product in a suitable purity) or by using an alternative route which was developed to be more efficient, as shown in Scheme 2.
6-Chloro-2-(isopentyloxy)nicotinic acid 17 was prepared by reaction of 2,6-dichloronicotinic acid with isopentanol (confirmation of the regiochemistry of the alkoxide addition was again obtained by removal of the remaining chlorine atom by hydrogenolysis and examination of the \(^1\)H NMR aryl region for characteristic coupling patterns). Condensation of the aminoester ethyl (1\(^R\),5\(^S\),6\(^R\))-3-azabicyclo[3.1.0]hexane-6-carboxylate (an alternative amino-acid function that had been identified in the original hit set) using conditions previously described followed by hydrolysis of the ester with sodium hydroxide gave the target compounds 20\(^a\)−ae. The latter two steps were combined without purification of the intermediate ester product.

Compounds bearing alternative carboxylic acid groups were prepared either using similar methods as described above (21−23) or by using Suzuki or Buchwald−Hartwig chemistry (24−28). (For these examples, particular care was taken to remove residual palladium from final compounds to avoid apparent activity due to metal contamination.)

Reverse carboxamide compounds 39\(^a\)−39\(^c\) were prepared from the appropriately substituted aminopyridine compound 37 as shown in Scheme 3 using cyclohexane carboxylic acid chloride, cyclohexylisocyanate, and cyclohexane sulfonyl chloride as electrophiles.

Thus, sequential displacements of halogen from 2,5-dichloro-3-nitropyridine by isopentoxide and ethyl (1\(^R\),5\(^S\),6\(^R\))-3-azabicyclo[3.1.0]hexane-6-carboxylate followed by reduction to the amine gave intermediate 37, which was then substituted further before hydrolysis to the carboxylic acid.

Sulfonamide replacement compound 43 was prepared from 2,6-dichloropyridine-3-sulfonyl chloride as shown in Scheme 4. Reaction of the commercially available 2,6-dichloropyridine-3-sulfonyl chloride with 4-aminoadamantan-1-ol to give 40 followed by sequential displacements of halogen by 3-methyl butoxide and ethyl (1\(^R\),5\(^S\),6\(^R\))-3-azabicyclo[3.1.0]hexane-6-carboxylate followed by hydrolysis to the carboxylic acid gave compound 43.

The des-carbonyl compound 47 was prepared by the route shown in Scheme 5 below. Compound 17 was reduced to alcohol 44 and then oxidized to give aldehyde 45, which was reacted with ethyl (1\(^R\),5\(^S\),6\(^R\))-3-azabicyclo[3.1.0]hexane-6-

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**Scheme 1. Route to R2 Substituted Compounds**

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Reagents and conditions: (i) Cyclohexylamine, DIPEA, DCM, RT. (ii) R2OH, NaH/DMF, RT, 22−99%, or potassium t-butoxide/THF, RT, 38−99%. (iii) Ethyl (S)-2-(piperidin-3-yl)acetate, DIPEA/PrCN, 110 °C, 23−99%. (iv) NaOH/Methanol, rt to 50 °C, 4−99%
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**Scheme 2. Route to R1 Substituted Compounds**

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Reagents and conditions: (i) Sodium hydride 60% in mineral oil, THF/DCM, 3-methyl butan-2-ol, RT, 73%. (ii) DIPEA/PrCN 110 °C, 42−75%. (iii) aq NaOH in methanol, rt, 38−85%.
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**Scheme 3. Route to 3-Amino-Substituted Compounds**

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Reagents: (i) 3-Methyl butanol, NaH, THF, rt, 93%. (ii) Amine, DIPEA, butyronitrile, 100 °C, 75%. (iii) Fe, NH\(_4\)Cl, aq ethanol, reflux, 74%. (iv) Acyl chloride, sulfonyl chloride or isocyanate, rt, 73−86%. (v) aq NaOH in methanol, rt, 38−85%.
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**Scheme 4. Route to 3-Amino-Substituted Compounds**

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Reagents and conditions: (i) 3-Methyl butanol, NaH, THF, rt, 93%. (ii) Amines, DIPEA, butyronitrile, 100 °C, 75%. (iii) Fe, NH\(_4\)Cl, aq ethanol, reflux, 74%. (iv) Acyl chloride, sulfonyl chloride or isocyanate, rt, 73−86%. (v) aq NaOH in methanol, rt, 38−85%.
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**Scheme 5. Route to Des-Carbonyl Compounds**

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Reagents and conditions: (i) Sodium hydride 60% in mineral oil, THF/DCM, 3-methyl butan-2-ol, RT, 73%. (ii) DIPEA/PrCN 110 °C, 42−75%. (iii) aq NaOH, methanol, or dioxane.
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carboxylate followed by addition of the hydroxyadamantyl amine by reductive amination and then hydrolysis of the ester to the carboxylic acid to give compound 47, albeit with poor recovery after preparative HPLC purification.

**RESULTS**

**Region R2.** Compounds 16a–16az with differing substituents in the core pyridine 2-position, R2, were intended to explore the topology and the nature of the small lipophilic pocket identified in the crystal structure of compound 3. These compounds were screened in the IDH1 (R132H) biochemical assay, the results of which are shown in Table 2.

The introduction of a heteroatom (i.e., N or O) to the substituent was poorly tolerated, as exemplified by the methyl ether compound 16s, the pyranyl compound 16t, and the dimethylamine compound 16w, which were all inactive (IC50 > 50 μM). These results were consistent with the X-ray crystallographic data from the original hit, which showed that the alkyl group at the 2-position occupies a lipophilic pocket devoid of any potential polar interactions. Reducing the length of the O-linked alkyl chain at the 2-position to an isobutyl 16n, ethyl 16u, or methyl group 16v all led to a 3-fold to >7-fold drop off in potency. Given the potencies observed for similarly sized moieties at this position, these data (and the data for 16x and 16y) are rather surprising and remain unexplained.

Attempts to reduce the conformational flexibility of the 2-substituent by introducing sp2 character or a small ring, compounds 16a, 16d, 16j, 16p, and 16q, all led to a decrease in the potency compared with compound 12. The introduction of a 1,1,1-trifluorobutoxy group 16h resulted in a 2-fold decrease in the activity toward IDH1 (R132H) compared with an isopentyl group, roughly equipotent with the butoxyl compound 16g. All this provided confirmation that the binding pocket of the IDH1 (R132H) enzyme preferred lipophilic, nonpolar groups at the 2-position of the pyridine ring and that, from the examples prepared, the isopentyl moiety was optimal.

The introduction of a variety of different substituted O-linked benzyl groups at this position bearing electron-donating or electron-withdrawing substituents is shown in Table 3. Unsubstituted benzyl compound 16z gave an IC50 of 8.6 μM, representing a marginal decrease in the potency compared with the isopentyl group at this position, 12. The addition of an electron-donating methyl group to the phenyl ring of the benzyl group had a significant impact on the activity of these compounds. For example, the ortho-methyl (16aa) and meta-substituted benzyl (16ab), compounds led to a 2-fold increase in the potency compared with the unsubstituted system compound 9z. However, the corresponding para-substituted compound 16ac was inactive against IDH1 (R132H). Methoxy-substituted benzyl systems, however, gave a marginal loss in potency for ortho- and meta-substituted benzyl groups, compounds 16ad and 16ae, while the para-methoxy substituted benzyl compound 16af was inactive. Interestingly, 16ad and 16ae contrast strongly with 16s and 16t, where an

**Table 2. Biochemical Screening Data for R2 Aliphatic Substituents**

| R2   | IC50 μM (SD) | R2   | IC50 μM (SD) |
|------|-------------|------|-------------|
| 12   | 6.4 (1.9)   | 16m  | 16 (4.4)    |
| 16a  | 8.0 (0.95)  | 16n  | 26 (12.7)   |
| 16b  | 7.6 (2.4)   | 16o  | 14 (7.5)    |
| 16c  | 9.7 (4.4)   | 16p  | 20 (6.9)    |
| 16d  | 7.2 (3.2)   | 16q  | 30 (3.3)    |
| 16e  | 12 (2.1)    | 16r  | 20 (12.8)   |
| 16f  | 12 (2.7)    | 16s  | >50         |
| 16g  | 12 (3.1)    | 16t  | >50         |
| 16h  | 15 (0.47)   | 16u  | >50         |
| 16i  | 12 (5.1)    | 16v  | >50         |
| 16j  | 14 (2.7)    | 16w  | >50         |
| 16k  | 14 (4.7)    | 16x  | >50         |
| 16l  | 12 (5.9)    | 16y  | >50         |

*IC50’s are mean values of a minimum of three replicates. Standard deviations are listed in parentheses.*
In order to understand the influence of linkers other than oxygen on the SAR, a small set of matched pairs were prepared in a similar manner to that shown in Scheme 1, using the appropriate starting materials with O, N, and S linker atoms. Full details of the synthesis are given in the Supporting Information.

Comparison of the IDH1 (R132H) activities of the O-isopentyl compound 12 and S-isopentyl compound 16ar showed only a small difference in potencies (Table 4).

### Table 3. IDH1 (R132H) Enzyme Assay Data for R2 Substitutions

| R2 | IC<sub>50</sub> [μM] (SD) |
|----|----------------------------|
| 12 | 6.4 (1.9) |
| 16z| 8.6 (3.7) |
| 16aa| 4.5 (1.3) |
| 16ab| 4.6 (0.08) |
| 16ac| >50 |
| 16ad| 13 (0.37) |
| 16ae| 12 (0.65) |
| 16af| >50 |
| 16ag| 5.6 (0.32) |
| 16ah| 10 (3.1) |

*IC<sub>50</sub>*'s are mean values of a minimum of three replicates. Standard deviations are listed in parentheses.

### Table 4. IDH1 (R132H) Enzyme Assay Data IC<sub>50</sub> (μM) for R2 and X Substitutions

| R2 | O | S | N |
|----|---|---|---|
| 12 | 6.4 (1.9) | 16ar | 10 (1.3) | 16av | >50 |
| 16e | 12 (2.1) | 16aw | >50 |
| 16c | 9.7 (4.4) | 16ax | >50 |
| 16z | 8.6 (3.7) | 16as | 39 (9.8) | 16ay | >50 |
| 16aa | 4.5 (1.3) | 16at | >50 | 16az | >50 |
| 16ag | 5.6 (0.32) | 16au | 21 (5.4) |

*IC<sub>50</sub>*'s are mean values of a minimum of three replicates. Standard deviations are listed in parentheses.

However, it was apparent that, in all other examples, the S-linkage was inferior to the O-linkage and that the N-linkage was not tolerated at all, perhaps as a result of the change from an H-bond acceptor to an H-bond donor.

Summarizing SAR in this region, having investigated a wide variety of small lipophilic groups in the R2 position, -O-isopentyl and -O-ortho-methylbenzyl were found to be the preferred substituents for this vector.

### Region R1

Compounds 20a–20af, with differing substituents on the pyridine 3-carboxamide, R1, were intended to explore topology and the nature of the more open and less well-defined pocket identified in the crystal structure of compound 9. The results of the investigation can be seen in Table 5. All new compounds were compared against the R1 cyclohexyl carboxamide compound 20ab, enzyme IC<sub>50</sub> 7.9 μM.

Aliphatic and cycloaliphatic groups, with or without the addition of heteroatoms or single bridge structures, failed to offer any increase in potency against IDH1 (R132H), except for 20f. Direct aryl attachments gave a range of inhibitory activities from unsubstituted phenyl (>50 μM) (not shown in Table 5) to 20f (2.9 μM), with compounds with a hydroxyl pendent group (direct or indirect) being the most potent, suggesting the
possibility of a positive interaction between the hydroxyl group and the protein structure.

Benzyl carboxamides gave a range of activities. The baseline benzyl substituent group \( 20y \) was marginally improved upon by simple monosubstitutions, and modest (up to 2-fold) improvements were obtained from the \( R - \alpha - \text{methyl} \) compound \( 20t \) and \( S - \alpha - \text{methyl} \) compound \( 20k \). Extending the aryl group further with \( R1 = \text{phenethyl}, 20i \), gave an additional small increase in enzyme potency.

The most significant improvements in IDH1 (R132H) inhibition came from the use of bulkier structures. Although the adamantyl carboxamide \( 20b \) was essentially inactive (>50 \( \mu \text{M} \)), substitution of the adamantyl group in the 1-position with hydroxyl \( 20a \) or methoxy \( 20c \) gave compounds with submicromolar activity. Extending the hydroxyadamantyl group further from the carboxamide link with a methyl substituted methylene \( 20d \) gave a similar submicromolar level of activity, while the addition of a difluoromethoxy group \( 20e \) gave a noticeable decrease in activity, albeit still at a submicromolar level.

**Region R3.** Simple replacement of the carboxylic acid group of \( 20b \) by ethyl ester (19a) dramatically reduced activity, as might be expected by elimination of the key interaction with Arg119. Although a number of alternative carboxylic acid scaffolds were tolerated, it was noted that the scaffolds in \( 20b, 21, \) and \( 22 \) were the most effective (Table 6). One exception to this trend was the thiazole carboxylic acid \( 26 \), which displayed submicromolar potency, although this is still some three-fold less active than the most potent compound \( 20a \).

With this information in hand, a set of compounds were then synthesized combining the more potent \( R2 \) and \( R3 \) components with the hydroxyadamantyl carboxamide \( R1 \) group to examine the additive nature of the group SAR. It is evident from the results in Table 7 that the component SAR is not truly additive and that the overall SAR is dominated by the \( R3 \) carboxylic acid group. It is logical to suggest that the rigid nature of the aza-bicyclohexane acid, which gave rise to the most potent inhibitors with a small subset of \( R1 \) and \( R2 \) groups, would be less tolerant of alternative \( R2 \) substitutions than the more flexible (and accommodating) piperidine acetic acid.

The X-ray crystal structure obtained for compound 9 indicated that there may be some potential for hydrogen bonding between the pyridine carboxamide \(-\text{NH}-\) with Tyr285
and -CO- with His132, in this latter case via water mediation. In order to understand further the importance of the role of the 3-carboxamido group, compounds were prepared by modifying the nature of the 3-carboxamide group by substitution, by reversal of the carboxamide, or by replacement with other groups.

A comparison of benzyl carboxamide compound 20y and cyclohexyl carboxamide compound 20ab with the N-methyl analogues, 20aa and 20ac, shows either no change or a small loss of potency on replacement of the carboxamide -NH- by -NMe-, suggesting that either any potential internal hydrogen bond to the pyridyl 2-oxygen atom added little to the potency of the compounds or the increased rotational freedom of the amide bond is beneficial.

Replacement of the pyridine carboxamide with the reversed carboxamide 39a or urea 39b resulted in complete loss of activity, as did direct replacement of the carbonyl group with a sulfonamide 43 (Figure 6). Further, reduction of the carbonyl group completely to yield 47 resulted in a complete loss of activity, suggesting that the carbonyl group has a role in hydrogen bonding and/or rotational stability. Only the reverse sulfonamide 39c (8.6 μM) was found to have a similar potency when compared to 20ab. Here, perhaps the differing geometry allows a strong hydrogen bond with His132 or reorientates the R1 lipophilic group improving contact with the enzyme, presenting an opportunity for further study.

Having established that compound 20a was a potent compound against IDH1 (R132H) in this series, we chose to re-examine selectivity against other related enzymes (Table 8).

While retaining some activity against an alternative mutant IDH1 (R132C) (0.47 μM), compound 20a was inactive against both wild type IDH1 and IDH2 and glucose-6-phosphate dehydrogenase (G6PD). There remained, however, considerable potency against the original (AZ) target for this series of compounds, 11-βHSD-1, at 0.009 μM.

Selected compounds with a range of enzyme inhibitions including a related but inactive analogue 16v were screened in a cellular assay (using mutated R132H isocitrate dehydrogenase 1 transfected HEK293T cells—see Experimental section) measuring the inhibition of production of 2-HG directly by mass spectrometry (Table 9).

Compounds 20a, 21, and 16ag show modest cellular activity for the inhibition of production of 2-HG while displaying no general cytotoxicity against HeLa cells (72-h proliferation assay). There was, however, a 3−10x fall off from the enzyme activity to the cell activity, which may be explained by the poor permeability and high efflux ratio found for compound 20a (Caco-2: \( P_{app} \) 2.56 × 10^-6 cm s^-1, Efflux ratio 16.1).

A series of surface entropy reduction mutations were made to R132H to enhance the repeatability of the crystallogenesis. In all five, additional surface residues were mutated (E80A, K81A, E240A, Q242A, and K243A) to alanine residues. These mutations produced a construct, R132H*, that behaved favorably during crystallogenesis and was subsequently used throughout this study.

Table 7. Matched Pair Combinations of R2 and R3 Substitutions, IDH1 R132H IC₅₀ μM

| R² | R³ | IC₅₀μM (SD) | IC₅₀μM' (SD) |
|----|----|-------------|-------------|
| 20a | 21 | 0.27 (0.22) | 1.2 (0.33) |
| 29 | 32 | 27 (15) | 2.3 (0.79) |
| 30 | 33 | 4.5 (4.7) | 0.69 (0.30) |
| 31 | 34 | 3.2 (2.0) | 2.3 (0.39) |

\(^{a}\)IC₅₀’s are mean values of a minimum of three replicates. Standard deviations are listed in parentheses.
The crystal structure of compound 20a complexed with IDH1 (R132H) homodimer in the presence of NADPH (PDB accession code 5L57) was obtained at 2.7 Å (Figure 7). The electron density for compound 20a and for the cofactor were found in both dimer subunits.

This crystal structure is very similar to that obtained with compound 9, with the inhibitor binding at the interface of the two components of the protein dimer. The ionic interaction between the carboxylic acid of compound 20a and Arg119 is unchanged from the crystal structure of compound 9, as is the π-stacking of the central pyridine ring with the side chain of Tyr285. The pyridyl 2-isopentoxide group occupies the same lipophilic pocket bounded by the residues Leu120, Val121, Trp124, Val255, and Met259 but perhaps makes better contacts. There is an internal hydrogen bond between the -NH of the pyridine 3-carboxamide and 2-O linker, but no hydrogen bond with the oxygen atom of Tyr285. The 3-carboxamide carbonyl group points in the general direction of His132 but again is too distant for direct hydrogen bonding. The pendent hydroxy adamantyl group is situated in the same area as in the structure of compound 9, making several lipophilic contacts but no direct hydrogen bonds from the hydroxyl group. Note that, as in the previous structure, solvent molecules were not resolvable at this resolution.

In this crystal structure there is sufficient electron density to assign several previously missing loops, including Tyr272−Val281 and Lys212−Lys218. Of these newly resolved residues, only Val281 interacts directly with the ligand, providing an additional lipophilic contact with the pyridyl ring.

The binding mode of 20a can be summarized in terms of four key pharmacophore features: hydrogen-bonding between the acidic moiety and Arg119; aromatic stacking between the pyridyl and Tyr285; lipophilic contacts between the isopentyl moiety and the enclosed dimeric pocket around Val255 and Table 8. Selectivity of Compound 20a against Related Enzymes

| Compd | Structure | Enzyme $IC_{50}$ μM (SD) | Cell IDH1 $IC_{50}$ μM (SD) | Hela cytotox $IC_{50}$ μM |
|-------|-----------|--------------------------|-----------------------------|---------------------------|
| 20a   | ![Image](image1) | 0.27 (0.22) | 2.3 (0.73) | >30 |
| 21    | ![Image](image2) | 1.3 (0.33) | 8.5 (2.3) | >30 |
| 16ag  | ![Image](image3) | 5.6 (0.32) | 16 (0.5) | >30 |
| 16v   | ![Image](image4) | >50 | >30 | >30 |

"IC$_{50}$s are mean values of a minimum of two replicates. Standard deviations are listed in parentheses."

Table 9. Enzyme and Cell Activities ($IC_{50}$ μM) for a Selected Set of Compounds

| Cmpd | Structure | Enzyme $IC_{50}$ μM (SD) | Cell IDH1 $IC_{50}$ μM (SD) | Hela cytotox $IC_{50}$ μM |
|-------|-----------|--------------------------|-----------------------------|---------------------------|
| 20a   | ![Image](image5) | 0.27 (0.22) | 2.3 (0.73) | >30 |
| 21    | ![Image](image6) | 1.3 (0.33) | 8.5 (2.3) | >30 |
| 16ag  | ![Image](image7) | 5.6 (0.32) | 16 (0.5) | >30 |
| 16v   | ![Image](image8) | >50 | >30 | >30 |

"IC$_{50}$s are mean values of a minimum of two replicates. Standard deviations are listed in parentheses. $IC_{50}$ Inhibition of production of 2-HG measured directly by mass spectrometry. $IC_{50}$ Proliferation assay in a non-IDH driven cell line.

![Figure 7](image9) (A) Crystal structure of compound 20a in IDH1 (R132H). PDB accession code 5L57; previously unresolved loops highlighted in magenta. (B) Protein:ligand interaction diagram.
Met 259; and looser lipophilic contacts around the adamantyl moiety. Of these interactions, only lipophilic filling of the Val255/Met259 pocket is a consistent feature. In the Sano bisimidazole 3 structure, this pocket is enlarged due to Trp267 adopting an alternative rotamer, allowing a bulky branched octyl moiety to be accommodated. Overall, the ligand occupies a similar binding location to 20a: when the binding sites are overlaid, the central phenol is observed to be somewhat displaced from the pyridyl of 20a, with the two imidazole moieties aligning loosely with the adamantyl and aza-bicyclohexane moieties of 20a. A notable difference between the structures is the significant repositioning of the Asn271−Gly286 loop, with Ser280−Gly284 now adopting a short helical structure. As a result, Tyr285 is significantly displaced and hence not available to π-stack to the phenol ring; instead, the side chain of Val281 now occupies a similar position. A further consequence is that Asp279 moves closer to the ligand and forms a direct hydrogen bond with the phenolic hydroxyl. The side chain of Arg119, which formed an ion pair with the acidic moiety of 20a, adopts a different rotamer and does not interact with 3.

Inhibitor 6 again binds in the dimer interface but displays some strikingly different interactions. Although Ser280-Gly284 forms a short helix similar to that in the Sano structure, most of this loop (Asn271−Asp279) is unresolved. The pyrazolopiperidine ring system of 6 overlays with the aza-bicyclohexane of 20a, with the fluorobenzyl moiety of 6 filling the Val255/Met259 lipophilic pocket. The pyrrole ring of 6 extends beyond the acidic moiety of 20a, in effect displacing the side chain of Arg119. However, the most notable difference is the location of the hydroxyethylanilino substituent, which extends between the Ser280−Gly284 helix and the Ile112−Pro127 loop into a region not occupied by either 3 or 20a. Direct protein:ligand hydrogen bonds, unique to 6, are formed between the carboxamide linker and the backbone atoms of Leu120 and Val281, and between the pyrrolo carbonyl and Ile128.

Recently, the binding mode of the Agios allosteric inhibitor 2-(2-(1H-benzo[d]imidazol-1-yl)-N-(3-fluorophenyl)-acetamido)-N-cyclopentyl-2-orthoacetamide (ML309) has been established by cryoelectron microscopy.27 Although the resolution is too low to allow accurate placement of the inhibitor, it is evident that the compound is occupying a
binding site at the dimer interface which is broadly similar to that seen for the above compounds.

Although the structural basis of inhibitor selectivity for mutant over wild type IDH1 is not fully understood, it is apparent that these inhibitors bind to a conformation of the protein in which the location of several catalytically important residues is significantly disrupted, including, for example, His132 and Tyr139 (involved in substrate binding) and Asp275 and Asp279 (involved in binding the catalytic magnesium ion). Hence, it is likely that allosteric inhibitors bind preferentially to an inactive conformation that is more accessible to the mutant protein than to wild type. For example, Deng et al.20 highlight differences in binding affinity for the catalytic magnesium ion between mutant and wild type IDH1.

Figure 10. Compound 20a induces myeloid differentiation in primary human IDH1 R132H AML cells \textit{ex vivo}. (A) Flow cytometry histograms for CD11b, CD15, and CD117, (B) cytospin preparations, (C) indicative colony morphologies, and (D) total colony numbers relative to vehicle-treated control cells from the indicated patients after 14 days \textit{ex vivo} methylcellulose culture with compound 20a 18.4 μM (red) or DMSO vehicle (blue). Error bars represent mean ± standard error of three independent experiments.
In summary, comparison of our crystal structures with those of other allosteric inhibitors reveals that the allosteric binding site is accessible to lipophilic ligands of diverse chemotypes, which share some common binding interactions, notably to the Val255/Met259 lipophilic pocket, but also achieve a number of unique polar and lipophilic interactions. The allosteric binding site displays varying degrees of induced fit, as well as differences in the overall packing of the dimers, to accommodate these diverse ligands. Taken together, the crystal structures may guide the future optimization of allosteric inhibitors and facilitate the identification of additional allosteric chemotypes through focused screening campaigns.

We next tested compound 20a in acute myeloid leukemia (AML) cells. Approximately 10% of patients carry IDH1 mutations, and inhibitors of mutant IDH1 and IDH2 are currently being evaluated in early phase trials. 28-30 As no immortalized human AML cell lines carrying native IDH1 mutations are available, we generated an AML cell line system using THP1 AML cells, whereby Myc-tagged IDH1 R132H could be inducibly expressed following the addition of doxycycline (Figure 9A). As expected, induced expression of this was inhibited in a dose-dependent manner by compound 20a, with IC_{50} 1.9 μM and complete reduction to baseline levels at ≥10 μM. Interestingly, treatment of cells with compound 20a had no effect on proliferation or differentiation (data not shown), suggesting limited off-target or nonspecific cellular toxicity.

We next tested the activity of compound 20a versus primary AML blast cells (cultured in semisolid medium) from three IDH1 R132H-mutated patients (BB171; BB340; BB539; BB = Biobank number) and four IDH1 wild type patients. Three doses of 20a reflecting the cellular IC_{50} 4×IC_{50} and 8×IC_{50} or DMSO vehicle control, were used. After 14 days in culture, primary cells exposed to doses commensurate with near-total (9.2 μM) or total (18.4 μM) 2-HG reduction in THP1 cells exhibited significant myeloid differentiation compared with vehicle-treated controls, as evidenced by upregulation of the myeloid differentiation markers CD11b and CD15, downregulation of the stem cell marker CD117 (Figure 10A), and induction of mature cellular morphology (Figure 10B). Interestingly, treatment of IDH1 R132H mutant primary AML cells with 20a led to an increase in clonogenic activity (Figure 10D). This "proliferative burst" is associated with release of the differentiation block and has previously been described following treatment of IDH2 mutant primary AML cells with the mutant IDH2 inhibitor AGI-67801.28 By contrast, and in keeping with 20a being selective for mutant IDH1, no differences in clonogenic potential or myeloid differentiation were observed in similarly treated IDH1 wild type AML blasts (Figure 10A, 10D).

**CONCLUSIONS**

As part of a collaborative program, a high throughput screen of 1.35 million compounds against isocitrate dehydrogenase IDH1 (R132H) led to the identification of three series of inhibitors, which, when resynthesized, reduced to a single series (having 20a). As no immortalized human AML cell lines carrying native IDH1 mutations are available, we generated an AML cell line system using THP1 AML cells, whereby Myc-tagged IDH1 R132H could be inducibly expressed following the addition of doxycycline (Figure 9A). As expected, induced expression of this was inhibited in a dose-dependent manner by compound 20a, with IC_{50} 1.9 μM and complete reduction to baseline levels at ≥10 μM. Interestingly, treatment of cells with compound 20a had no effect on proliferation or differentiation (data not shown), suggesting limited off-target or nonspecific cellular toxicity.

We next tested the activity of compound 20a versus primary AML blast cells (cultured in semisolid medium) from three IDH1 R132H-mutated patients (BB171; BB340; BB539; BB = Biobank number) and four IDH1 wild type patients. Three doses of 20a reflecting the cellular IC_{50} 4×IC_{50} and 8×IC_{50} or DMSO vehicle control, were used. After 14 days in culture, primary cells exposed to doses commensurate with near-total (9.2 μM) or total (18.4 μM) 2-HG reduction in THP1 cells exhibited significant myeloid differentiation compared with vehicle-treated controls, as evidenced by upregulation of the myeloid differentiation markers CD11b and CD15, downregulation of the stem cell marker CD117 (Figure 10A), and induction of mature cellular morphology (Figure 10B). Interestingly, treatment of IDH1 R132H mutant primary AML cells with 20a led to an increase in clonogenic activity (Figure 10D). This "proliferative burst" is associated with release of the differentiation block and has previously been described following treatment of IDH2 mutant primary AML cells with the mutant IDH2 inhibitor AGI-67801.28 By contrast, and in keeping with 20a being selective for mutant IDH1, no differences in clonogenic potential or myeloid differentiation were observed in similarly treated IDH1 wild type AML blasts (Figure 10A, 10D).

**EXPERIMENTAL SECTION**

Chemical Experimental. All reagents obtained from commercial sources were used without further purification. Anhydrous solvents were obtained from Sigma-Aldrich Chemical Co. Ltd. or Fisher Chemicals Ltd. and used without further drying. Solutions containing products were either passed through a hydrophobic frit or dried over anhydrous MgSO_4 or Na_2SO_4 and filtered prior to evaporation of the solvent under reduced pressure. Thin layer chromatography (TLC) was conducted with 5 cm × 10 cm plates coated with Merck type 60 F254 silica gel to a thickness of 0.25 mm. Chromatography was performed on Biotage SNAP HP-Sil cartridges using a CombiFlash Companion machine. Proton (1 H) NMR spectra were recorded on a 300 MHzBruker spectrometer at ambient temperature. Solutions were typically prepared in either deuterochloroform (CDCl_3) or deuterated dimethyl sulfoxide (DMSO-d_6) with chemical shifts referenced to deuterated solvent as an internal standard. 1H NMR data are reported indicating the chemical shift (d), the multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad; dd, doublet of doublets; etc.), the coupling constant (J) in Hz, and the integration (e.g., 1H). Deuterated solvents were obtained from the Sigma-Aldrich Chemical Co., Goss, or Fluorochem. LCMS spectra with UV detection were recorded on a Waters Acuity UPLC. Mass spectrometry was performed on a Waters Acuity SQD quadrupole spectrometer running in dual ES' and ES" modes. High pH runs were conducted at pH 10, and low pH runs were conducted at pH 4, with a run time of 2 min. The column temperature was 40 °C, and the flow rate was 0.6 mL/min. Further details, including solvent gradients, are given in the Supporting Information. Details of the preparative HPLC instrument and the solvent gradient used to purify compounds are also given in the Supporting Information. All compounds were 95% purity, as determined by examination of both the LCMS and 1H NMR spectra unless otherwise indicated. When Cl or Br was present, expected isotopic distribution patterns were observed.

Preparation of Compound 20a. 6-Chloro-2-(isopentyl)-3-pyridine Carboxylic Acid (17). 3-Methylbutan-1-ol (9.93 mL, 91.2 mmol) was added to a stirred suspension of sodium hydride (60% dispersion in mineral oil) (4.17 g, 104.2 mmol) in a mixture of DCM (200 mL) and THF (100 mL) at 0 °C. After 20 min of stirring, 2,6-dichloropyridine-3-carboxylic acid (5.0 g, 26.0 mmol) was added portionwise over 10 min and the resultant mixture allowed to stir at room temperature for 96 h. To the mixture was added water (300 mL), and the aqueous layer was collected. The mixture was reextracted once more with water (150 mL), and the combined aqueous layers were then acidified to pH 1 using conc HCl. The acidic aqueous was then extracted with DCM 3(150 mL) and EtOAc (100 mL). The combined organic solution was dried over MgSO_4 and concentrated in vacuo to afford a residue that was purified on a 100g SNAP column using a 0–5% MeOH:DCM eluent to afford 6-chloro-2-isopentylpyridine-3-carboxylic acid 17 (4.69 g, 19.2 mmol, 73.9%) as a white solid. 1H NMR (300 MHz, CDCl_3) δ 6.92–11.28 (m, 1H), 8.37 (d, J = 8.01 Hz, 1H), 6.46 (t, J = 6.55 Hz, 2H), 1.76 (br d, J = 3.96, 6.22 Hz, 3H), 0.98 (br d, J = 6.31 Hz, 6H). High pH RT = 0.77 min, [M- H]^− = 242.1.

6-[[1R,5S,6R]-6-(Ethoxycarbonyl)-3-azabicyclo[3.1.0]hexan-3-yl]-2-(isopentyl)-3-pyridine Carboxylic Acid (18). To a stirred solution of 6-chloro-2-isopentylpyridine-3-carboxylic acid 17 (2.52 g, 10.35...
The combined organic solvents were washed with water (10 mL) and Aminoadamantan-1-ol (0.10 mmol) was then added, and the mixture and triethylamine (40 μL) was stirred for 2 h at 50 °C, generating a transfection mix. Meanwhile, HEK293T cells (180,000 cells/ml) were then mixed with 2 mL of the donor antibody (anti-IDH1 R132C) and subsequently seeded into a 96-well plate. The plate was then incubated at room temperature for 2 h before measuring the TR-FRET signal on a Pheraster FS Microplate Reader.  

11β-HSD1 Cellular Mode of Action Assay. 20 μL of Lipofectamine 2000 (Thermofisher #11668019) was added to 980 μL of OptiMEM reduced serum media (Thermofisher #31985062). Separately 16 μg of pcDNA3.1 mammalian expression vector (Thermo scientific #V79020) carrying isocitrate dehydrogenase 1 mutated at R132H was added to 1000 μL of OptiMEM reduced serum media. Following 5 min incubation, the solutions were mixed and incubated for 30 min at RT, generating a transfection mix. Meanwhile, HEK293T cells cultured in assay media (DMEM (#D6546), 10%FBS (Sigma #F7524), 2 mM l-glutamine (Sigma #G7513), and 1% Penicillin Streptomycin (Sigma #P0721)) were detached and placed in 3% formaldehyde and stained with Hoechst, allowing a nuclear count to be performed on the cell insulin; 2HG concentrations were normalized to this value.  

Biochemical Assays. All proteins used in the biochemical assays were produced by the Protein Expression Facility at the Manchester Institute of Biotechnology. The activity of the various dehydrogenase enzymes was measured by coupling NADPH consumption/production to a diaphorase/resazurin-based Amplett detection system, measuring resorufin production.  

All assays were performed in 384-well black plates (Corning #3575) in a reaction volume of 25 μL. IDH1 R132H was incubated at a final concentration of 32 nM with compounds in the presence of 20 mM Tris pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 0.03% BSA (w/v) buffer, and 1 μM α-ketoglutarate and 50 μM NADPH substrates. IDH1 R132C was incubated at a final concentration of 100 nM with compounds in the presence of 20 mM Tris pH 7.5, 10 mM MgCl₂, 150 mM NaCl, 0.03% BSA (w/v) buffer and 0.2 μM isocitrate, and 50 μM NADPH substrates. IDH2 was incubated at a final concentration of 9 nM with compounds in the presence of 20 mM Tris pH 8.0, 1 mM MgCl₂, 150 mM NaCl, 0.03% BSA (w/v) buffer with 0.2 μM isocitrate, and 50 μM NADPH substrates. G6PD was incubated at a final concentration of 7 nM with compounds in the presence of 20 mM Tris pH 8.0, 10 mM MgCl₂, 0.03% BSA (w/v) buffer with 0.5 mM glucose-6-phosphate, and 50 μM NADPH substrates.  

All reactions were initiated by the addition of NADPH/NADP. The assays were allowed to proceed at 26 °C for 30 min (IDH1 R132C, IDH1, and G6PD) or 60 min (IDH1, R132H, and IDH2) before terminating with the addition of 5 μL of Amplett detection system (The G6PD assay was stopped using 2.5 μL of 0.5% SDS (w/v) prior to the addition of Amplett). Following incubation at room temperature for 30 min, the fluorescence signal at Ex544/Em590 nm was measured using the BioTek Synergy 2 multimode plate reader.
Mass spectrometry detection was made using an API4000 QTrap (AB Sciex, Framingham, MA) operating in negative ion mode with multiple reaction monitoring mass ion transitions 147/85 (2-HG) and 150/88 (D3-2-HG) IS. Data was processed using Analyst Software (Applied Biosystems) and signal intensities obtained by standard peak integration methods. Quantitations were performed by comparison against a standard curve generated from multiple dilutions of growth medium spiked with 1 mM 2-HG stock in water to known concentrations. Final 2-HG quantification was derived from a mean of two biological replicates. The lower limit of accurate quantification (LLQ) was set at a level twice the peak area measured in conditioned medium, to ensure robust and confident discrimination of 2-HG from low-level background peaks which were observed to coelute during development of the method.

**Generation of Stable THP1-IDH1-WT/R132H Clones.** Ectopic human IDH1 wild type and IDH1 R132H were stably overexpressed in THP1 AML cells using standard site-directed mutagenesis and lentiviral transduction techniques. Briefly, the coding sequence of human IDH1 (transcript NCBI accession NM_001282387) was PCR amplified from THP1 cDNA with addition of sequence encoding a Myc epitope tag, and ligated into the pGEMT-Easy vector (Promega). Mutant forms were generated by overlap extension PCR and expression constructs ligated into the pTRE2 minigene. Lentiviruses were generated in 293FT cells using standard techniques, and was used to spinoculate THP1 cells previously stably transduced with EF1α rtTAadv IR-ES_Neo (i.e., the tetracycline transactivator component). Successfully transduced cells were selected by culture for at least 5 days in 6 μg/mL blasticidin. Ectopic construct expression was confirmed in lysates by Western blotting for the Myc epitope tag and full length IDH1, using standard techniques. Vinculin was used as a loading control. Primary antibodies used were: anti-IDH1 (DH21; rabbit; Cell Signaling, Beverly MA); anti-MYC-TAG (9B11/mouse; Cell Signaling); anti-VINCULIN (hVIN-1/mouse; Sigma-Aldrich).

**Primary AML Patient Samples.** Primary material was obtained from bone marrow and peripheral blood samples donated to the Manchester Cancer Research Centre (MCRC) Tissue Biobank, following informed written consent and with approval of the South Manchester Research Ethics Committee. Samples were collected, and IDH mutation status was determined as previously described.1,2,3 Samples selected for use contained ≥80% blasts.

**Cell Culture and Clonogenic Assays.** Suspension cell lines were cultured in RPMI (Sigma-Aldrich) containing 10% tetracycline-free FBS and 1% penicillin/streptomycin (Sigma). Primary AML cells were rapidly thawed in a 37 °C water bath, washed twice in DAMP solution (PBS with 4 μg/mL DNAse, 2.5 mM MgCl2, 16.4 mM trisodium citrate), and seeded in 200 μL serum-free StemSpan (Stem Cell Technologies) at 2 × 104 cells per condition in methylcellulose (Methocult H4230; Stem Cell Technologies) final volume 1 mL, with the following cytokines at the specified final concentration: IL-3, 20 ng/mL; IL-6, 20 ng/mL; IL-11, 10 ng/mL; FLT3L, 50 ng/mL; SCF, 50 ng/mL; TPO, 50 ng/mL (all from Peprotech); G-CSF, 50 ng/mL (Phenetrex); and, Epo 4U/mL (Janssen Cilag). Compound 20a or DMSO vehicle was added at 1:1000 for the desired final concentration. Plates were cultured at 37 °C and 5% CO2. After 14 days, colonies were scored, counted, and photographed before being washed out of methylcellulose by ≥10× dilution in PBS and centrifugation. Cultures were recovered to be subjected to counting by Trypan Blue exclusion and flow cytometry.

**Flow Cytometry Immunophenotyping.** Cells were suspended in staining medium (SM) buffer (5.2 g of phenol red free RPMI powder, 15 mL of FBS, 5 mL of 1 M Hepes, 1 mL of 500 mM EDTA made up to 1L with ddH2O) with antibodies targeting cell surface markers of myeloid differentiation status. Antibody cocktails [and clone] used were: anti-CD15-FITC [H98]; anti-CD11b-PE [ICR44]; and anti-CD117-APC [104D2] (all from Biolegend). All were used at 1:50 concentration. Cells were stained for ≥30 min in the dark at 4 °C before being resuspended in fresh SM buffer and analyzed on an LSR FortessaTM flow cytometer (BD Biosciences). Data were analyzed using FlowJo for Mac v10.1r5.

**LC-MS/MS measurement of 2-HG.** Extracellular and intracellular 2-HG were measured by hydrophilic interaction liquid chromatography (HILIC) LC-MS/MS, on media supernatant removed from cell cultures or cell lysates, respectively. Cryopreserved media samples were thawed and diluted 1:10 with water containing deuterated (D3)-2-HG as an internal standard (IS). Solid phase extractions were performed using an Oasis MAX 96-well-plate (Waters) containing 10 mg of Oasis MAX sorbent per well. Plates were conditioned with 750 μL of methanol and 750 μL of water before buffered test samples were loaded and pulled through under vacuum. Plates were washed with 750 μL of water, 750 μL of 50% methanol, and 100% methanol before extracts were eluted with 2 × 250 μL 1% formic acid in methanol for cell lysates, pelleted cells were washed twice with ice-cold PBS before sequential quenching with −80 °C 80% methanol:20% water containing 0.5 μg/mL D3-2-HG IS; transfer to dry ice bed; sonication on a Biorupter (Diagenode) at high power for 5 min (at 30 s on/30 s off); and subsequent incubation at −80 °C for ≥20 min. Debris was removed by centrifugation at 14,000g, and supernatant was used for LC-MS/MS. Eluates were dried under nitrogen gas, reconstituted in initial conditions (88:2:10 acetonitrile:water:50 mM ammonium acetate; pH 8.5), and injected into the LC-MS.

**Chromatographic separation of 2-HG from coextracted interferences was performed using high pressure liquid chromatography (Agilent 1200, Agilent) with a Luna HILIC 5 μm column (150 × 3.0 mm) (Phenetrex). The flow rate was 750 μL/min using a gradient starting at 95% mobile phase A, moving to 80% over 180 s before rising back to 95% over 60 s. This was followed by a hold to equilibrate at 95% phase A for a further 150 s. The column temperature was ambient, and the sample compartment was chilled to 10 °C. Mobile phase A was acetonitrile:50 mM ammonium acetate (90:10 v/v) pH 8.5, and mobile phase B was acetonitrile:water:50 mM ammonium acetate (50:40:10 v/v/v) pH 8.5.

Mass spectrometry detection was made using an API4000 QTrap (AB Sciex) operating in negative ion mode with multiple reaction monitoring mass ion transitions 147/85 (2-HG) and 150/88 (D3-2-HG IS). Data was processed using Analyst Software (Applied Biosystems) and signal intensities obtained by standard peak integration methods. Quantitations were performed by comparison against a standard curve generated from multiple dilutions of growth medium spiked with 1 mM 2-HG stock in water to known concentrations. Final 2-HG quantification was derived from a mean of two biological replicates. The lower limit of accurate quantification (LLQ) was set at a level twice the peak area measured in conditioned medium, to ensure robust and confident discrimination of 2-HG from low-level background peaks which were observed to coelute during development of the method.

**IDH1 Cloning and Mutagenesis.** IDH1 (NP_005887) cDNA was cloned into the NdeI and EcoRI sites of bacterial expression vector pET22b (Merck). Disease mutation R132H was first introduced by site directed mutagenesis using the following oligos: Top 5′ GATTATCATTTGCTCATCAGCTATG 3′ and Bottom 3′ CATAGGCAATGACCAATGATACT 5′. Surface mutations E890A and K314A were introduced using oligos: Top 5′ GCCGGAC-ATTAACCCGCGATGCACGGCAGTGGTACATCGCCG 3′ and Bottom 3′ CGATGTCATCAATTGCGAGGACATCAATTGCGACATCGTCGGGCTCCGGCCGATATTGGTGCCCGG-S′. Mutations were E240A, Q242A, and K243A were then produced using oligos: Top 5′ GTAAAAACGCAGTTTGGACGCGGGCGCATCAGTTGTTACATCGCCG 3′ and Bottom 3′ CTGATTCCTTCAATTGCGAGGACATCAATTGCGACATCGTCGGGCTCCGGCCGATATTGGTGCCCGG-S′. Site directed mutations were made using a two-step PCR method using Phusion HF enzyme (NEB). Step 1 involved single top or bottom primer PCR reactions, and these were then combined 50:50 in step 2. Reaction conditions were: Step 1: 98 °C, 1 min, followed by 10 cycles of 98 °C, 10 s, 55 °C, 30 s, 72 °C, 5 min. Step 2: 98 °C, 1 min followed by 18 cycles of 98 °C, 10 s, 55 °C, 30 s, 72 °C, 5 min. The lower limit of accurate quantification (LLQ) was set at a level twice the peak area measured in conditioned medium, to ensure robust and confident discrimination of 2-HG from low-level background peaks which were observed to coelute during development of the method.
Accession Codes

Atomic coordinates and experimental data for the cocystal structures of 20a (PDB SLS7) and 9 (PDB SLS8) in complex with mIDH will be released upon article publication.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

IDH, isocitrate dehydrogenase; AML, acute myeloid leukemia; NADP, nicotinamide adenine dinucleotide phosphate; α-KG, α-ketoglutarate; 2-HG, 2-hydroxyglutarate; TET2, Tet methylcytosine dioxygenase 2; DNA, deoxyribonucleic acid; ICT, isocitrate; AZ, AstraZeneca; CRUK-Mif, Cancer Research UK Manchester Institute; HTS, high throughput screen; SPR, surface plasmon resonance; 11β-HSD1, 11β-hydroxysteroid dehydrogenase Type 1; PDB, Protein Databank; SAR, structure activity relationship; DIPEA, diisopropyl ethylamine; HPLC, high performance liquid chromatography; NMR, nuclear magnetic resonance; G6PD, glucose-6-phosphate dehydrogenase; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; DCM, dichloromethane; LCMS, liquid chromatography–mass spectrometry; DMF, dimethylformamide; EToAc, ethyl acetate

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ASSOCIATED CONTENT

* Supporting Information is available free of charge on the Journal of Medicinal Chemistry website at DOI: 10.1021/acs.jmedchem.6b01320.

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Summary of LC−MS methods and solvent gradients; preparative HPLC instrument and solvent gradients; purity data; detailed chemical experimental information for all compounds described; selected 1H and 13C NMR spectra; selected LCMS and HRMS spectra; protein purification and crystallographic methods (PDF)

Molecular Formula Strings (CSV)
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