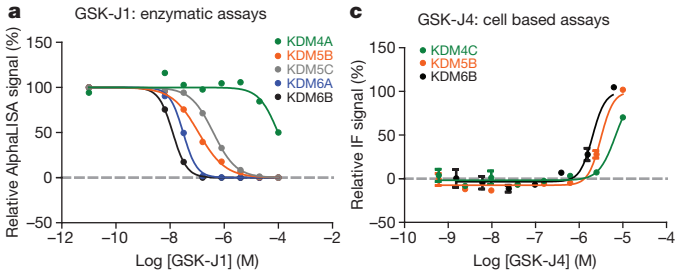
Inhibition of demethylases by GSK-J1/J4

ARISING FROM L. Kruidenier *et al*. *Nature* **488,** 404–408 (2012); doi:10.1038/nature11262

The recent publication[1] of the first highly potent and specific inhibitor GSK-J1/J4 of the H3K27me3/me2-demethylases JMJD3/KDM6B and UTX/KDM6A provides a potential tool compound for this histone demethylase subfamily[1]. This inhibitor was used in tissue culture assays to conclude that the catalytic activities of the KDM6 proteins are required in inflammatory responses[1]; the generation of the inhibitor is intriguing, because it provides a strategy for generating sub-type-specific inhibitors of the 27-member jumonji family and for the future treatment of various types of disease[2–6]. Here we show that the inhibitor is not specific for the H3K27me3/me2-demethylase subfamily *in vitro* and in tissue culture assays. Thus, the inhibitor cannot be used alone for drawing conclusions regarding the specific role of H3K27me3/me2-demethylase activity in biological processes or disease. There is a Reply to this Brief Communications Arising by Kruidenier, L. *et al. Nature* **514,** http://dx.doi.org/10.1038/nature13689 (2014).



b

Enzyme	Enzymatic assays, IC_{50} (M)			
	GSK-J1	GSK-J2	GSK-J4	GSK-J5
KDM2B	2.1×10^{-5}	8.3×10^{-6}	2.1×10^{-6}	2.4×10^{-5}
KDM3A	$> 7.5 \times 10^{-5}$ *	$> 1.0 \times 10^{-4}$ *	8.5×10^{-6} *	$> 1.0 \times 10^{-4}$ *
KDM3B	$> 7.5 \times 10^{-5}$	$> 1.0 \times 10^{-4}$ *	6.9×10^{-6} *	$> 1.0 \times 10^{-4}$ *
KDM4A	$> 5.0 \times 10^{-5}$	$> 1.0 \times 10^{-4}$ *	7.5×10^{-6} *	$> 1.0 \times 10^{-4}$ *
KDM4B	7.3×10^{-5}	$> 1.0 \times 10^{-4}$ *	3.8×10^{-6} *	$> 1.0 \times 10^{-4}$ *
KDM4C	3.4×10^{-5}	$> 1.0 \times 10^{-4}$	5.5×10^{-6}	$> 1.0 \times 10^{-4}$
KDM5A	6.8×10^{-6} *	$> 2.0 \times 10^{-5}$ *	ND	$> 5.0 \times 10^{-5}$ *
KDM5B	1.7×10^{-7}	3.3×10^{-5}	9.7×10^{-6}	$> 2.5 \times 10^{-5}$
KDM5C	5.5×10^{-7}	$> 7.5 \times 10^{-5}$ *	1.5×10^{-5}	$> 1.0 \times 10^{-4}$ *
KDM6A	5.3×10^{-8}	$> 1.0 \times 10^{-4}$	6.6×10^{-6}	$> 1.0 \times 10^{-4}$
KDM6B	2.8×10^{-8}	4.9×10^{-5} *	8.6×10^{-6} *	$> 1.0 \times 10^{-4}$ *
PHF8	2.8×10^{-5} *	1.7×10^{-5} *	4.2×10^{-6}	$> 2.5 \times 10^{-5}$

n = 3 unless otherwise specified. **n* = 2.

d

Enzyme	Cell based assays, IC_{50} (M)			
	GSK-J1	GSK-J2	GSK-J4	GSK-J5
KDM4C	ND	ND	7.3×10^{-6}	$> 1.0 \times 10^{-5}$ *
KDM5B	ND	ND	3.1×10^{-6}	$> 1.0 \times 10^{-5}$ *
KDM6B	1.7×10^{-5} *	$> 1.0 \times 10^{-4}$ *	3.1×10^{-6}	$> 2.5 \times 10^{-5}$

n = 2 unless otherwise specified. **n* = 1.

Figure 1 | GSK-J1/J4 inhibition of several histone demethylase subfamilies. **a**, Assessment of the inhibitory potential of GSK-J1 towards the indicated jumonji enzymes by AlphaLISA based assays. **b**, Inhibitory potential of GSK-J1, GSK-J2, GSK-J4 and GSK-J5 towards the indicated enzymes as assessed by AlphaLISA based assays. IC_{50} values are indicated as means. The deviation of the mean was always less than twofold. ND, not determined. *n* equals the number of replicates. **c**, Assessment of inhibitory potential of GSK-J4 in cell-based assays in which the indicated enzymes were transfected, and their activity measured by induced loss of H3K27me2 (KDM6B), H3K4me2 (KDM5B) and H3K9me3 (KDM4C). **d**, Inhibitory activity of the indicated compounds towards the indicated enzymes in cell-based assays. IC_{50} values are indicated as mean, and the deviation from the mean was always less than twofold. ND, not determined.

The jumonji demethylases are dependent on two cofactors, 2-oxoglutarate (also known as α-ketoglutarate) and Fe^{2+} for enzymatic activity. The compound published by Kruidenier *et al.*[1], GSK-J1 is a competitive inhibitor of the two cofactors, but not of the substrate, with a half-maximum inhibitory concentration (IC_{50}) of 60 nM towards KDM6B as measured in an AlphaScreen assay. By performing *in vitro* assays on a number of other jumonji demethylases, including the closely related JMJD2/KDM4 subfamily and 160 other proteins, Kruidinier *et al.*[1] concluded that GSK-J1 is specific for the KDM6 subfamily. However, we noted that GSK-J1 was not tested on the JARID1/KDM5 subfamily, which contains the four demethylases with the closest homology in the catalytic domain to KDM6B and KDM6A (ref. 3). As shown in Fig. 1a, b, we tested the inhibitory activity of GSK-J1 towards 12 different jumonji demethylases. In agreement with the published data[1], our results show that GSK-J1 is a highly potent inhibitor of KDM6B and KDM6A. Moreover, and also in agreement with Kruidenier *et al*[1], the other tested demethylases, except for KDM5B and KDM5C, were only marginally or not significantly inhibited *in vitro*. However, our results show that GSK-J1 only is fivefold to tenfold more potent towards KDM6B and KDM6A as compared to KDM5B and KDM5C. As a control for these experiments, we used GSK-J2, an isomer of GSK-J1 that does not have any specific activity[1]. Taken together, these results show that GSK-J1 is a potent inhibitor of jumonji proteins with activity towards H3K27me3/me2 (KDM6) and H3K4me3/me2 (KDM5) *in vitro*.

The highly polar GSK-J1 compound is restricted from entering into cells, and Kruidenier *et al.*[1] therefore changed the acid group in GSK-J1 and GSK-J2 to an ester, thereby generating GSK-J4 and GSK-J5, respectively[1]. In a mass-spectrometry based *in vitro* assay, GSK-J4 was shown to have an $IC_{50} > 50\ \mu M$[1]. In a more sensitive AlphaLISA assay, we found that GSK-J4 has half-maximum inhibitory concentration (IC_{50}) towards KDM6B and KDM6A of 8.6 μM and 6.6 μM, respectively (Fig. 1b). GSK-J4 was also found to inhibit the catalytic activity of the other tested demethylases with similar potency (Fig. 1b). Kruidenier *et al.*[1] did not report on the IC_{50} value of GSK-J4 towards different jumonji demethylases in transfected cells, however, they showed an IC_{50} value of 9 μM towards the production of TNF-α in lipopolysaccharide-stimulated macrophages. We tested the inhibitory effect of the four GSK compounds in cells transfected with KDM6B, KDM5B and KDM4C, respectively, and as shown in Fig. 1c, d, GSK-J4 shows very similar IC_{50} values towards the 3 demethylases, representing 3 different subfamilies. Taken together, our results show that GSK-J1 and GSK-J4 inhibit demethylases in addition to KDM6B and KDM6A. Therefore, this compound cannot be used alone for demonstrating a role for H3K27 demethylation in biological processes.

Methods

AlphaLISA assays were essentially performed as described in the protocol provided by the manufacturer (PerkinElmer). The enzymes used were: KDM2B (amino acids 1–650), KDM3A (amino acids 2–1,322), KDM3B (amino acids 842–1,761), KDM4A (amino acids 1–350), KDM4B (amino acids 2–500), KDM4C (amino acids 1–349), KDM5A (amino acids 1–1,090), KDM5B (amino acids 1–809), KDM5C (amino acids 2–1,560), KDM6A (amino acids 919–1,401), KDM6B (amino acids 1,043–1,643), and PHF8 (amino acids 1–1,024). Substrates and assay conditions can be provided upon request.

To measure the inhibitory activity of the tested compounds in cell-based assays, U2OS cells were transfected with epitope tagged versions of KDM6B (amino acids

1,026–1,682), KDM5B (amino acids 1–752) or full length KDM4C. Transfected cells were incubated with the indicated concentration of compounds, and the activity of the demethylase towards substrate in transfected cells was measured using antibodies specific for H3K27me2 (Abcam Ab24684), H3K4me2 (Milipore 07-030) and H3K9me3 (Abcam Ab8898).

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Author Contributions B.H., J.M.N. and H.R.H. contributed equally to this manuscript. B.H., J.M.N., H.R.H., M.J.L. and D.V.L. performed experiments and analysed data. T.B., M.L., L.-O.G., P.B. and K.H. analysed data. K.H. wrote the manuscript with input from the other authors.

Competing Financial Interests B.H., J.M.N., H.R.H., D.V.L, T.B., M.L., L.-O.G. and P.B. are employees of EpiTherapeutics Aps. K.H. is a co-founder, stockowner and consultant of EpiTherapeutics Aps. M.J.L. has no competing interests to declare.

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Kruidenier *et al.* reply

REPLYING TO B. Heinemann *et al. Nature* **514,** http://dx.doi.org/10.1038/nature13688 (2014)

We welcome the accompanying Comment[1] by Heinemann *et al.*, in which the authors use an extensive panel of sensitive KDM assays to independently confirm our results[2] that GSK-J1 is a potent KDM6 inhibitor. Additionally, Heinemann *et al.*[1] demonstrate that GSK-J1 has some, albeit weaker, activity towards KDM5B and KDM5C, for which we only had preliminary data available at the time of our original publication. As our jumonji assay portfolio expands, we have continued to update the GSK-J1 activity profile on the SGC website (http://www.thesgc.org/chemical-probes/GSKJ1); this includes KDM5 inhibition activity by GSK-J1 similar to that reported by Heinemann. In conclusion, GSK-J1 remains the most selective KDM inhibitor yet disclosed and thus a valuable chemical tool.

Heinemann *et al.*[1] also show a broader, weak micromolar KDM inhibitory activity of the ester pro-drug version of GSK-J1, GSK-J4. GSK-J4 is not itself a chemical tool for direct KDM inhibition, but was designed specifically to enable efficient intracellular delivery of GSK-J1 into macrophages. In our work, the intracellular conversion of ester pro-drug is complete within 15 min after which levels of intracellular GSK-J4 are negligible ([GSK-J4] = 150 nM; [GSK-J1] = 11.8 μM). This renders the activity profile of GSK-J4 irrelevant and the biological effects in macrophages will be exclusively driven by the activity of GSK-J1. For other cell systems, it is essential to assess the ability to convert GSK-J4 to GSK-J1 before conducting and interpreting biological studies.

Despite the refinement of the selectivity profile of GSK-J1, our conclusion that KDM6 enzymatic activity is a key determinant of lipopolysaccharide responses in macrophages stands and was independently verified using short interfering RNA (siRNA) mediated knockdown of KDM6 enzymes. GSK-J1 remains a useful chemical probe for studying the catalytic function of KDM6 and the additional KDM5 activity may provide new opportunities for its use.

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