

SUPPLEMENTARY FIGURES

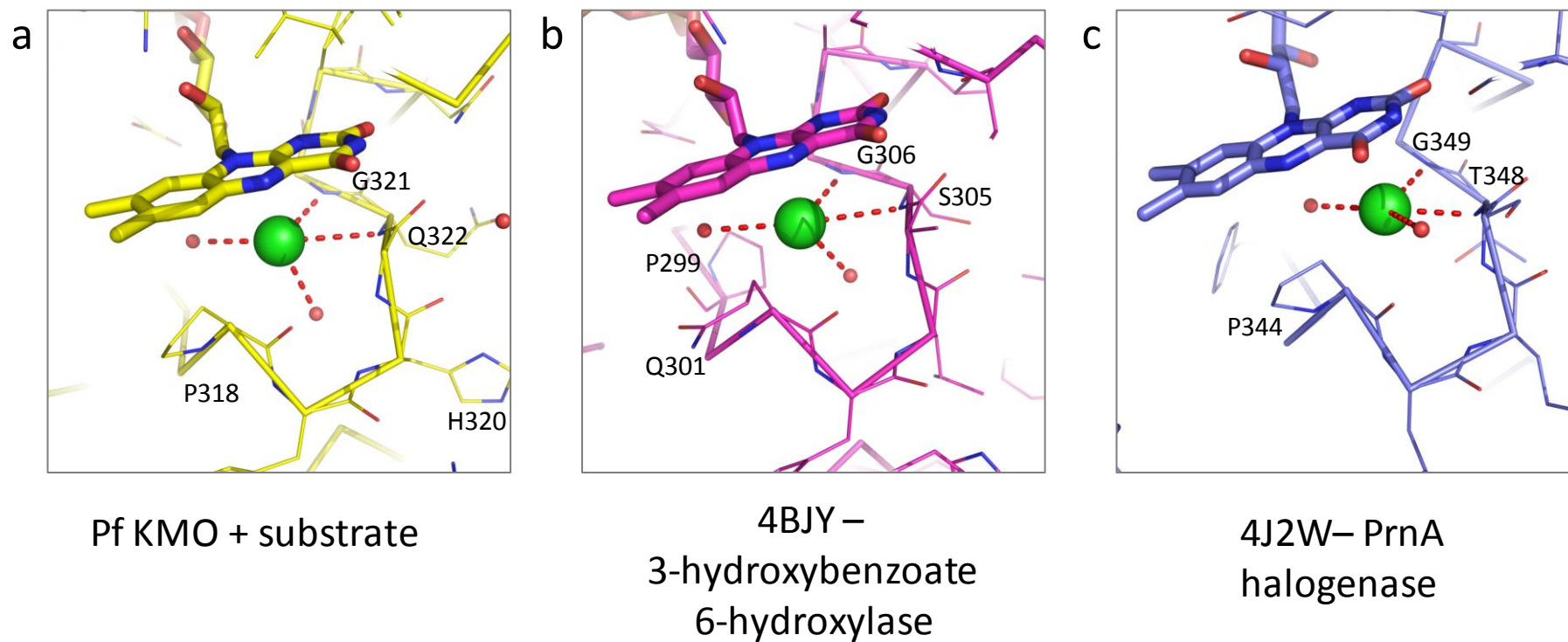
KMO protein sequence alignment (Boxed residues are within 4Å of UPF648 and are all conserved except Tyr 323)

1	MDSSVIQRKKVAVIGGGLVGS	LQACFLAKRNFQIDVYEARE	DRVATFTRG--RSINL	ALSHRGRQAL	Human_O15229							
1	-----MSESVAIIGAGLVGC	LAALAFSKEGYNVTLYDFRQDPR	LDTTKNKNLKSINLAI	SARGIDAL	Saccharomyces_P38169							
1	-MTATDNARQVTIIGAGLAGT	LVARLLARNGWQVNLFFERRPDPRI	ETGARG--RSINL	LAERGAHAL	Pseudomonas_Q84HF5							
					53							
67	KAVG--LEDQIVSQGIPMRAR	MIHSLSGKKSAPYG-TKSQY	ILSVSRENLNKD	LLTAAEKYPNVKMH	Human_O15229							
63	KSIDPDACEHILQDMIPMKGR	MIHDLKGRQESQLYG-LHGEA	INSINRSVNLNS	LLDELEKS-TTELK	Saccharomyces_P38169							
66	RLAG--LEREVLAEAVMMRGR	MVHVPGTTPPNLQPYGRDD	SEVWWSINRDR	LNRI	LLDGAEAA-GASIH	Pseudomonas_Q84HF5						
		83	97	104								
132	FNHRLKCNPEEG---MITV	LGSD-KVPKDVTC	DLIVGCDGAYS	TVRS	HLMKKPRFDYSQYIPHGYM	Human_O15229						
129	FGHKLVKIEWTDDKQICHFAI	GEDLKTPEHTEKYDFVI	GCDGAYSATRS	QMQRKVEMDF	SOEYMNLRVI	Saccharomyces_P38169						
131	FNLG	LDSVDFARQLT	LSNV	SGER---	LEKRFHLLIGADGCNSAVR	QAMASVVDLGEHLETPHGYK	Pseudomonas_Q84HF5					
						169						
196	ELTIPP-----KNGDYAME	PNYLHIWPRN	TFMMIALPNM	NKSFTCT	IMPFE-----FEKL	Human_O15229						
197	ELYIPPTTEEFKPNYGCNFAI	APDHLHIWPRHK	FMLIALANS	DGSGFTST	FGSKDQ-----ISDL	Saccharomyces_P38169						
195	ELQITP-----EASAQFN	LEPNALHIWPH	GDYMCIAL	ENLDRSET	VTLFLHHQSPAAQPASPCFAQL	Pseudomonas_Q84HF5						
		221	230	232	246							
248	LTSN-DVVDFFQKYFPDAI	FLIGEKL	LVQDFLL	PAQPMISVK	CSSFHFKS-HCVLLGDA	AHAIVFEF	Human_O15229					
256	ITSKSRVREFLIENFPDI	INIMLDDAVKRF	ITYPKESLVC	VNCKPYD	PGGKAILLGDA	AHAMVFEF	Saccharomyces_P38169					
257	VDGH-AARRFFQRQFPDLS	FMLDS--LEQDFE	HHPTGKL	LATLRLTTW	HVGG-QAVLLGDA	AHPMVEFH	Pseudomonas_Q84HF5					
							321/322/323					
314	GGMNA	GFEDCLV	DELMDKF	SNDLSLC	LPVFSRLRI	PDDHATSDLS	SMYNIEMRAHVNS	SWFIFQKN	Human_O15229			
324	GGMNC	GFEDVRI	LMALLK	KHSGDRS	RAFTEYTQ	TRHKDLV	SITELAKRNY	KEMSHDVT	SKRFLLRKK	Saccharomyces_P38169		
321	GGMNC	ALEDAVAL	AHLQSA	ADNAS-ALAA	FTAQRQ	PDALAIQAMA	LENYVEMS	SKVASPTY	LLERE	Pseudomonas_Q84HF5		
										324		
382	MERFL	HAIMPSTFI	PLYTMVTF	-SRIRY	HEAVQR	WHWQKKV	VINKGLFF	LGLSLI	ATSSTY	LLIHYMSPR	Human_O15229	
392	LDALF	SIIMKDK	WIPLYT	MISFRSDI	YSRALER	AGKQTRI	LKFL	ESLT	LGMLSIG	GGYKLFKFL	TRER	Saccharomyces_P38169
388	LGQ	IMAQRQ	PTRFIP	RYSMVTF	-SRLPYA	QAMARGQIQE	QLKFAVAN	HSDLT	SINL	DAVEHEVTRCL	Pseudomonas_Q84HF5	
											394	
449	SFL	RLRRP	WNWIA	HFRNTT	CFPAK	AVDSLE	QISNLI	SR			Human_O15229	
460	S										Saccharomyces_P38169	
455	PP	LSHLC									Pseudomonas_Q84HF5	

Residues deleted in yeast crystal structure construct

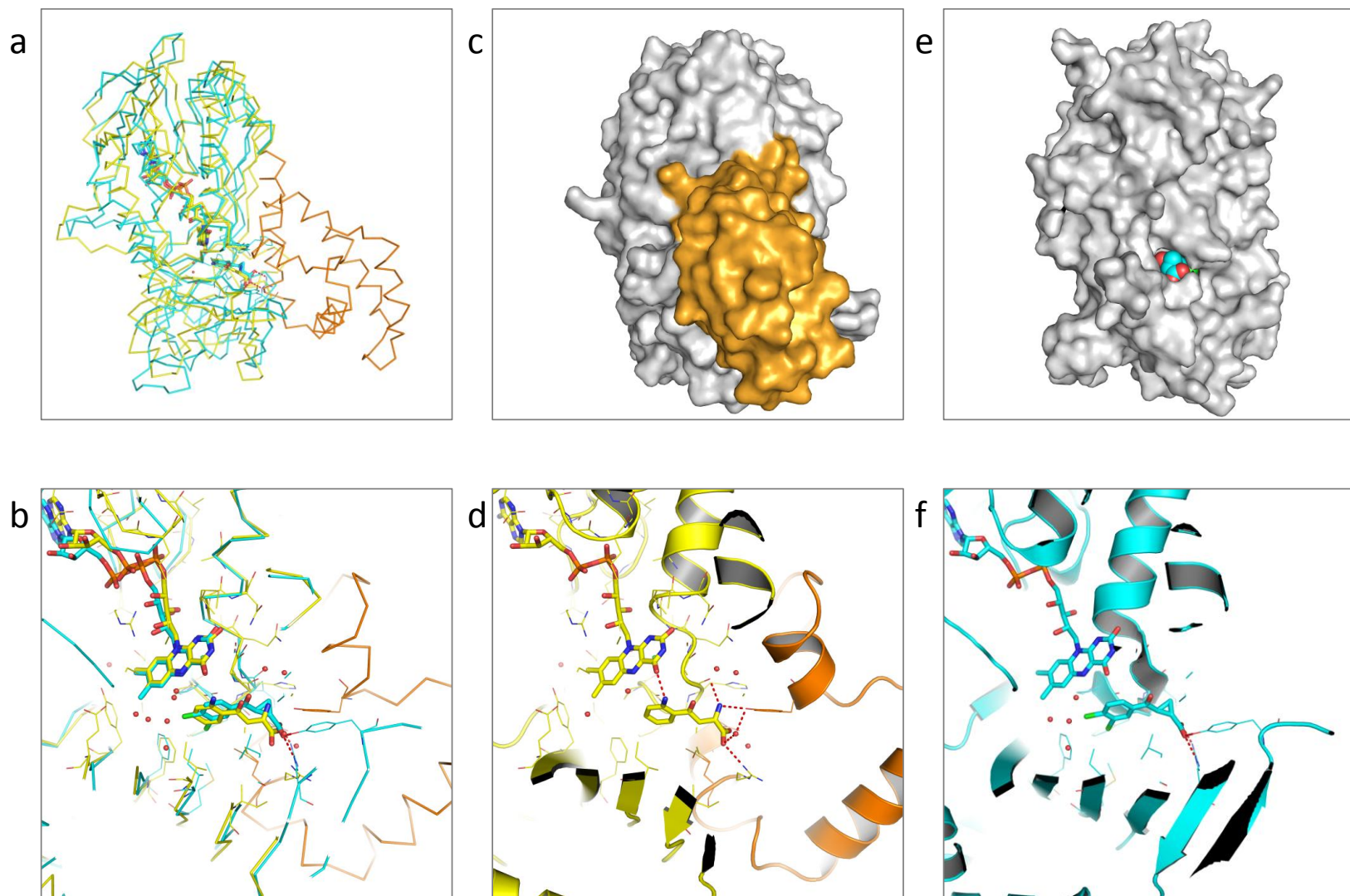
Sequence identities: human/yeast 38%, human/pseudomonas 35%, yeast/pseudomonas 35%

Supplementary Figure 1 Alignment of KMO sequences of Human, Scacharmyces and Pseudomonas fluorescens

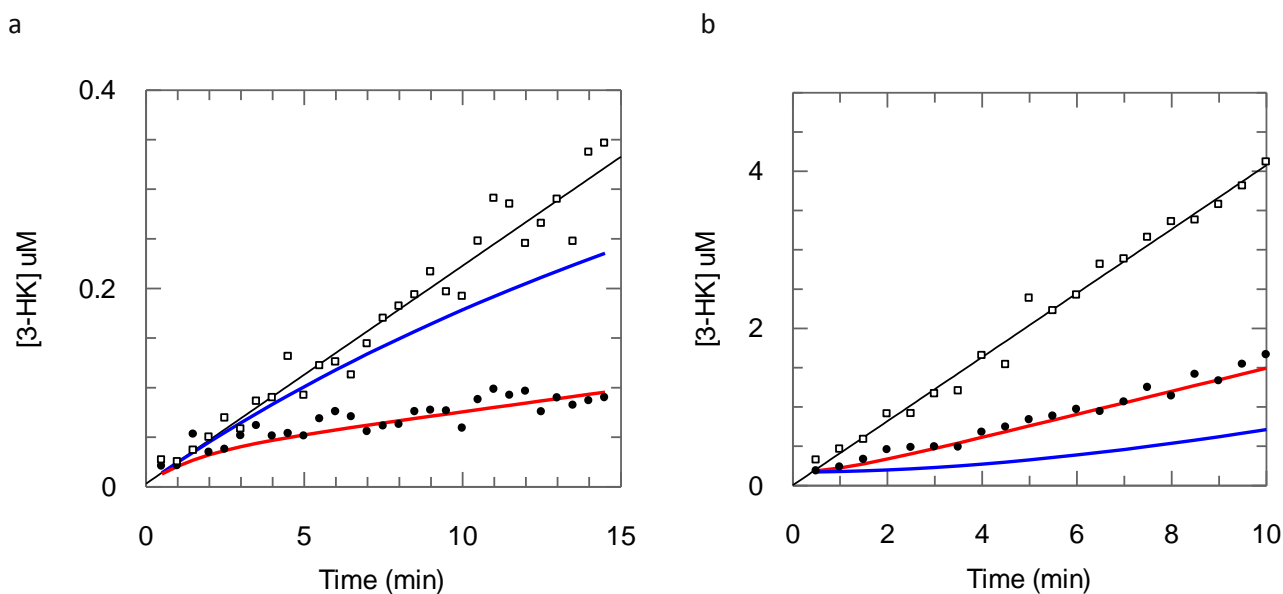


Supplementary Figure 2 Similarity of chloride ion proximal to catalytic flavin of three FAD-dependent enzymes

(a) Pf-KMO, **(b)** 3-hydroxybenzoate6-hydroxylase (PDB: 4BJY) and **(c)** PrnA halogenase (PDB: 4J2W)

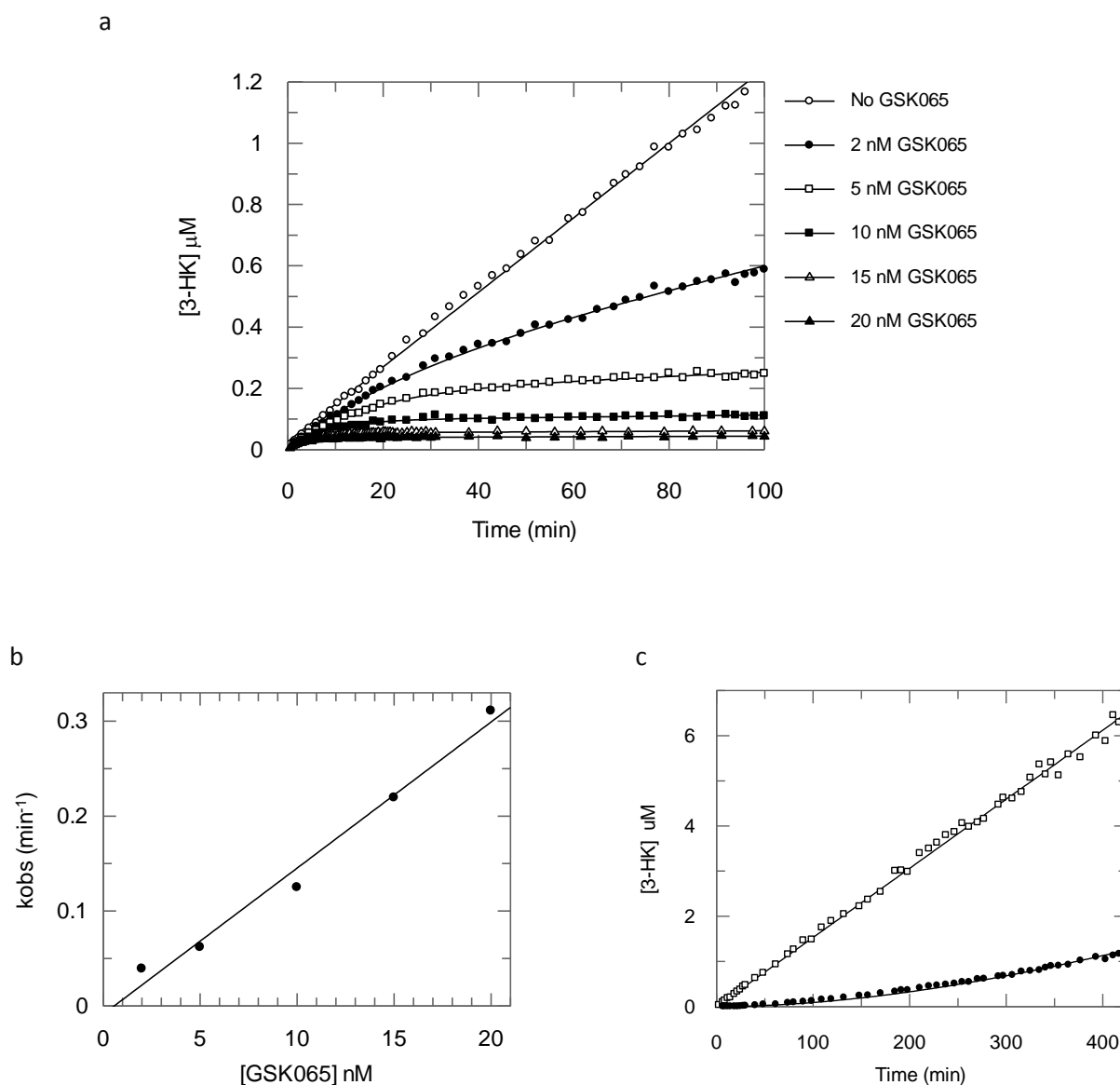


Supplementary Figure 3 Comparison of Pf-KMO-Kyn substrate complex with *Saccharomyces cerevisiae* KMO-394UPF inhibitor complex (cyan carbons, PDB 4J36)
(a) Structural overlay of yeast (cyan) and Pf structures (domain1 and 2 in yellow, domain 3 in orange)). Domain 3 is not present in the protein crystallised yeast structure.
(b) Enlarged view of (a) focused on the active site. **(c)** Pf-KMO-Kyn substrate complex shown as surface representation with orange colouring to highlight domain 3 **(d)** Enlarged view of the Pf-KMO active site of (c) without protein surface **(e)** Yeast structure in the same orientation as (b) highlighting the substrate capping effect of domain 3 **(f)** Enlarged view of the active site of *Saccharomyces cerevisiae* (c) without protein surface.



Supplementary Figure 4. Simulations of association and dissociation timecourses for inhibition of KMO by GSK428(A2)

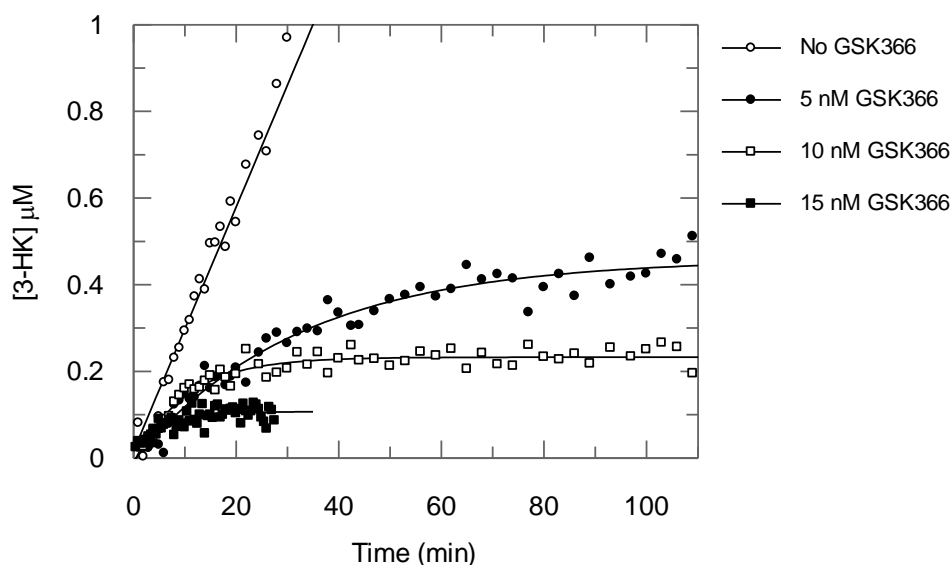
(a) Onset of inhibition for GSK428(A2). The uninhibited timecourse (open symbols) was fitted to a straight line to give the uninhibited rate ($V_i = 0.022 \mu\text{M}/\text{min}$), the gradient of the last 5 minutes of the timecourse in the presence of 10 nM GSK428(A2) (closed symbols) was taken as the steady-state rate ($V_s = 0.004 \mu\text{M}/\text{min}$). These two values were used to simulate timecourses for onset of inhibition with $k_{\text{obs}} = 0.062 \text{ min}^{-1}$ (blue) and $k_{\text{obs}} = 0.62 \text{ min}^{-1}$ (red), using Equation 1 (Supplementary Methods). **(b)** Dissociation of GSK428(A2). The uninhibited timecourse (open symbols) was fitted to a straight line to give the uninhibited rate, the gradient of the last 5 minutes of the timecourse in the presence of GSK428(A2) (closed symbols) was taken as the steady-state rate after equilibration of inhibitor binding ($V_s = 0.147 \mu\text{M}/\text{min}$). This value was used to simulate timecourses for recovery of activity with $k_{\text{obs}} = 0.1 \text{ min}^{-1}$ (blue) and $k_{\text{obs}} = 1.0 \text{ min}^{-1}$ (red), using Equation 1 with V_i set to zero.



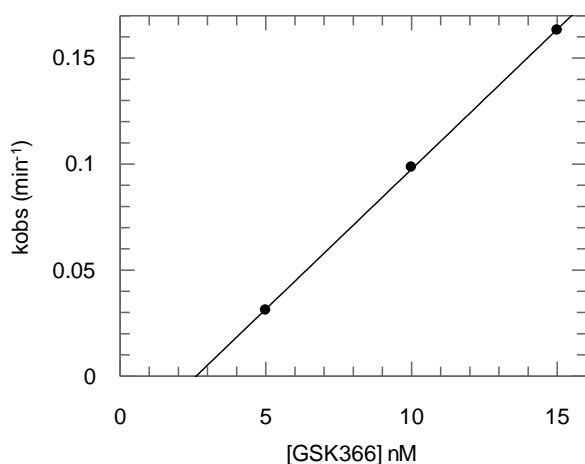
Supplementary Figure 5. Detailed kinetic analysis of inhibition of KMO by GSK065(C1)

(a) Onset of inhibition timecourses measured in a range of concentrations of GSK065(C1). k_{obs} at each inhibitor concentration was obtained by fitting timecourses to Equation 1 (Supplementary Methods). (b) Plot of the resulting k_{obs} values against [GSK065(C1)]; k_{on} was obtained from a linear fit of the data. $k_{\text{on}} = 6.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (average of two determinations). (c) Dissociation of GSK065(C1) from KMO. Recovery of activity timecourse was measured after adding a high concentration of L-Kyn to a pre-equilibrated mixture of KMO and inhibitor (closed symbols). [GSK065(C1)] = 5 nM after addition of L-Kyn. The uninhibited timecourse was generated in an equivalent way, omitting the inhibitor (open symbols). k_{off} for the inhibitor was obtained by simultaneously fitting both timecourses to a competitive binding model (Supplementary Methods), with k_{on} fixed at $6.3 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ (see above). $k_{\text{off}} = 3.2 \times 10^{-5} \text{ s}^{-1}$ (average of two determinations).

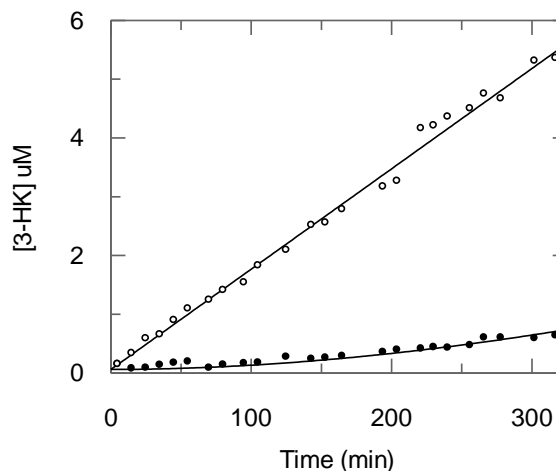
a



b

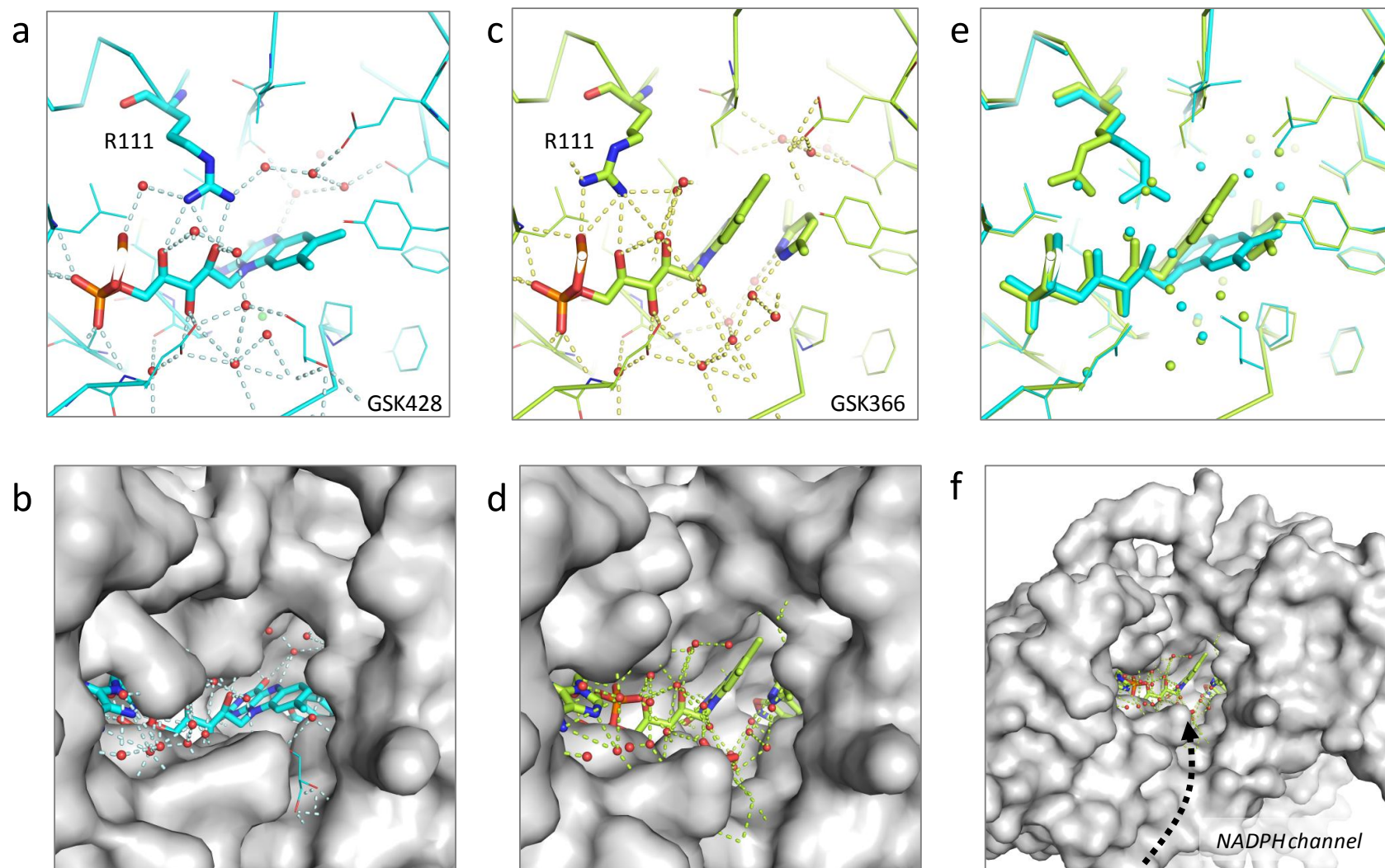


c



Supplementary Figure 6. Detailed kinetic analysis of inhibition of KMO by GSK366(C2)

(a) Onset of inhibition timecourses measured in a range of concentrations of GSK366(C2). k_{obs} at each inhibitor concentration was obtained by fitting timecourses to Equation 1 (Supplementary Methods). (b) Plot of the resulting k_{obs} values against [GSK366(C2)]; $k_{\text{on}} = 1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ was obtained from a linear fit of the data. (c) Dissociation of GSK366(C2) from KMO. A recovery of activity timecourse was measured after adding a high concentration of L-Kyn to a pre-equilibrated mixture of KMO and inhibitor (closed symbols). [GSK366(C2)] = 5 nM after addition of L-Kyn. The uninhibited timecourse was generated in an equivalent way, omitting the inhibitor (open symbols). k_{off} for the inhibitor was obtained by simultaneously fitting both timecourses to a competitive binding model (Supplementary Methods), with k_{on} fixed at $1.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$ (see above). $k_{\text{off}} = 1.6 \times 10^{-5} \text{ s}^{-1}$ (average of two determinations).

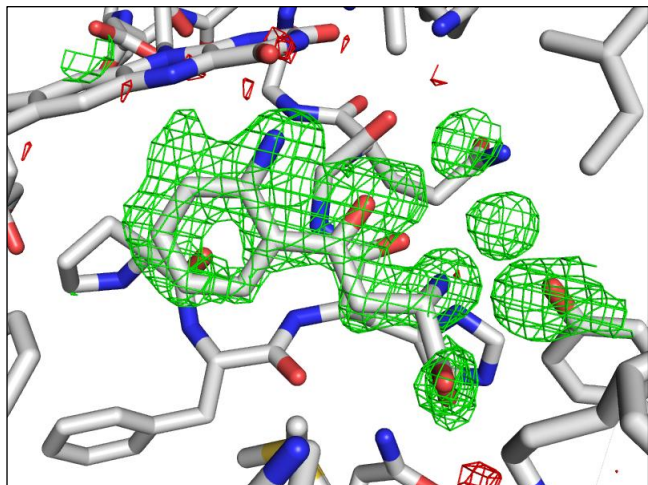


Supplementary Figure 7. Comparison of Pf-KMO complexes of GSK428 (cyan) and GSK366(C2) (pale green) viewed from solvent channel and putative NADPH access site

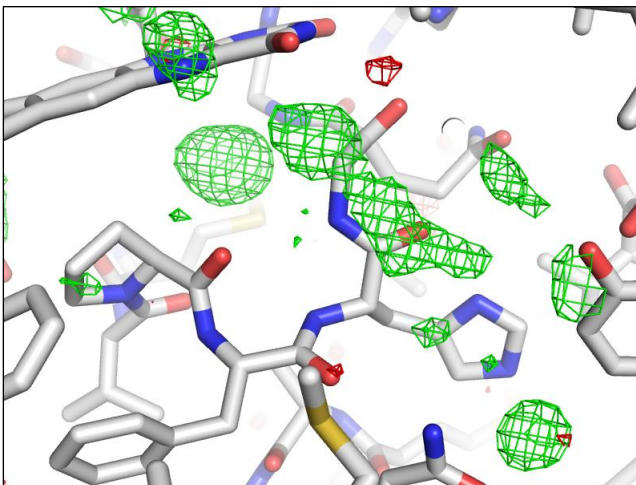
(a-b) Enclosed active site of GSK428(A2) with flavin in *in* position, substrate is not visible from solvent channel **(c-d)** Same views as (a-b) but using GSK366(C2) complex **(e-f)** Overlay of structures of GSK428(A2) (cyan) and GSK366(C2)(pale green). Movement of R111 visible.

L-kynurenine complex

A subunit

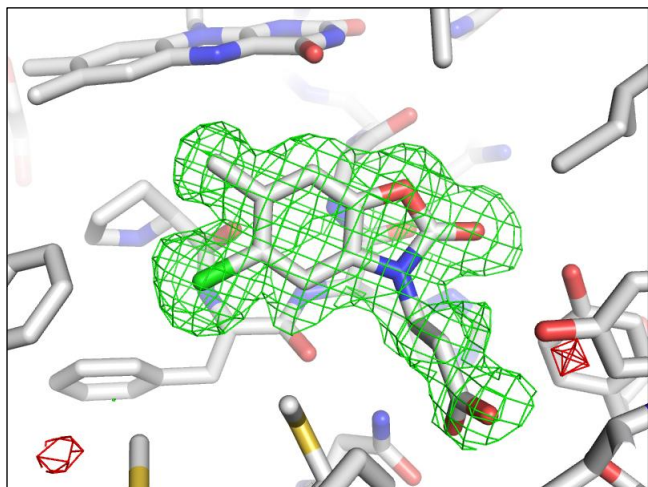


B subunit (ligand not bound)

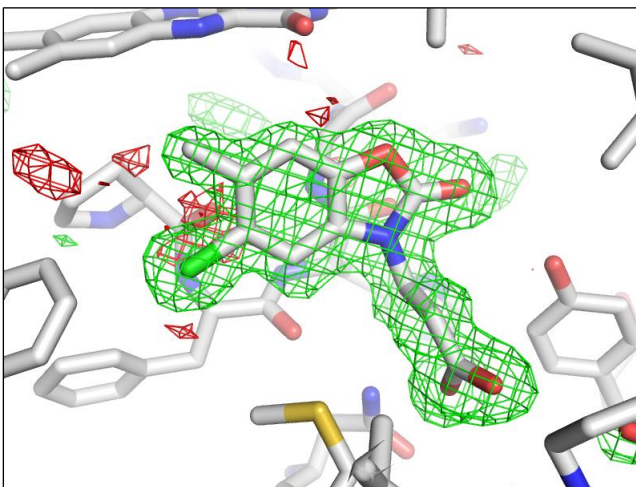


GSK428(A2) complex

A subunit



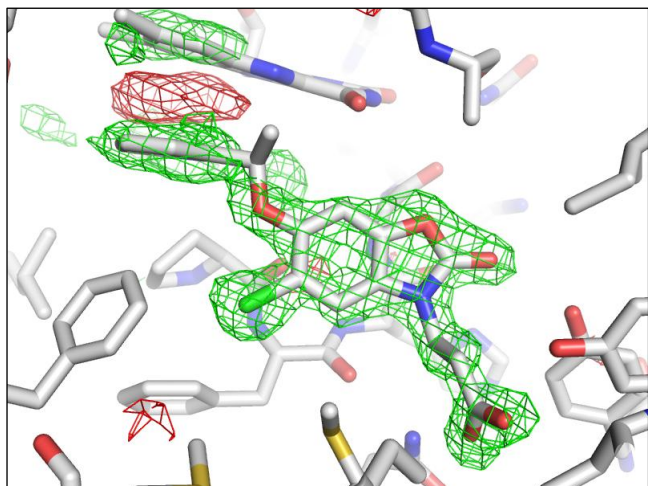
B subunit

**Supplementary Figure 8. Electron density difference maps in the inhibitor binding sites.**

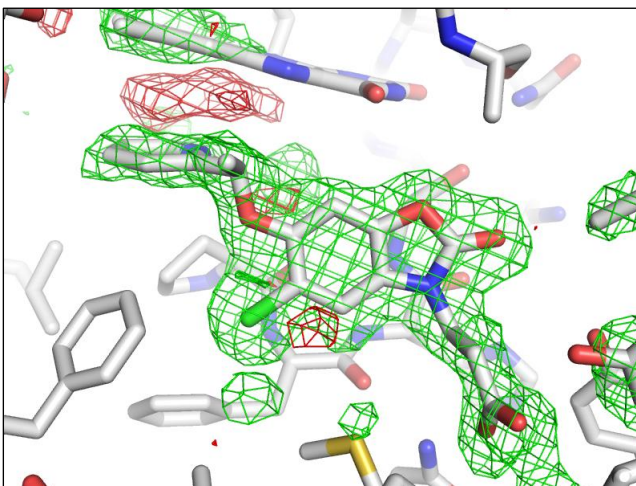
Initial $F_o - F_c$ electron density maps contoured at 3σ (green) and -3σ (red) superposed on the refined protein-inhibitor complex structures. Maps calculated after preliminary refinement of an unliganded protein model against the structure factors of the protein-inhibitor complex.

GSK775(B2) complex

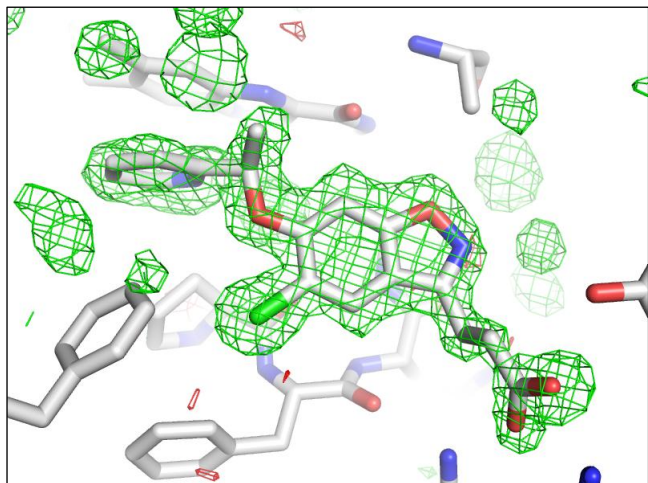
A subunit



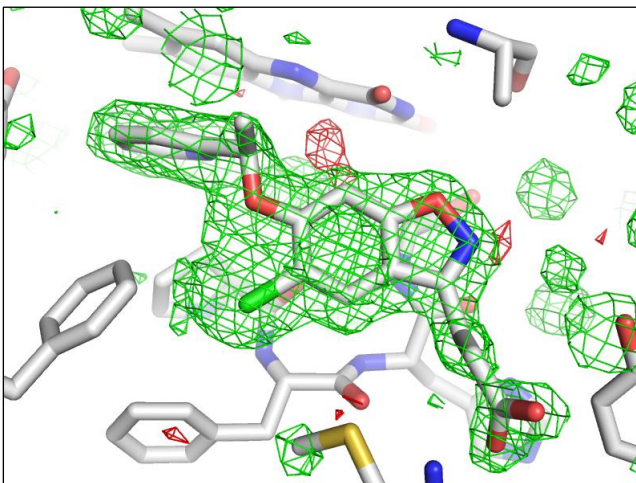
B subunit

**GSK065(C1) complex**

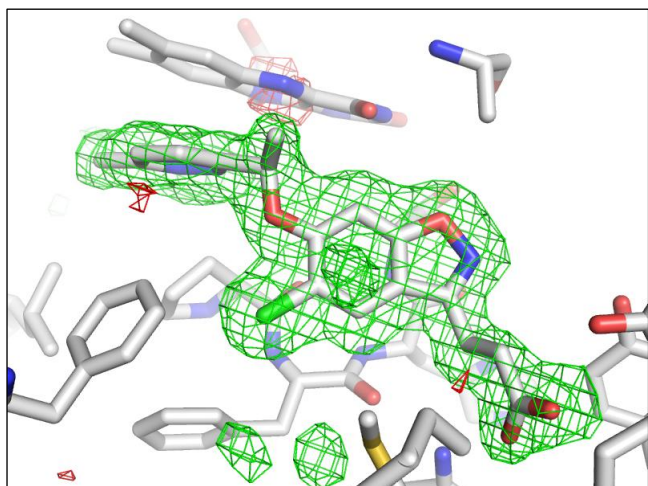
A subunit



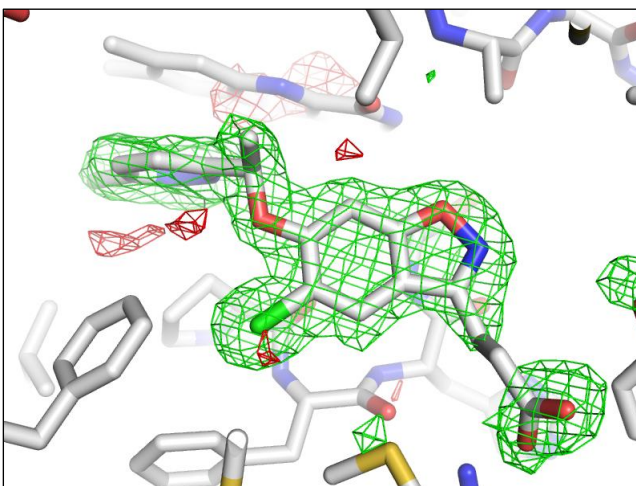
B subunit

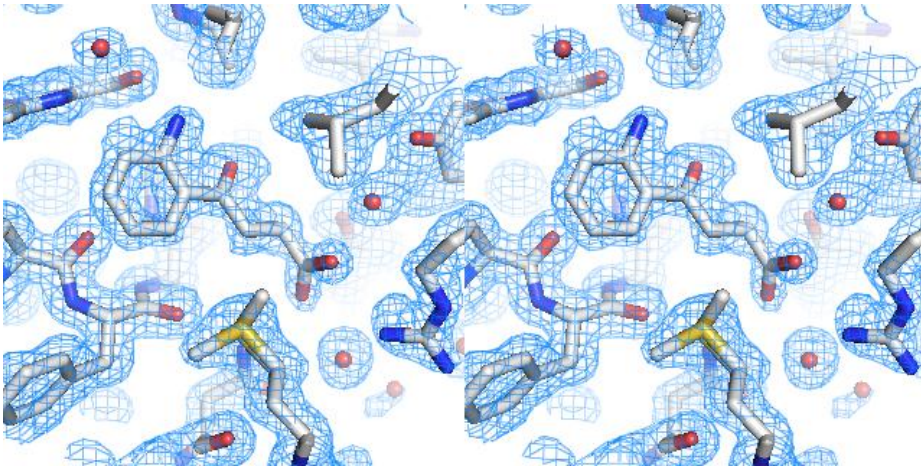
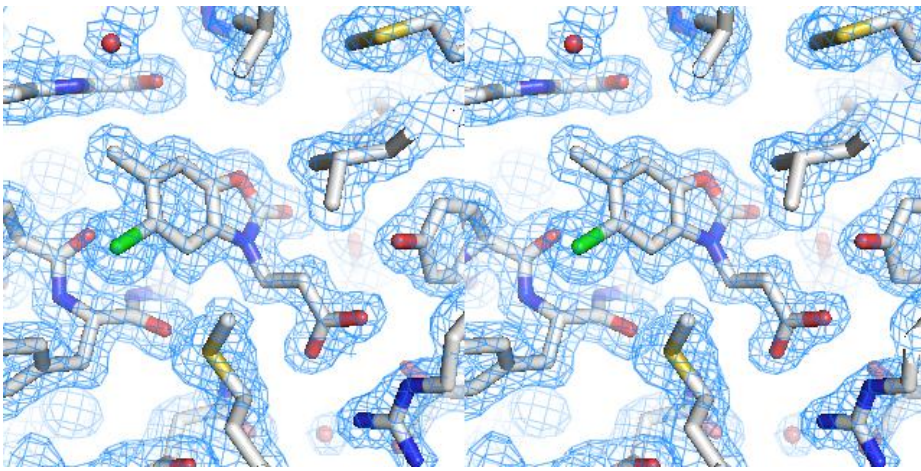
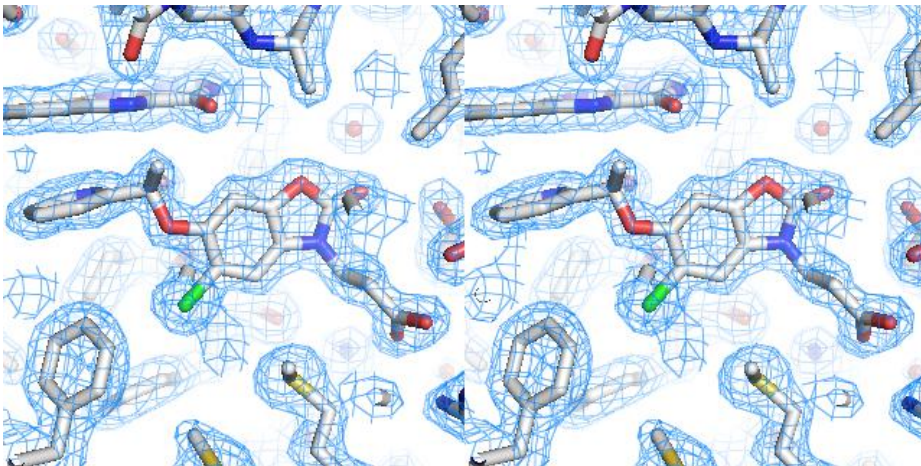
**GSK366(C2) complex**

A subunit



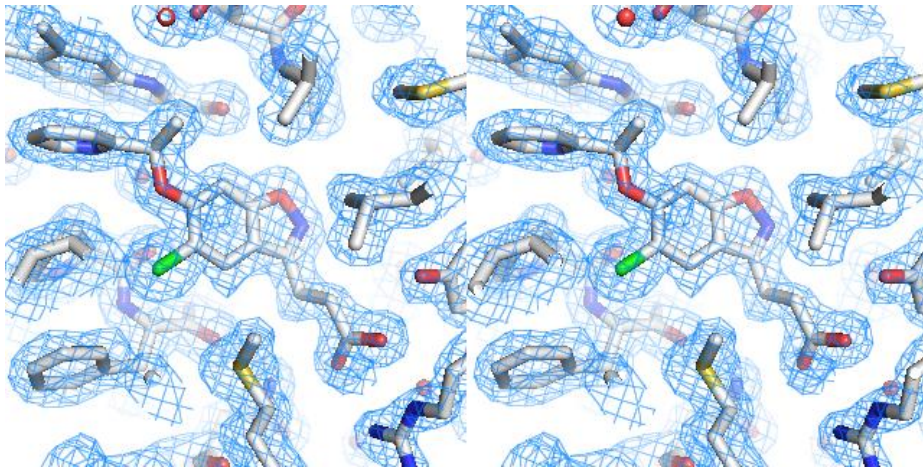
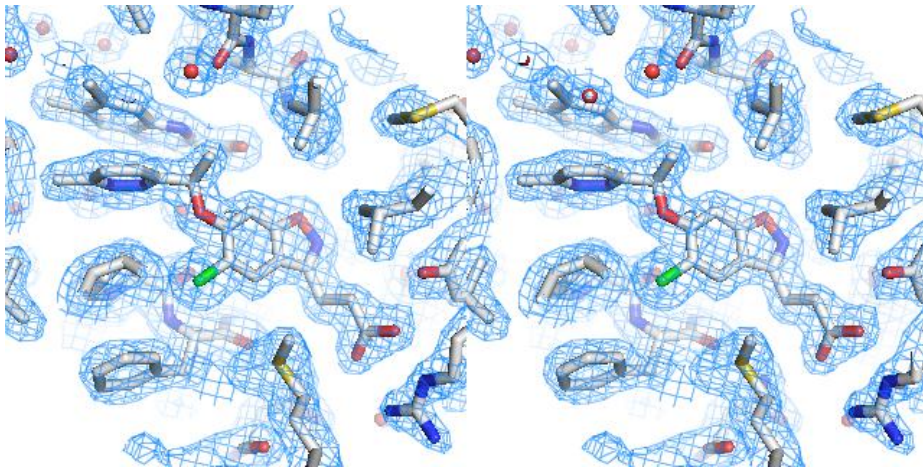
B subunit

**Supplementary Figure 8 cont. Electron density difference maps in inhibitor binding sites.**

L-kynurenine complex (A subunit)**GSK428(A2) complex (A subunit)****GSK775(B2) complex (A subunit)**

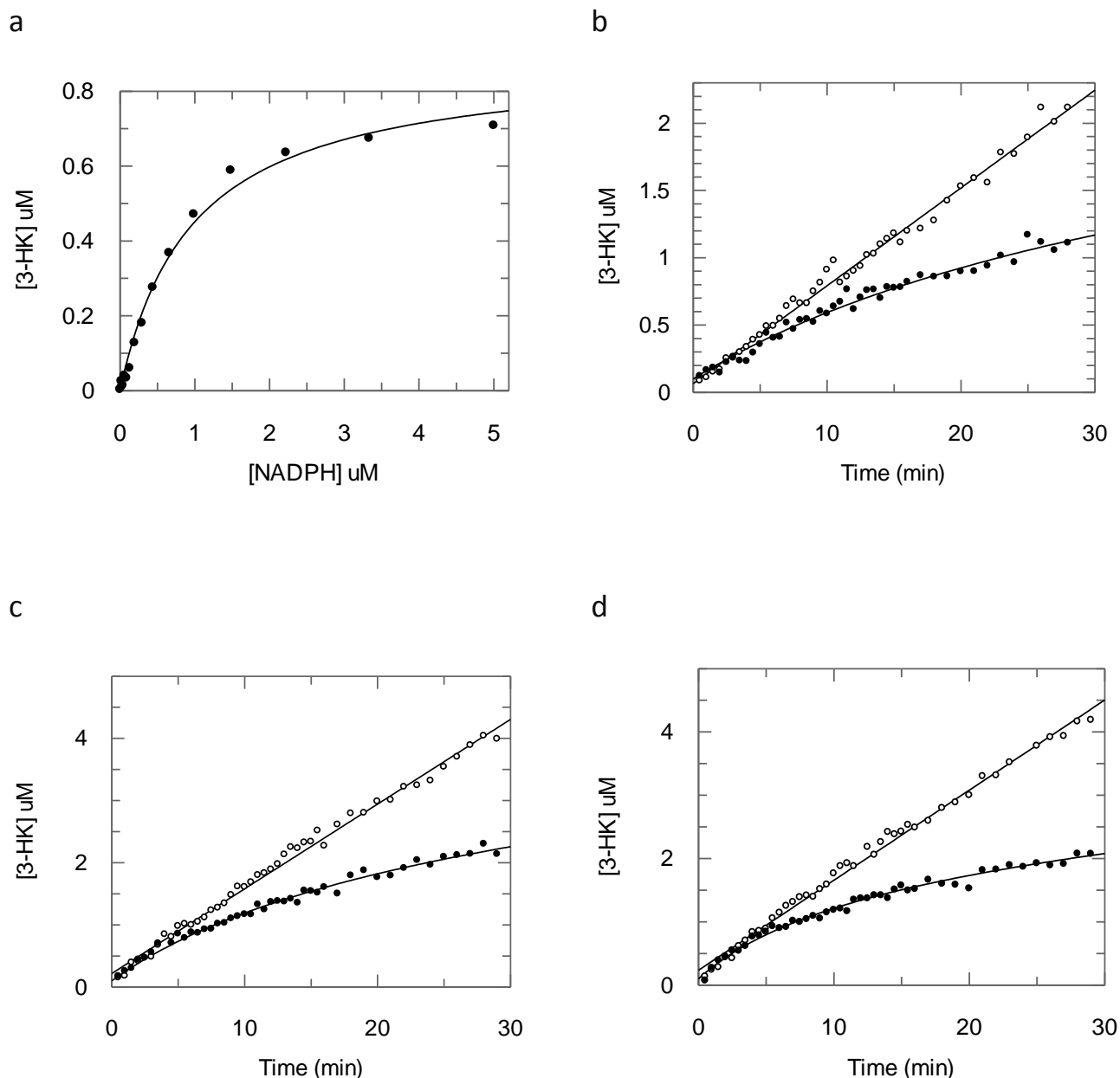
Final $2F_o - F_c$ electron density maps contoured at 1σ (blue) superposed on the coordinates of the corresponding fully refined crystal structure.

Supplementary Figure 8 cont. Stereoimages of ligand density.

GSK065(C1) complex (A subunit)**GSK366(C2) complex (A subunit)**

Final $2F_o - F_c$ electron density maps contoured at 1σ (blue) superposed on the coordinates of the corresponding fully refined crystal structure.

Supplementary Figure 8 cont. Stereoimages of ligand density.



Supplementary Figure 9. Binding kinetics of GSK775(B2) at sub-saturating [NADPH]

(a) Determination of K_M for NADPH against human KMO, at 10 μM Kynurenine. An NADPH regeneration system was used as described in Lowe et al. (2014)¹. Data were fitted to the Michaelis-Menten equation with $K_M = 1.0$ μM . **(b-d)** Onset of inhibition timecourses (closed symbols) measured with 10 nM GSK775(B2) at different concentrations of NADPH. k_{obs} values were obtained by fitting to an equation describing onset of inhibition under non-pseudo first order conditions. The corresponding uninhibited timecourses are shown with open symbols and were fitted to a straight line. **(b)** 0.3 μM NADPH, $k_{\text{obs}} = 0.013$ min^{-1} ; **(c)** 1 μM NADPH, $k_{\text{obs}} = 0.035$ min^{-1} ; **(d)** 5 μM NADPH, $k_{\text{obs}} = 0.069$ min^{-1} .

SUPPLEMENTARY TABLES

Supplementary Table 1. X-ray data collection and refinement statistics.

	Apo	L-kynurenine	GSK428(A2)
Data collection			
Beam line/detector	Diamond IO4-1/Pilatus 6M	Diamond IO4-1/Pilatus 6M	ESRF ID30B/Pilatus 6M
Space Group	P2 ₁	P2 ₁	P2 ₁
Resolution (Å)	134-1.94 (1.98-1.94)	68-1.50 (1.58-1.50)	50-1.62 (1.63-1.62)
Observations	230137 (10056)	482557 (68538)	384501 (3269)
Unique reflections	71621 (3201)	155570 (22560)	116150 (1076)
Redundancy	3.2 (3.1)	3.1 (3.0)	3.3 (3.0)
Completeness (%)	96.4 (86.4)	99.7 (99.6)	95.9 (89.1)
Mean I/ σ	16.9 (2.5)	8.1 (1.7)	13.7 (1.9)
R _{merge}	0.052 (0.578)	0.062 (0.614)	0.057 (0.603)
CC _{1/2}	0.993 (0.542)	0.998 (0.495)	0.998 (0.725)
Refinement			
Resolution (Å)	134-1.94 (1.99-1.94)	68-1.50 (1.54-1.50)	50-1.63 (1.67-1.63)
Reflections	71566 (4680)	155537 (11388)	115718 (7959)
R _{work} /R _{free}	0.201/0.231 (0.280/0.323)	0.189/0.210 (0.246/0.264)	0.166/0.186 (0.246/0.266)
Total number of atoms	7725	8222	8200
Protein atoms	6993	7003	7000
Ligand atoms	120	135	190
Waters	612	1084	1010
Average B-factors (Å ²)			
Protein (main chain/side chain)	29.6/38.0	24.1/30.8	21.9/29.2
Ligand atoms	19.7	18.3	21.9
Waters	38.5	41.0	40.1
RMS deviations from ideal values			
Bond lengths (Å)	0.010	0.010	0.010
Bond angles (°)	0.96	0.96	0.97

Values for the highest resolution shell are given in parentheses

Supplementary Table 1 cont. X-ray data collection and refinement statistics.

	GSK775(B2)	GSK065(C1)	GSK366(C2)
Data collection			
Beam line/detector	ESRF ID30B/Pilatus 6M	ID23-1/Pilatus 6M	ID30A-1/Pilatus 2M
Space Group	P2 ₁	P2 ₁	P2 ₁
Resolution (Å)	67-1.76 (1.77-1.76)	50-1.68 (1.74-1.68)	49-1.75 (1.81-1.75)
Observations	305615 (3236)	362111 (27281)	328445 (30375)
Unique reflections	92637 (964)	108786 (10114)	95918 (9341)
Redundancy	3.3 (3.4)	3.3 (2.7)	3.4 (3.3)
Completeness (%)	98.0 (96.0)	99.1 (94.5)	99.8 (99.7)
Mean I/ σ	12.6 (2.0)	12.9 (1.6)	13.4 (1.5)
R _{merge}	0.057 (0.713)	0.052 (0.634)	0.051 (0.772)
CC _{1/2}	0.998 (0.670)	0.999 (0.621)	0.999 (0.611)
Refinement			
Resolution (Å)	67-1.76 (1.81-1.76)	50-1.68 (1.72-1.68)	45-1.75 (1.79-1.75)
Reflections	92615 (6545)	108768 (7465)	95896 (7054)
R _{work} /R _{free}	0.177/0.206 (0.216/0.237)	0.169/0.198 (0.267/0.289)	0.176/0.203 (0.233/0.260)
Total number of atoms	7985	8078	7989
Protein atoms	6972	7008	6991
Ligand atoms	156	154	174
Waters	857	916	824
<i>Average B-factors (Å²)</i>			
Protein (main chain/side chain)	32.0/39.4	25.9/33.7	34.1/42.3
Ligand atoms	26.1	18.7	24.7
Waters	45.0	42.1	48.9
<i>RMS deviations from ideal values</i>			
Bond lengths (Å)	0.010	0.010	0.009
Bond angles (°)	0.98	0.99	0.96

Values for the highest resolution shell are given in parentheses

Supplementary references

1. Lowe, D.M. et al. Lead discovery for human kynurenine 3-monooxygenase by high-throughput RapidFire mass spectrometry. *J Biomol Screen* **19**, 508-15 (2014).