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Supporting Information

Design and Synthesis of Hsp90 Inhibitors with B-Raf and PDHK1 Multi-Target Activity

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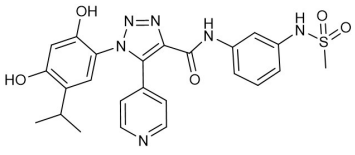
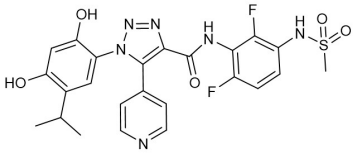
Figure S1: Chemical structures of the designed compounds.



4a



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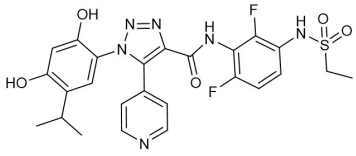

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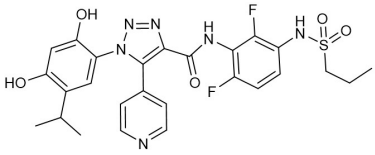

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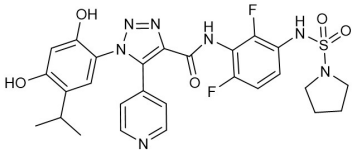
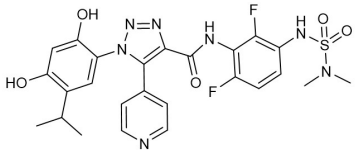


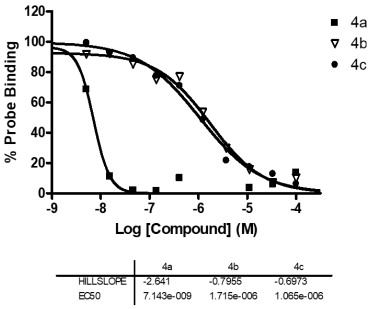
4e



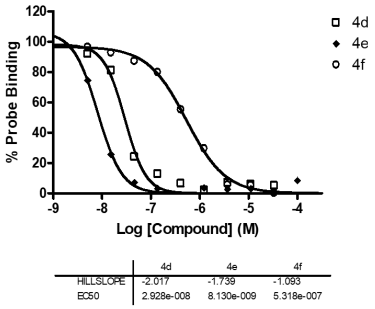
4f

Figure S2: Dose-response curves of compounds **4a-4e** and the controls tested for the inhibition of the three targets. Titration curves are not reported for compounds that resulted to be inactive in the assays.

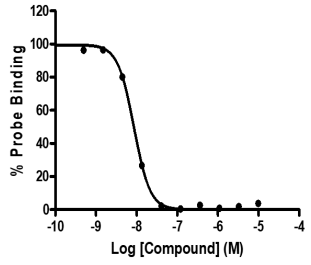
Hsp90



	4a	4b	4c
HILLSLOPE	-2.641	-0.7955	-0.6973
EC50	7.143e-009	1.715e-006	1.065e-006

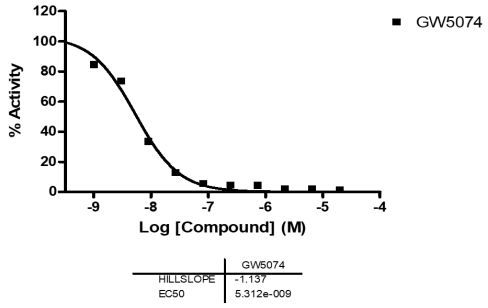


	4d	4e	4f
HILLSLOPE	-2.017	-1.739	-1.093
EC50	2.928e-008	8.130e-009	5.318e-007

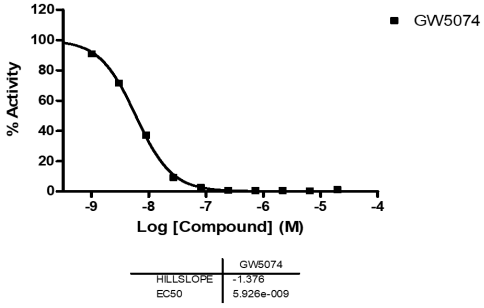


	Geldanamycin
HILLSLOPE	-2.200
EC50	8.607e-009

B-Raf^{WT} / B-Raf^{V600E}

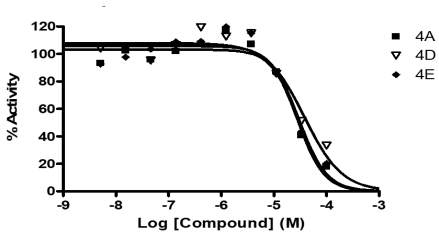


	GW5074
HILLSLOPE	-1.137
EC50	5.312e-009

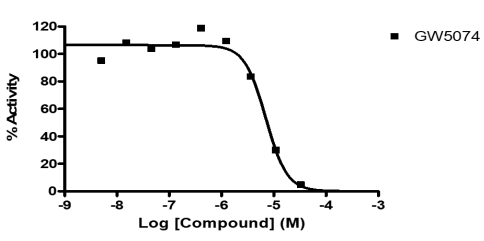


	GW5074
HILLSLOPE	-1.376
EC50	5.926e-009

PDHK1



	4A	4D	4E
HILLSLOPE	-1.732	-1.219	-1.628
EC50	2.870e-005	3.829e-005	2.940e-005



	GW5074
HILLSLOPE	-2.142
EC50	7.065e-006

Figure S3. Schematic representation of the main hydrogen bond interactions sampled by MD for the complexes Hsp90-**4a**, Hps90-**4b** and Hsp90-**4e** (left panels), and their corresponding representative binding modes (right panels). The 3D illustration of each ligand-protein complex was created with UCSF Chimera (version 1.14).

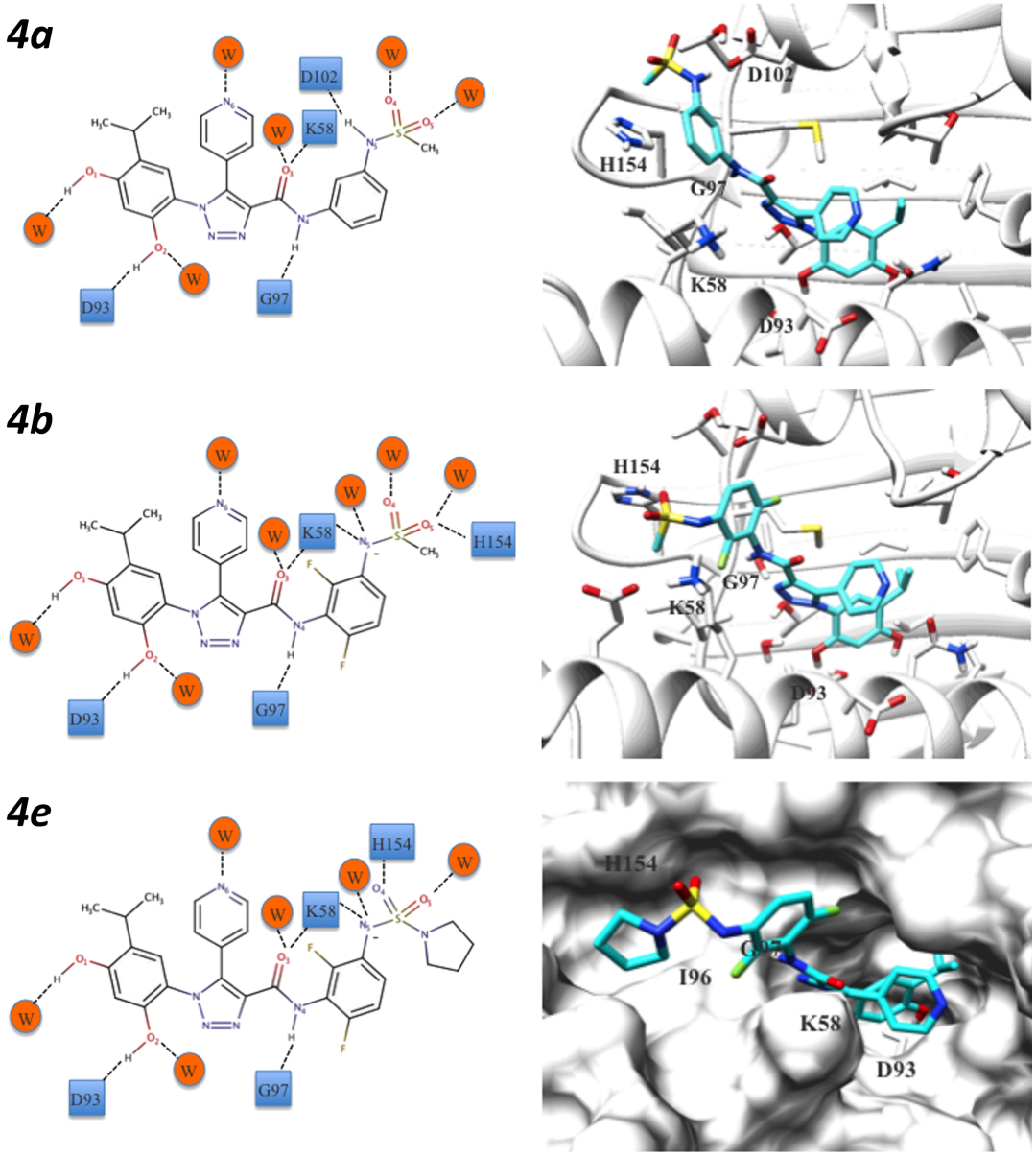


Figure S4: Schematic representation of the main hydrogen bonds observed between compound **4e** and B-Raf in the docked (panels *a* and *b*) and MD complexes (panel *c*). The docked ligand is shown in cyan in the *c* panel, while the ligand resulting from MD is represented as green sticks, their RMSD being higher than 6Å.

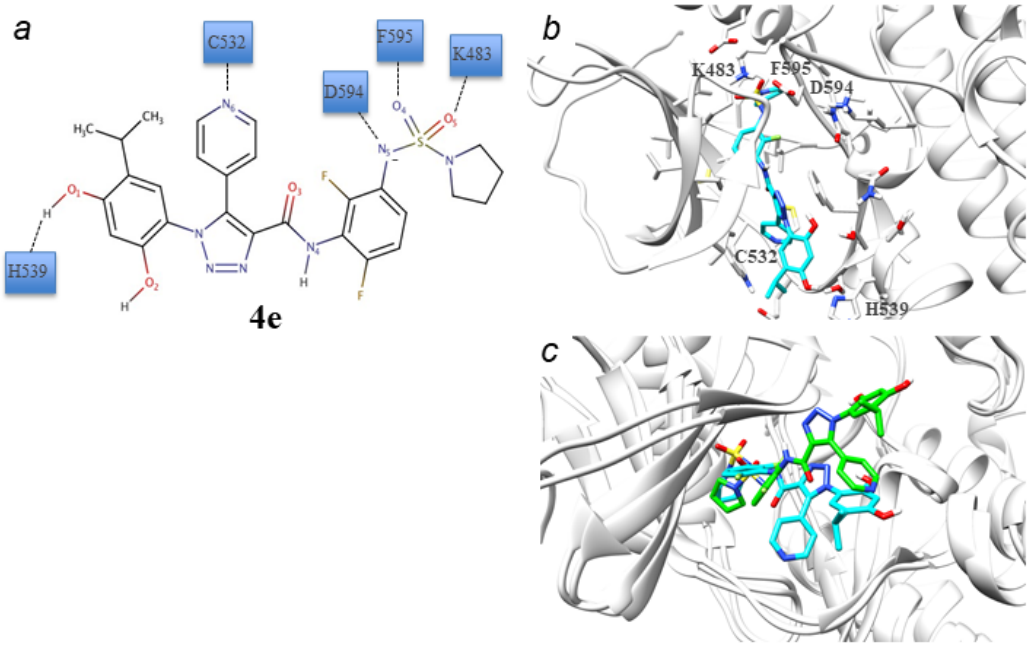


Figure S5. Schematic representation of the main hydrogen bond interactions sampled by MD of complexes PDHK1-**4a** and PDHK1-**4e** (left panels), and their corresponding representative binding modes (right panels). The 3D illustration of each ligand-protein complex was created with UCSF Chimera (version 1.14).

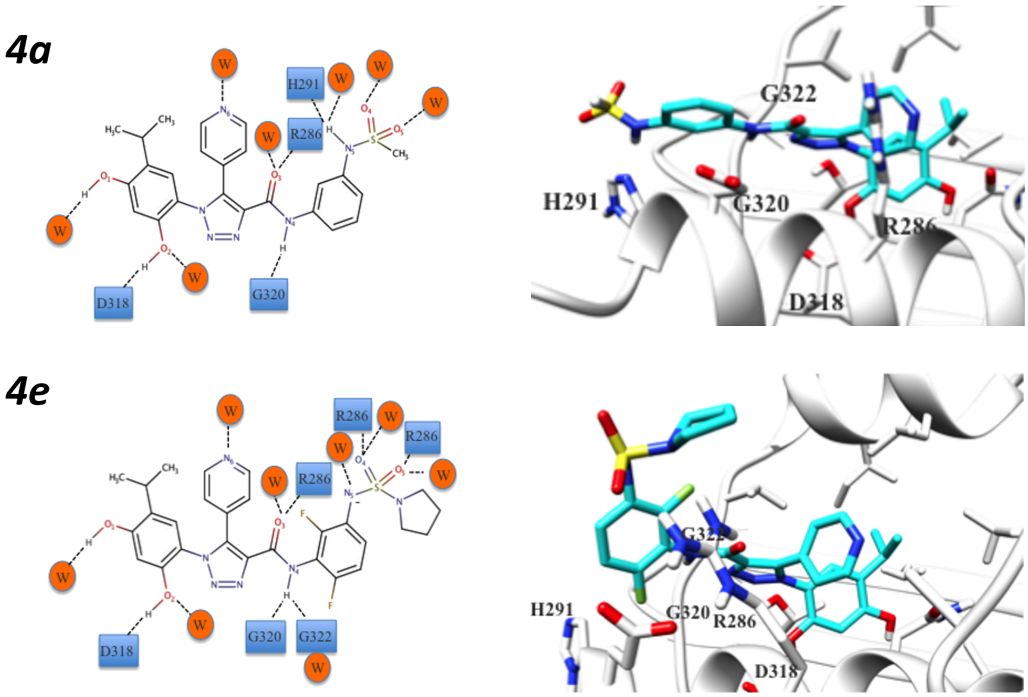


Table S1. pKa predictions made for compounds **4a-4f** with Epik (Schrödinger Suite 2020-1).

Compound ID	pKa
4a	8.1
4b	7.3
4c	7.3
4d	7.4
4e	6.8
4f	6.5

Table S2. IFD docking scores (kcal/mol) of compounds **4a-4f** into Hsp90α (PDB code 2VCI), B-Raf^{WT} (PDB code 3D4Q) and PDHK1 (PDB code 2Q8G).

Compound ID	Hsp90α	B-Raf^{wt}	PDHK1
4a	-11.7	-9.8	-10.5
4b	-10.8	-9.4	-9.5
4c	-8.7	-10.9	-8.7
4d	-11.1	-12.0	-9.1
4e	-11.0	-12.5	-10.1
4f	-11.0	-9.4	-9.0

Table S3. Results of the cytotoxicity predictions made with the CLC-pred webserver (A.A. Lagunin et al. DOI: https://doi.org/10.1371/journal.pone.0191838). Only cytotoxic activities predicted with a probability score Pa higher than 0.3 are reported in the table. The probability of a compound to be classified as not cytotoxic (*i.e.*, inactive) is represented by the Pi value. Compounds with a Pa value higher than the corresponding Pi can be considered as potentially cytotoxic for a given cancer cell line. Cytotoxic activities with Pa≥0.3 were not detected on normal human cell lines.

Compound ID	***Pa***	***Pi***	***Cell line ID***	***Cell line name***	***Tissue - Organ***
4a	0.592	0.011	NCI-H460	Non-small cell lung carcinoma	Lung
4a	0.375	0.095	NCI-H187	Small cell lung carcinoma	Lung
4a	0.361	0.164	SK-MEL-1	Metastatic melanoma	Skin
4a	0.366	0.171	NCI-H838	Non-small cell lung cancer. 3 stage	Lung
4a	0.307	0.129	U-266	Plasma cell myeloma	Blood
4b	0.502	0.02	NCI-H460	Non-small cell lung carcinoma	Lung
4b	0.304	0.146	A2058	Melanoma	Skin
4b	0.335	0.212	NCI-H838	Non-small cell lung cancer. 3 stage	Lung
4b	0.325	0.235	SK-MEL-1	Metastatic melanoma	Skin
4c	0.471	0.023	NCI-H460	Non-small cell lung carcinoma	Lung
4c	0.347	0.094	A2058	Melanoma	Skin
4d	0.432	0.038	A2058	Melanoma	Skin
4d	0.42	0.031	NCI-H460	Non-small cell lung carcinoma	Lung
4d	0.302	0.069	SK-MEL-28	Melanoma	Skin
4d	0.314	0.108	PA-1	Ovarian carcinoma	Ovarium
4d	0.314	0.258	SK-MEL-1	Metastatic melanoma	Skin
4e	0.4	0.035	NCI-H460	Non-small cell lung carcinoma	Lung
4e	0.308	0.087	HCT-116	Colon carcinoma	Colon
4e	0.317	0.251	SK-MEL-1	Metastatic melanoma	Skin
4f	0.396	0.036	NCI-H460	Non-small cell lung carcinoma	Lung
4f	0.369	0.059	HCT-116	Colon carcinoma	Colon
4f	0.309	0.269	SK-MEL-1	Metastatic melanoma	Skin

Table S4. Percentages of occurrence and hydrogen bonding distances between Hsp90 and ligands **4a, 4b** and **4e** in the 100 ns molecular dynamics simulations.

Hydrogen bonds with protein amino acids	*Occur. (%)*	*Dist. (Å)*	*Hydrogen bonds with waters*	*Occur. (%)*	*Dist. (Å)*
			4a		
O_2H----D93	99.5	2.7	O_4---Wat	106	2.8
N_5H----D102	94	2.8	O_1H---Wat	93	2.8
N_4-H----G97	80	2.8	O_5---Wat	87	2.8
O_3---K58	5	2.8	N_6---Wat	66	2.8
O_3---Wat	120		O_2---Wat	38	2.8
			4b		
O_2H----D93	99.6	2.7	O_5---Wat	162	2.8
N_4-H----G97	36	2.9	O_3---Wat	96	2.8
O_3----K58	17	2.8	O_1H---Wat	92	2.8
N_5----K58	11	2.9	N5---Wat	87	2.8
O_5---H154	7	2.9	N6---Wat	72	2.8
O_4---Wat	164	2.8	O_2---Wat	37	2.8
			4e		
O_2H----D93	99	2.7	O_4---Wat	130	2.8
N_4-H----G97	44	2.9	O_3---Wat	94	2.8
O_3----K58	23	2.8	O_1H---Wat	92	2.8
N_5----K58	16	2.9	N_5---Wat	75	2.8
O_4---H154	15	2.8	N_7---Wat	66	2.8
O_5---H154	7	2.9	O_2---Wat	37	2.8
O_5---Wat	176	2.8	N_6---Wat	36	2.8

Note: Hydrogen bonds with % occurrence < 5% are not reported. The hydrogen bond distances reported in Å are related to the distance between the donor and acceptor heteroatoms.

Table S5: Percentages of occurrence and hydrogen bonding distances between PDHK1 and ligands **4a** and **4e** in the 100 ns molecular dynamics simulations.

Hydrogen bonds with protein amino acids	***Occur. (%)***	***Dist. (Å)***	***Hydrogen bonds with waters***	***Occur. (%)***	***Dist. (Å)***
4a					
O_2H----D318	99.6	2.6	O_1H---Wat	94	2.8
O_3---R286	43	2.8	O_3---Wat	66	2.8
N_5H----H291	30	2.9	N_6---Wat	50	2.8
N_4-H----G320	4	2.9	N_5H---Wat	38	2.8
			O_2---Wat	19	2.8
			O_4---Wat	99.5	2.8
			O_5---Wat	96	2.8
4e					
O_2H----D318	99.6	2.6	O_4---Wat	141	2.8
O_5---R286	34	2.8	O_3---Wat	113	2.8
O_3----R286	15	2.8	O_1H---Wat	93	2.7
N_4H----G320	9	2.9	N_5---Wat	93	2.8
N_4H----G322	5	2.8	N_7---Wat	61	2.8
O_4---R286	5	2.8	N_6---Wat	36	2.8
			N_4H---Wat	22	2.8
			O_2---Wat	11	2.8
			O_5---Wat	146	2.8

Note: Hydrogen bonds with % occurrence < 5% are not reported. The hydrogen bond distances reported in Å are related to the distance between the donor and acceptor heteroatoms.

Chemoinformatic analyses

Chemoinformatic analyses were performed for selected compounds of PDHK1,[1] Hsp90[2] and B-Raf,[3-5] with the aim of identifying privileged substructures and chemical frameworks, useful for the design of multi-target ligands. Chemoinformatic analyses, which included 2D similarity estimations, were performed by using the RDKit Python libraries (https://www.rdkit.org - release 2020-9). In particular, 2D similarity estimations were performed with two different types of fingerprints (*i.e.,* MACCS and ECFP4) to take into account different aspects of chemical similarity. Records that provided similarity values above commonly accepted thresholds (*i.e*., MACCS ≥ 0.8 and ECFP4 ≥ 0.3) were retained and visually inspected.[6] Interestingly, the 2D fingerprints-based estimations performed between Hsp90 and PDHK1 selected ligands, highlighted several records with similarity values above threshold both in terms of MACCS and ECFP4 fingerprints (**Table A**), clearly demonstrating that these targets present overlapping chemical spaces.
Slightly different results were obtained in the comparisons of Hsp90 and PDHK1 ligands, with known B-Raf inhibitors, which provided a number of records with a high degree of ECFP4 similarity. These results showed that B-Raf ligands overall present similar structural connectivity, with respect to Hsp90 and PDHK1 known inhibitors, several of the observed ECFP4 values resulting above accepted thresholds in the analyses (**Table A**).

Table A: Top20- scoring 2D similarity records obtained from the comparison of selected Hsp90, PDHK1 ad B-Raf ligands. Ligands are reported by means of their identifiers in the ChEMBL database.[7]

Hsp90 (Target A) vs PDHK1 (Target B)			
Target A ligand	***Target B ligand***	***MACCSfp similarity score***	***ECFP4fp similarity score***
CHEMBL252164	CHEMBL252370	0.952	1
CHEMBL3234764	CHEMBL252370	0.817	1
CHEMBL3234768	CHEMBL252370	0.812	1
CHEMBL3234774	CHEMBL252370	0.775	1
CHEMBL3235113	CHEMBL252370	0.766	1
CHEMBL3235112	CHEMBL252370	0.757	1
CHEMBL3234767	CHEMBL252370	0.781	0.947
CHEMBL3234765	CHEMBL252370	0.781	0.947
CHEMBL3234766	CHEMBL252370	0.781	0.947
CHEMBL3234775	CHEMBL252370	0.764	0.946
CHEMBL3234773	CHEMBL252370	0.733	0.946
CHEMBL3234771	CHEMBL252370	0.781	0.921
CHEMBL3234776	CHEMBL252370	0.764	0.921
CHEMBL3234772	CHEMBL252370	0.743	0.919
CHEMBL3235115	CHEMBL252370	0.743	0.895
CHEMBL3235118	CHEMBL252370	0.756	0.875
CHEMBL3235108	CHEMBL252370	0.808	0.872
CHEMBL3234779	CHEMBL252370	0.746	0.872
CHEMBL3234778	CHEMBL252370	0.736	0.872
CHEMBL3235116	CHEMBL252370	0.761	0.868
Hsp90 (Target A) vs B-Raf (Target B)			
Target A ligand	***Target B ligand***	***MACCSfp similarity score***	***ECFP4fp similarity score***
CHEMBL3234763	CHEMBL524445	0.609	0.429
CHEMBL3234763	CHEMBL498344	0.627	0.4

CHEMBL3234763	CHEMBL521562	0.627	0.4
CHEMBL3234760	CHEMBL498487	0.636	0.372
CHEMBL3234761	CHEMBL498487	0.636	0.366
CHEMBL3235118	CHEMBL524445	0.6	0.36
CHEMBL3234762	CHEMBL497671	0.632	0.357
CHEMBL3234758	CHEMBL498487	0.636	0.341
CHEMBL3234763	CHEMBL498317	0.634	0.341
CHEMBL3234763	CHEMBL497671	0.6	0.341
CHEMBL3234759	CHEMBL498487	0.636	0.333
CHEMBL3234762	CHEMBL498487	0.623	0.333
CHEMBL3235114	CHEMBL1822255	0.64	0.321
CHEMBL3234763	CHEMBL524640	0.627	0.319
CHEMBL3234763	CHEMBL498343	0.627	0.319
CHEMBL3234763	CHEMBL498487	0.638	0.318
CHEMBL3234762	CHEMBL525191	0.6	0.31
CHEMBL3235112	CHEMBL1822255	0.633	0.309
CHEMBL3235113	CHEMBL1822255	0.628	0.309
CHEMBL3234764	CHEMBL1822255	0.622	0.309

B-Raf (Target A) vs PDHK1 (Target B)

Target A ligand	***Target B ligand***	***MACCSfp similarity score***	***ECFP4fp similarity score***
CHEMBL498468	CHEMBL192894	0.625	0.462
CHEMBL498467	CHEMBL192894	0.6	0.423
CHEMBL1822258	CHEMBL3354226	0.608	0.419
CHEMBL1822262	CHEMBL3354226	0.608	0.405
CHEMBL1822256	CHEMBL3354226	0.608	0.405
CHEMBL1822257	CHEMBL3354226	0.608	0.405
CHEMBL1822261	CHEMBL3354226	0.6	0.405
CHEMBL1822259	CHEMBL3354226	0.608	0.395
CHEMBL1822265	CHEMBL3354226	0.627	0.372
CHEMBL1822097	CHEMBL3354226	0.616	0.372
CHEMBL1822260	CHEMBL3354226	0.608	0.372
CHEMBL1822264	CHEMBL3354226	0.6	0.372
CHEMBL1822092	CHEMBL3354226	0.6	0.372
CHEMBL1822096	CHEMBL3354650	0.629	0.36
CHEMBL1822097	CHEMBL3354225	0.622	0.356
CHEMBL1822095	CHEMBL3354226	0.616	0.356
CHEMBL1822071	CHEMBL3354650	0.685	0.354
CHEMBL1822072	CHEMBL3354650	0.667	0.354
CHEMBL1822097	CHEMBL3354650	0.625	0.354
CHEMBL1822096	CHEMBL3354226	0.6	0.348

Moreover, 2D fingerprints-based estimations specifically focussing on the amide substituents of the selected phenyl-triazole and phenyl-isoxazole inhibitors allowed to identify significant similarity with some of the phenyl-3-sulfonamide and phenyl-3-sulfamide chemical moieties of B-Raf ligands (**Table B**).

Table B: Top20- scoring 2D similarity records obtained from the comparison of amide substituents of the selected Hsp90, PDHK1 ad B-Raf ligands. As shown in the table several substituents have

reported ECFP4 values above commonly accepted similarity thresholds.[6] Amide substituents are identified by means of their respective ChEMBL IDs.

Hsp90 (Target A) vs PDHK1 (Target B)			
Target A ligand	***Target B ligand***	***MACCSfp similarity score***	***ECFP4fp similarity score***
CHEMBL3234772	CHEMBL252370	1	1
CHEMBL3235112	CHEMBL252370	0.692	0.889
CHEMBL3235113	CHEMBL252370	0.69	0.889
CHEMBL3235115	CHEMBL3354229	0.579	0.833
CHEMBL3235115	CHEMBL3354655	0.564	0.769
CHEMBL3235115	CHEMBL3354651	0.511	0.714
CHEMBL3235115	CHEMBL3354650	0.415	0.714
CHEMBL3235118	CHEMBL252370	0.439	0.7
CHEMBL3235117	CHEMBL3354229	0.356	0.692
CHEMBL3234759	CHEMBL3354228	0.667	0.684
CHEMBL3235116	CHEMBL3354229	0.674	0.667
CHEMBL3235115	CHEMBL3354656	0.595	0.667
CHEMBL3234759	CHEMBL3354227	0.605	0.619
CHEMBL3234759	CHEMBL3354652	0.59	0.619
CHEMBL3234772	CHEMBL3354651	0.413	0.615
CHEMBL3235113	CHEMBL3354651	0.574	0.571
CHEMBL3235114	CHEMBL3354229	0.538	0.571
CHEMBL3235112	CHEMBL3354651	0.438	0.571
CHEMBL3234758	CHEMBL3354228	0.595	0.565
CHEMBL3234759	CHEMBL3354654	0.511	0.545
B-Raf (Target A) vs Hsp90 (Target B)			
Target A ligand	***Target B ligand***	***MACCSfp similarity score***	***ECFP4fp similarity score***
CHEMBL1822093	CHEMBL3234759	0.233	0.37
CHEMBL1822258	CHEMBL3234759	0.18	0.345
CHEMBL1822093	CHEMBL3234758	0.279	0.323
CHEMBL1822261	CHEMBL3234759	0.234	0.321
CHEMBL1822262	CHEMBL3234759	0.219	0.321
CHEMBL1822081	CHEMBL3234759	0.188	0.321
CHEMBL1822083	CHEMBL3234759	0.188	0.321
CHEMBL1822082	CHEMBL3234759	0.188	0.321
CHEMBL1822257	CHEMBL3234759	0.18	0.321
CHEMBL1822260	CHEMBL3234759	0.18	0.321
CHEMBL1822107	CHEMBL3234759	0.148	0.321
CHEMBL1822098	CHEMBL3234759	0.19	0.31
CHEMBL1822102	CHEMBL3234759	0.188	0.31
CHEMBL1822259	CHEMBL3234759	0.18	0.31
CHEMBL1822095	CHEMBL3234759	0.148	0.31
CHEMBL1822258	CHEMBL3234758	0.226	0.303
CHEMBL1822109	CHEMBL3234759	0.286	0.3
CHEMBL2047870	CHEMBL3234759	0.188	0.3
CHEMBL1822093	CHEMBL3234759	0.233	0.37
CHEMBL1822258	CHEMBL3234759	0.18	0.345
B-Raf (Target A) vs PDHK1 (Target B)			

Target A ligand	*Target B ligand*	*MACCSfp similarity score*	*ECFP4fp similarity score*
CHEMBL1822259	CHEMBL3354224	0.377	0.519
CHEMBL2047869	CHEMBL3354224	0.35	0.519
CHEMBL1822258	CHEMBL3354224	0.377	0.5
CHEMBL2047878	CHEMBL3354224	0.411	0.481
CHEMBL2047877	CHEMBL3354224	0.411	0.481
CHEMBL2047876	CHEMBL3354224	0.393	0.481
CHEMBL1822260	CHEMBL3354224	0.377	0.481
CHEMBL2047879	CHEMBL3354224	0.411	0.464
CHEMBL2047876	CHEMBL3354226	0.357	0.458
CHEMBL2047878	CHEMBL3354226	0.351	0.458
CHEMBL2047877	CHEMBL3354226	0.351	0.458
CHEMBL2047869	CHEMBL3354653	0.391	0.452
CHEMBL1822259	CHEMBL3354653	0.354	0.452
CHEMBL2047870	CHEMBL3354224	0.339	0.448
CHEMBL1822088	CHEMBL3354224	0.415	0.444
CHEMBL2047879	CHEMBL3354226	0.351	0.44
CHEMBL2047870	CHEMBL3354653	0.4	0.438
CHEMBL1822258	CHEMBL3354653	0.354	0.438
CHEMBL1822094	CHEMBL3354224	0.471	0.429

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