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Proteine off-target potenzialmente responsabili di effetti avversi di farmaci individuate mediante il software SPILLO-PBSS e validazioni sperimentali

Presenting Author: *Alessandro Di Domizio*

SPILLO® PROJECT

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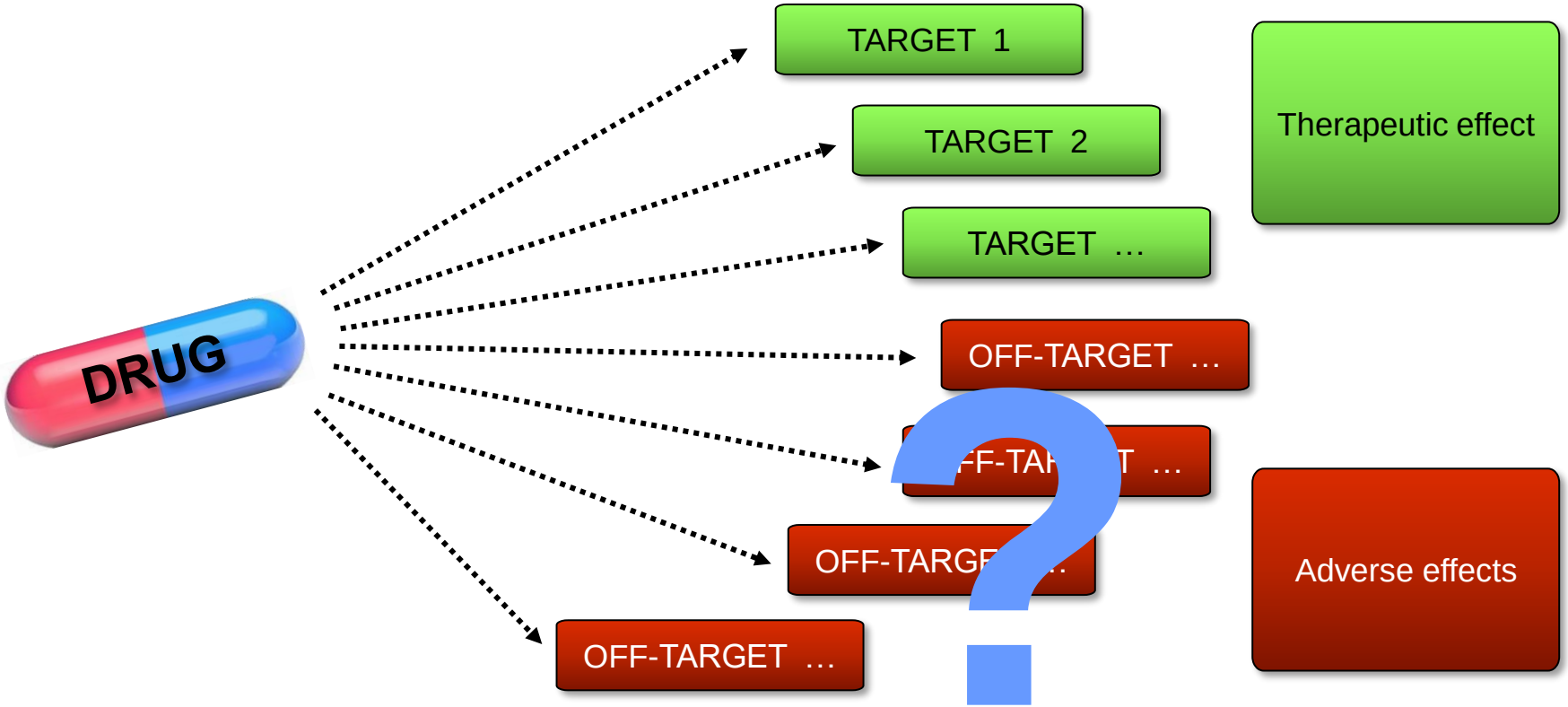
THE OFF-TARGET PROBLEM



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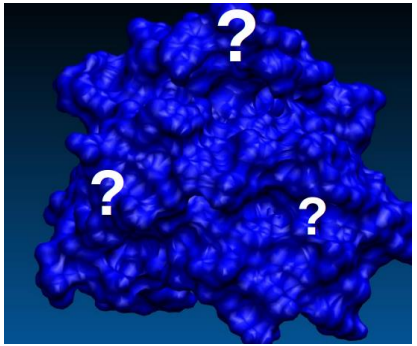
The binding site identification problem



- Proteins: **DYNAMIC** systems and fluctuate in solution (*in vivo* and *in vitro*)

BUT

- Protein structures: **STATIC** (e.g., crystal structure by X-ray diffraction)
- Drug **binding-sites** often **hidden**, **distorted**, or even **totally closed** (protein structures obtained in the absence of "our" drug)

Traditional software (e.g., Molecular docking):

- Static analysis of the surface of the protein to search for potential binding pockets
- Failures in identifying binding sites if hidden, distorted, or closed
- Failures in identifying off-target proteins

Alternatively…. (e.g., Molecular dynamics simulations):

- 1 week/protein * 20000 proteins –> **380** years
- 1 day/protein * 20000 proteins -> **54** years

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SPILLO-potential binding sites searcher (SPILLO-PBSS)

- New way of applying the Statistical thermodynamics laws
- **Apparently non-realistic model** (2nd virtualization level)
- **Interpenetration** between bodies is "**allowed**" (strongly forbidden by traditional software)

-> Includes the flexibility of the system

-> Unique ability in recognizing **hidden**, **distorted**, or even **totally closed** binding sites

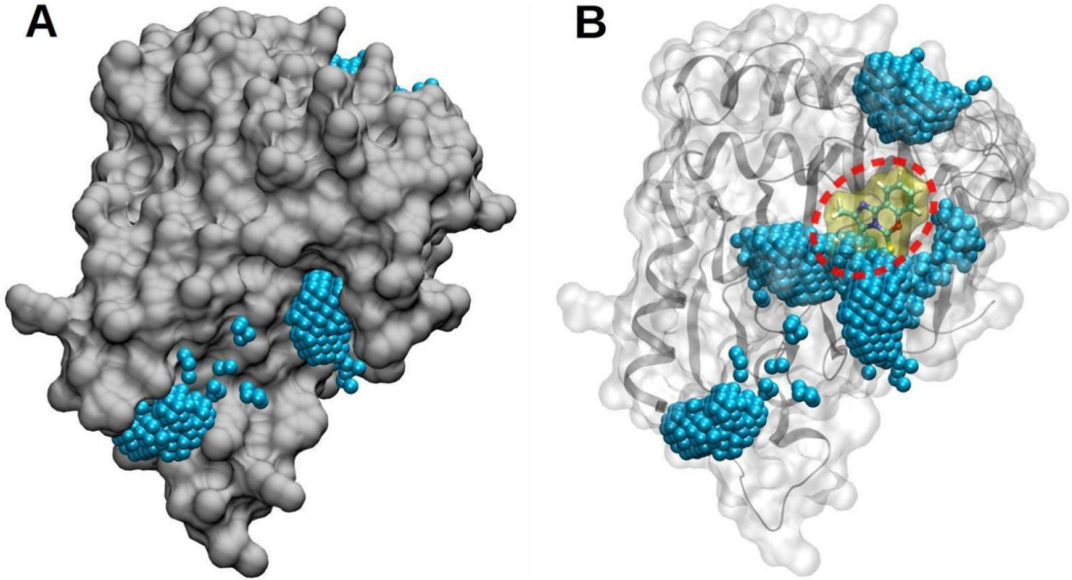
Journal of COMPUTATIONAL CHEMISTRY

Software News and Updates

SPILLO-PBSS: Detecting hidden binding sites within protein 3D-structures through a flexible structure-based approach

Alessandro Di Domizio, Alessandro Vitriolo, Giulio Vistoli, Alessandro Pedretti,

First published: 01 September 2014 | https://doi.org/10.1002/jcc.23714 | Citations: 4



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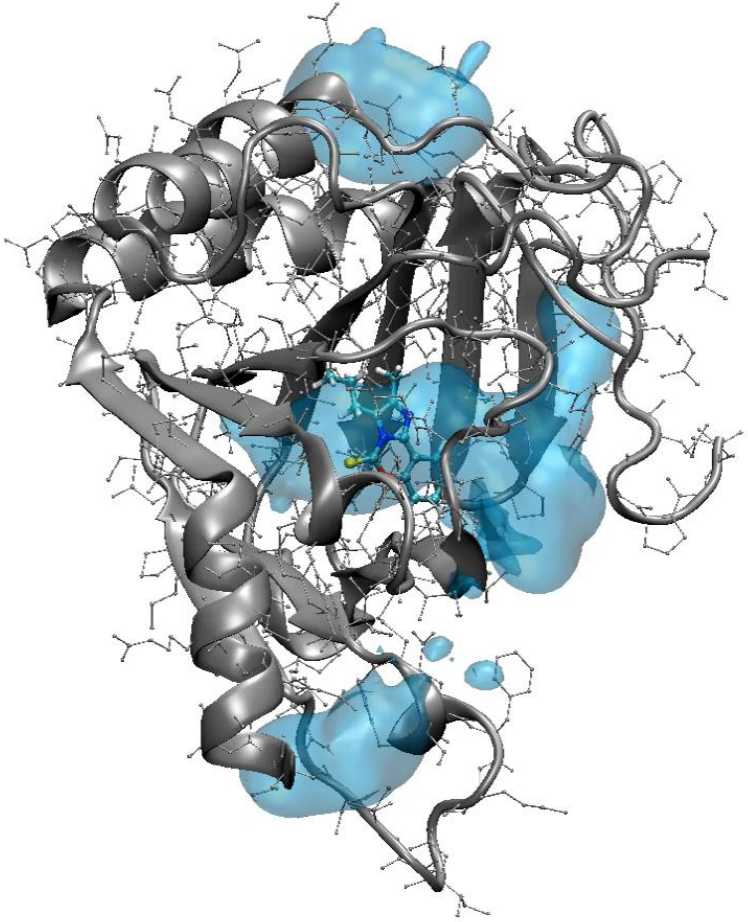

SPILLO-PBSS:

- **Unlike Molecular Docking**, SPILLO-PBSS allows not just the analysis of known binding sites, but the identification of totally unknown binding sites, not identifiable by other software solutions.
- **Unlike QSAR**, both the ligand and the protein are taken into account
- **Unlike QSAR**, totally unknown off-target proteins can be identified

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SPILLO-potential binding sites searcher (SPILLO-PBSS)

- **Rapid** identification of **off-target** proteins of any small molecule within the **whole structural proteome** of *Homo sapiens* or other organisms (*e.g., Mus musculus,* and *Rattus norvegicus*).

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EXPERIMENTAL VALIDATIONS

To **validate** the approach while ensuring **maximum objectivity** and **reliability**:

- Application to scientific **problems still waiting to be solved**
- *In silico* predictions have been **experimentally confirmed** (***in vitro*** and/or ***in vivo***) **by academic research centres** and published in peer-reviewed scientific journals

Collaborations:

UNIVERSITÀ DEGLI STUDI DI MILANO

UNIVERSITÀ DEGLI STUDI DI MILANO BICOCCA

UNIVERSITÀ DI PARMA

UNICAM Università di Camerino

UNIVERSITÀ DI CAMERINO

Queen Mary University of London

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FINASTERIDE

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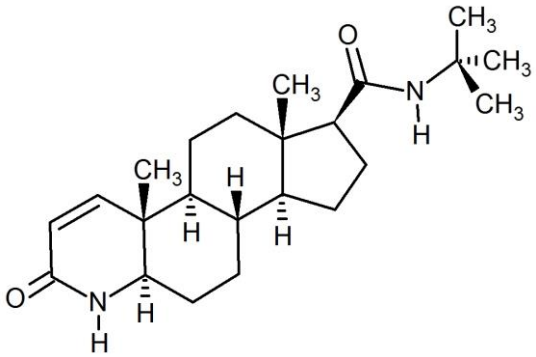
FINASTERIDE



Finasteride

(approved in the 90's)

- **Indications**:
  - Benign prostate hyperplasia (BPH)
  - Androgenetic alopecia (AGA)
  - Other androgen-dependent conditions
- **Molecular mechanism**:
  - Type II 5-alpha reductase (5α-R) inhibition

  (blocks the conversion of testosterone into dihydrotestosterone (DHT))
- **Reported adverse effects**:
  - Sexual complaints (decreased or loss of libido, disorders of ejaculation, erectile dysfunction, testicular atrophy, orgasmic disorders, and hypogonadism)
  - Psychological complaints (self-harm, slow cognition and psychological pathology, changes in emotional effect, and sleep disturbances)
  - Physical complaints (rash and metabolic abnormalities)

Biomolecular mechanism?

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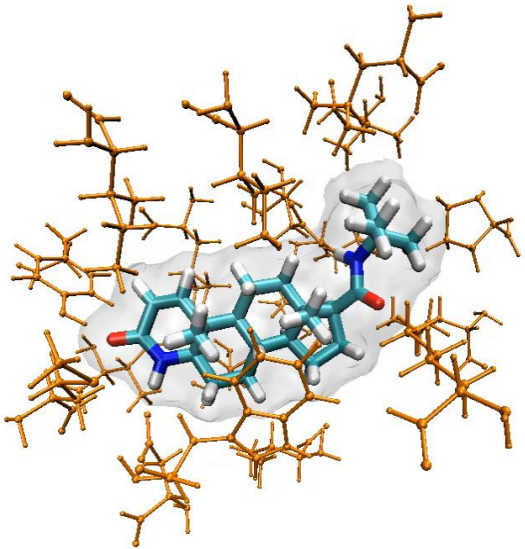
FINASTERIDE

INPUT



Finasteride Reference Binding Site (RBS)

INPUT

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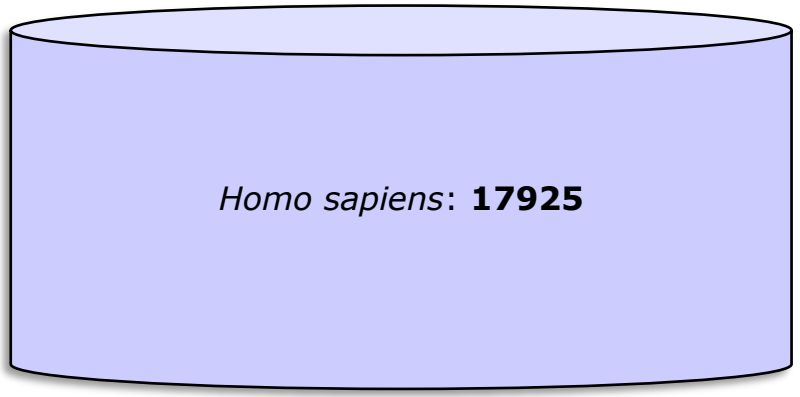

All experimental (X-ray, solution NMR) protein 3D structures available from the **RCSB Protein Data Bank** (*January 2019*), without sequence redundancies.

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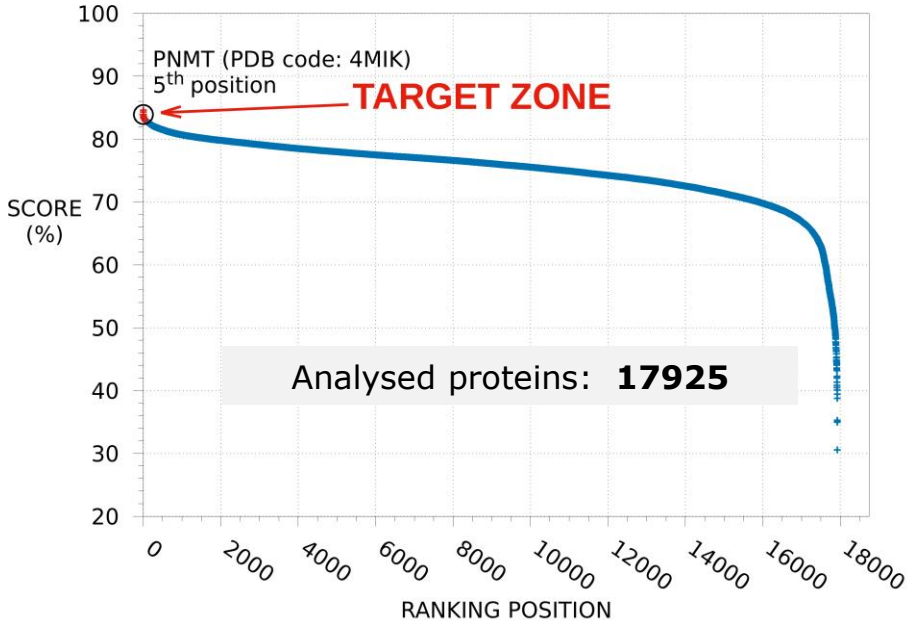
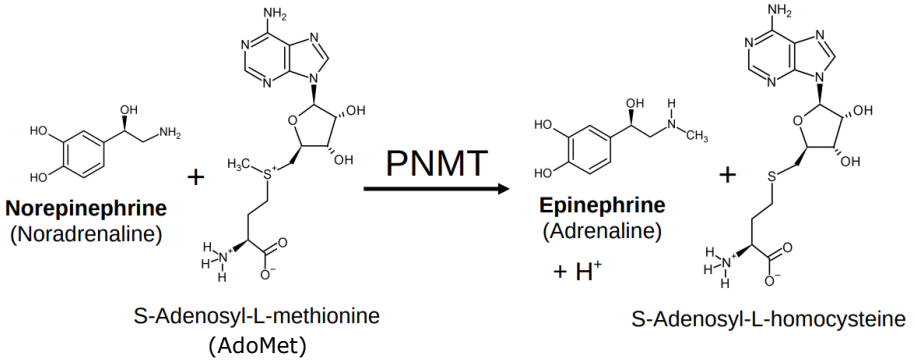
OUTPUT

Ranking of the protein database



Among the **top-10** targets of MV1035 identified by SPILLO-PBSS:

- **Phenylethanolamine N-methyltransferase (PNMT)**



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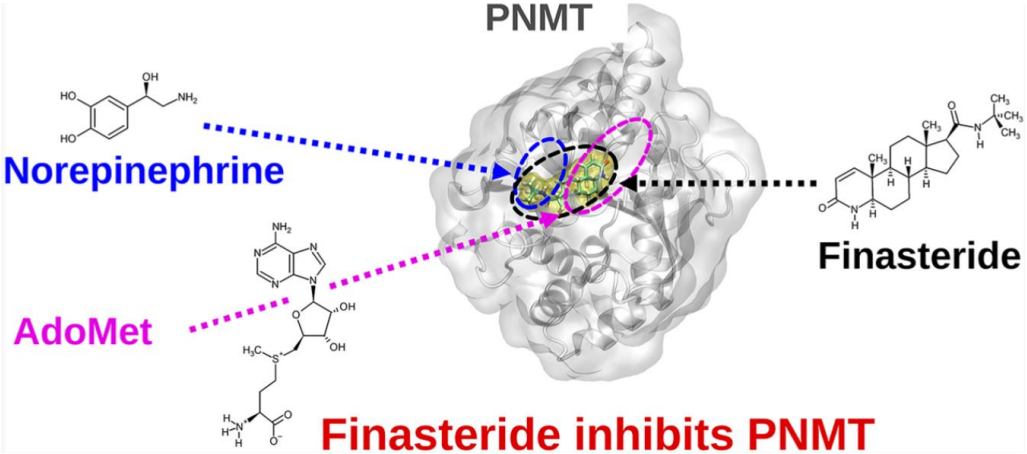
FINASTERIDE

In silico hypothesis: inhibitory activity of finasteride on PNMT, the enzyme that catalyses the formation of the stress hormone epinephrine (adrenaline)



Literature data indicate that PNMT activity perturbation may be correlated with sexual and psychological side effects.

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Inhibitory interaction confirmed *in vitro*

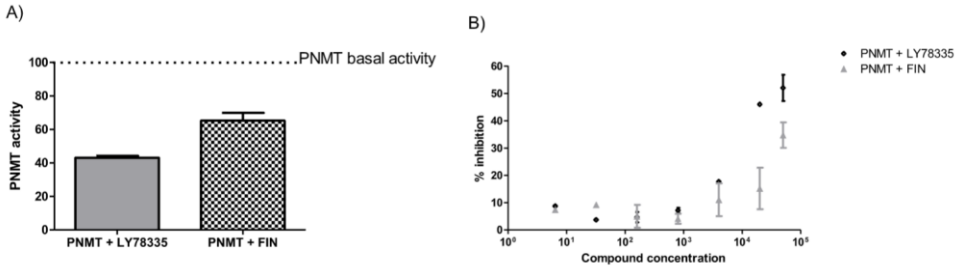

Figure 4. hPNMT activity. Human recombinant enzyme activity was detected by fluorescence in the presence of a vehicle (DMSO—basal PNMT activity) of LY78335 or FIN. Panel A: PNMT activity in the presence of a high-affinity inhibitor LY78335 (50 μM) or FIN (50 μM). The columns represent the mean ± SEM of duplicate reactions. Panel B: dose–response curve in the presence of a high-affinity inhibitor LY78335 (black rhombus; from 6.4 nM to 50 μM) or FIN (gray triangle; from 6.4 nM to 50 μM). The symbols represent the mean ± SEM of duplicate reactions.

Inhibitory interaction confirmed *in vivo*

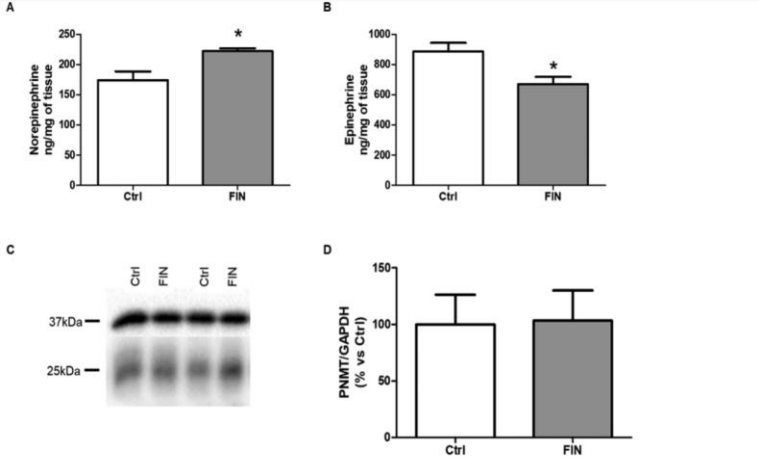

Figure 5. Adrenal catecholamine and PNMT protein levels. Catecholamine levels were detected by liquid chromatography tandem mass spectrometry analyses in control (Ctrl; $n = 6$) and FIN-treated animals (FIN, $n = 6$). Adrenal PNMT protein levels were detected by western blotting in control (Ctrl; $n = 6$) and FIN-treated animals (FIN, $n = 6$). Panel (A) norepinephrine levels; panel (B) epinephrine levels; panel (C) representative blot of PNMT (25 kDa) and GAPDH (37 kDa); panel (D) quantification of PNMT protein levels. The columns represent the mean ± SEM after normalization for the starting tissue (panel A and B) or for GAPDH (panel D). Data were analyzed by Student's *t*-test. *$p \leq 0.05$ vs Ctrl.

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Journal of Medicinal Chemistry

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Article

Three-Dimensional Proteome-Wide Scale Screening for the 5-Alpha Reductase Inhibitor Finasteride: Identification of a Novel Off-Target

Silvia Giatti, Alessandro Di Domizio, Silvia Diviccaro, Eva Falvo, Donatella Caruso, Alessandro Contini, and Roberto Cosimo Melcangi*

Cite This: J. Med. Chem. 2021, 64, 4553−4566

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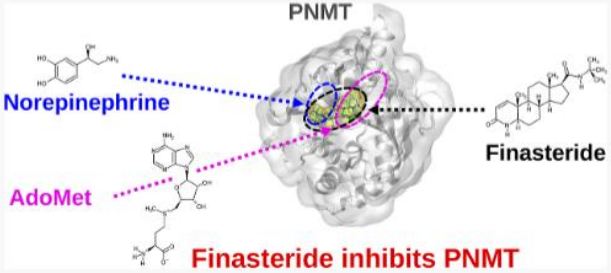
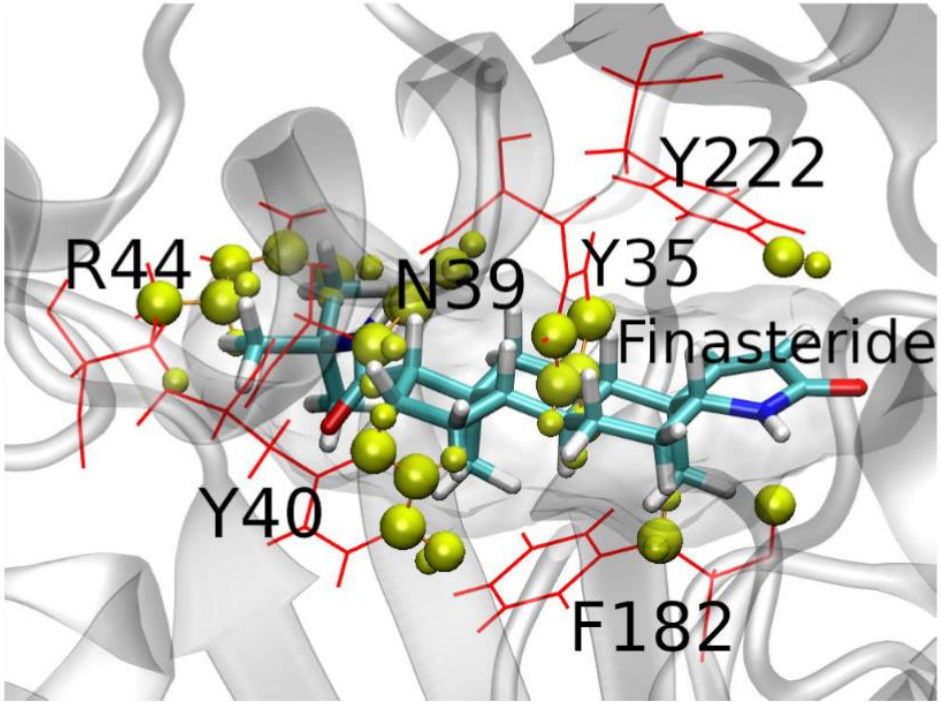


ABSTRACT: Finasteride, a 5-alpha reductase (5α-R) inhibitor, is a widely used drug for treating androgen-dependent conditions. However, its use is associated with sexual, psychological, and physical complaints, suggesting that other mechanisms, in addition to 5α-R inhibition, may be involved. Here, a multidisciplinary approach has been used to identify potential finasteride off-target proteins. SPILLO-PBSS software suggests an additional inhibitory activity of finasteride on phenylethanolamine *N*-methyltransferase

FINASTERIDE



Many steric clashes (in yellow)
Binding site **not identifiable**
by other methods

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BORTEZOMIB and CARFILZOMIB

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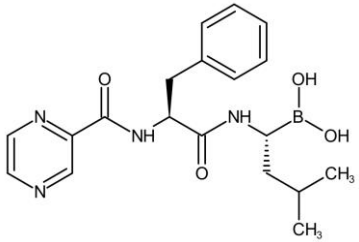
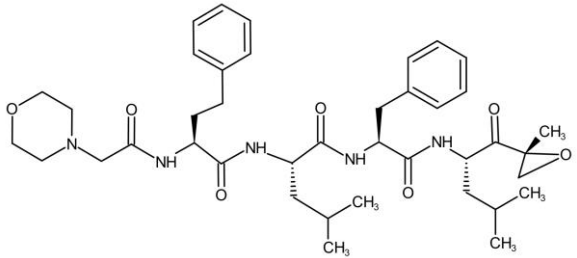
BORTEZOMIB and CARFILZOMIB



Frequently used as first line therapy

Bortezomib (BTZ)
(approved in 2003)



Carfilzomib (CFZ)
(approved in 2012)

Biomolecular mechanism?

- **Indications**:
  - Multiple myeloma
- **Molecular mechanism**:
  - Proteasome inhibition
- **<u>Different</u> reported adverse effects**:
  - Bortezomib: Peripheral neuropathy (**PN**) in ~ 50% of patients **Dose-limiting toxicity**
  - Carfilzomib: **Less severe PN**

- NOTE: same target and same mechanism of action
- NOTE: same binding site (catalytic sites of the proteasome)

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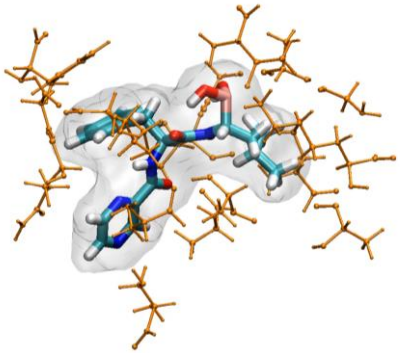
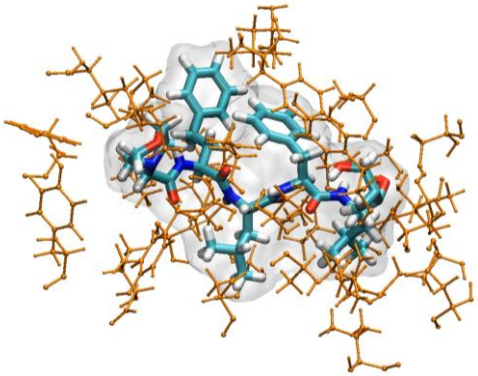
BORTEZOMIB and CARFILZOMIB

INPUT

Bortezomib



Carfilzomib



Reference Binding Sites (RBSs)

INPUT

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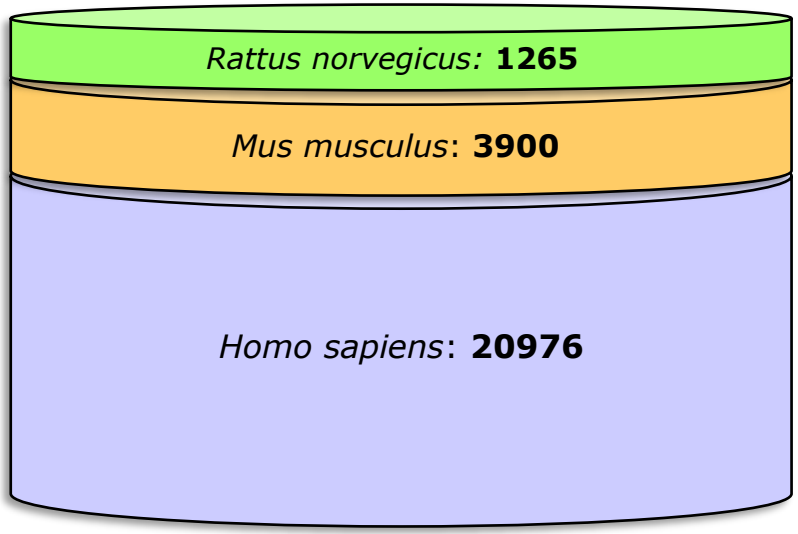

All experimental (X-ray, solution NMR) protein 3D structures available from the **RCSB Protein Data Bank** (*August 2019*), without sequence redundancies.

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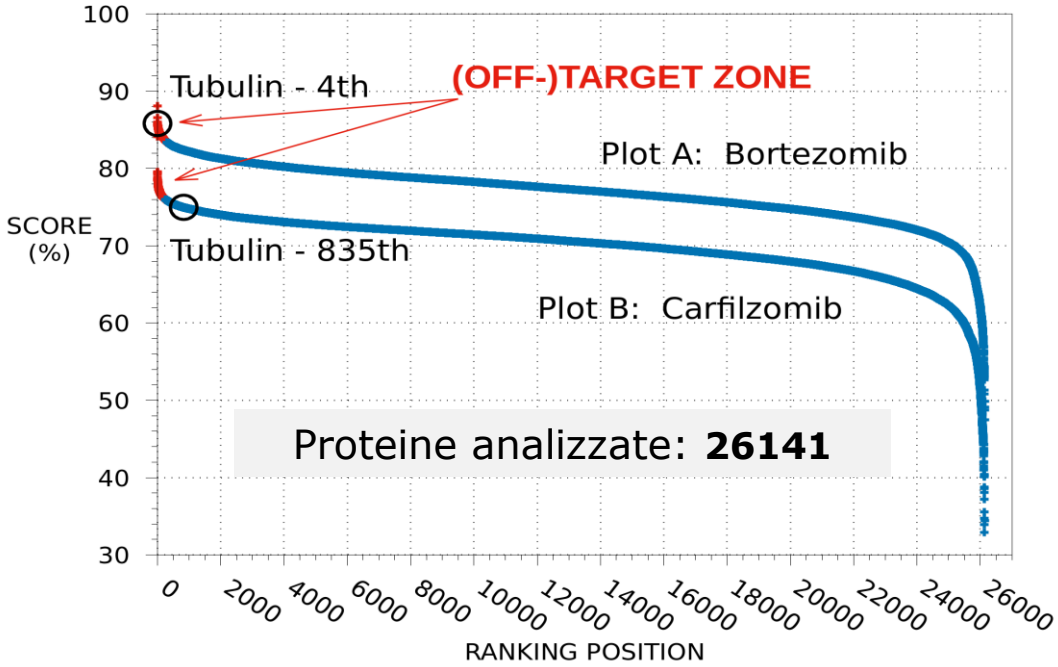
BORTEZOMIB and CARFILZOMIB

OUTPUT



Identified

off-target interaction:

Tubulin-Bortezomib

but not

Tubulin-Carfilzomib

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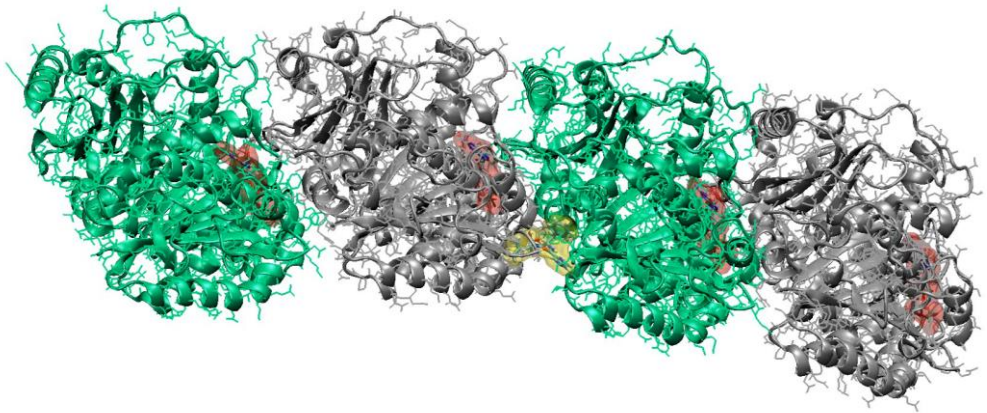
In silico hypothesis:

- direct interaction Tubulin-Bortezomib
  - variation of the GTPase activity of α-tubulin
    - reduction of the 'microtubule catastrophe' and consequent increment of microtubules polymerization (perturbation of the microtubule dynamics)



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BORTEZOMIB and CARFILZOMIB

Hypotheses confirmed *in vitro*

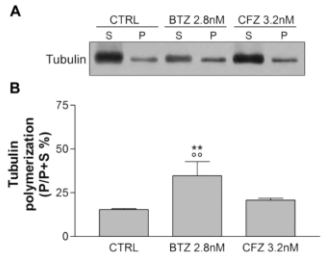


Figure 6. Tubulin polymerization in DRG neurons. Representative images (**A**) and quantification graphs (**B**) of anti-tubulin immunoblotting in neurons, not treated (CTRL) or treated for 48 h with 2.8 nM BTZ and 3.2 nM CFZ. Graphs represent the percentage of polymerized tubulin (present in the pellet fraction P) compared to total tubulin (free tubulin, present in the substrate fraction S, added to polymerized tubulin). $^{**}p < 0.01$ versus CTRL; $^{\circ\circ}p < 0.01$ versus CFZ 3.2 nM.

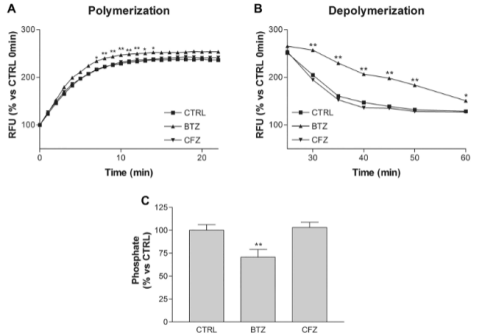


Figure 8. Tubulin polymerization and depolymerization in vitro with DMSO buffer. (**A**) In vitro cell-free tubulin polymerization in presence of 1 μM BTZ or 1 μM CFZ. (**B**) In vitro cell-free tubulin depolymerization in presence of 1 μM BTZ or 1 μM CFZ. (**C**) Phosphate quantification after tubulin polymerization assay. Graphs are represented as mean percentage ± SD compared to untreated CTRL (arbitrarily set to 100%). $^{*}p < 0.05$; $^{**}p < 0.01$ versus CTRL and versus CFZ.

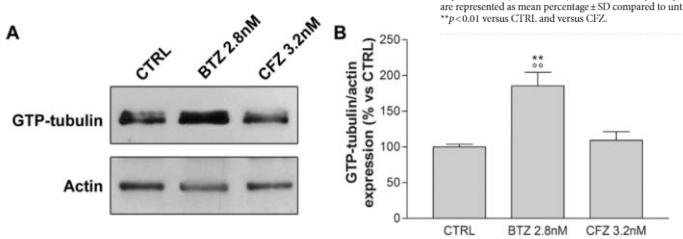
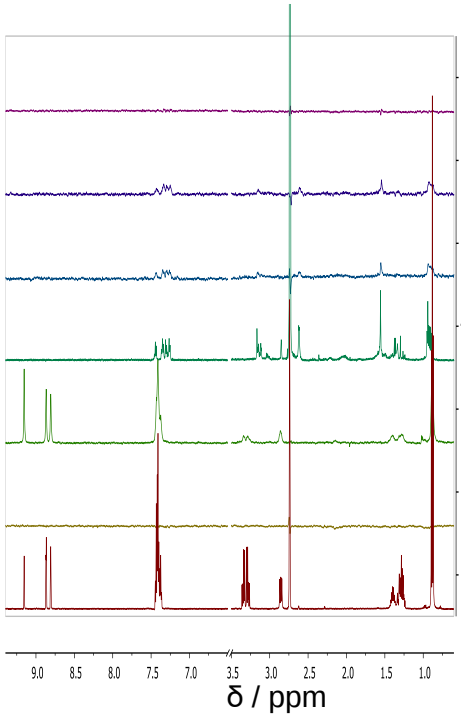


Figure 7. GTP-tubulin in sensory neurons. Representative image (**A**) and quantification graph (**B**) of GTP-tubulin immunoblotting in neurons not treated (CTRL) or treated for 48 h with 2.8 nM BTZ and 3.2 nM CFZ. Data are normalized respect to actin value and expressed as percentage respect control. $^{**}p < 0.01$ versus CTRL; $^{\circ\circ}p < 0.01$ versus CFZ 3.2 nM.

Direct interaction Tubulin-BTZ (but not Tubulin-CFZ) confirmed by NMR



G E-F

F Spettro STD NMR CFZ+ α/β tubulina

E Spettro STD NMR CFZ

D Spettro H1NMR CFZ

C Spettro STD NMR BTZ+ α/β tubulina

B Spettro STD NMR BTZ

A Spettro H1NMR BTZ

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Tubulin binding potentially clears up Bortezomib and Carfilzomib differential neurotoxic effect

A. Malacrida[1,2,6✉], S. Semperboni[1,2,6], A. Di Domizio[3,4], A. Palmioli[2,5], L. Broggi[1], C. Airoldi[2,5], C. Meregalli[1,2✉], G. Cavaletti[1,2] & G. Nicolini[1,2]

Proteasome inhibitors (PIs) represent the gold standard in the treatment of multiple myeloma. Among PIs, Bortezomib (BTZ) is frequently used as first line therapy, but peripheral neuropathy (PN), occurring approximately in 50% of patients, impairs their life, representing a dose-limiting toxicity. Carfilzomib (CFZ), a second-generation PI, induces a significantly less severe PN. We investigated possible BTZ and CFZ off-targets able to explain their different neurotoxicity profiles. In order to identify the possible PIs off-targets we used the SPILLO-PBSS software that performs a structure-based in silico screening on a proteome-wide scale. Among the top-ranked off-targets of BTZ identified by SPILLO-PBSS we focused on tubulin which, by contrast, did not turn out to be an off-target of CFZ. We tested the hypothesis that the direct interaction between BTZ and microtubules would inhibit the tubulin alfa GTPase activity, thus reducing the microtubule catastrophe and consequently furthering the microtubules polymerization. This hypothesis was validated in a cell-free model, since BTZ (but not CFZ) reduces the concentration of the free phosphate released during GTP hydrolysis. Moreover, NMR binding studies clearly demonstrated that BTZ, unlike CFZ, is able to interact with both tubulin dimers and polymerized form. Our data suggest that different BTZ and CFZ neurotoxicity profiles are independent from their proteasome inhibition, as demonstrated in adult

Small molecule: MV1035

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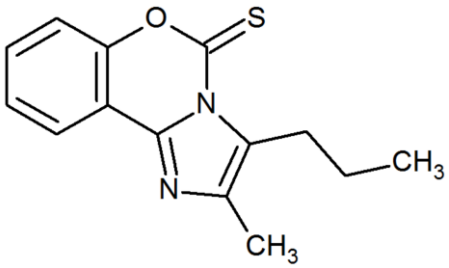
Small molecule: MV1035



Small molecule: MV1035

Glioblastoma (GBM, grade IV glioma):

- The most aggressive brain cancer
- Poor prognosis (median survival of only 15 months after diagnosis)
- Resistance to antineoplastic drugs (e.g., Temozolomide (TMZ))

➢ **MV1035** (a new molecule):

- was found to **reduce migration** and **invasiveness** of U87 glioblastoma (GBM) cell line

Biomolecular mechanism?

NOTE: **Unknown target(s)** responsible for the **positive effect**

NOTE: **Unknown off-targets** responsible for possible **toxic effects**

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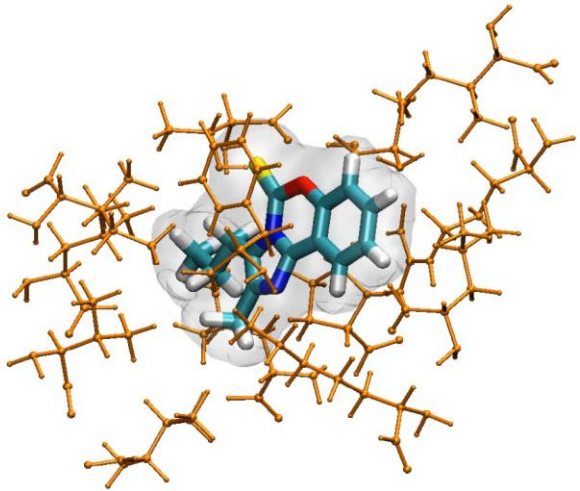
Small molecule: MV1035

INPUT



MV1035 Reference Binding Site (RBS)

INPUT

Database: **14537** protein 3D structures

All experimental (X-ray, solution NMR) protein 3D structures available from the **RCSB Protein Data Bank** (*September 2017*), without sequence redundancies.

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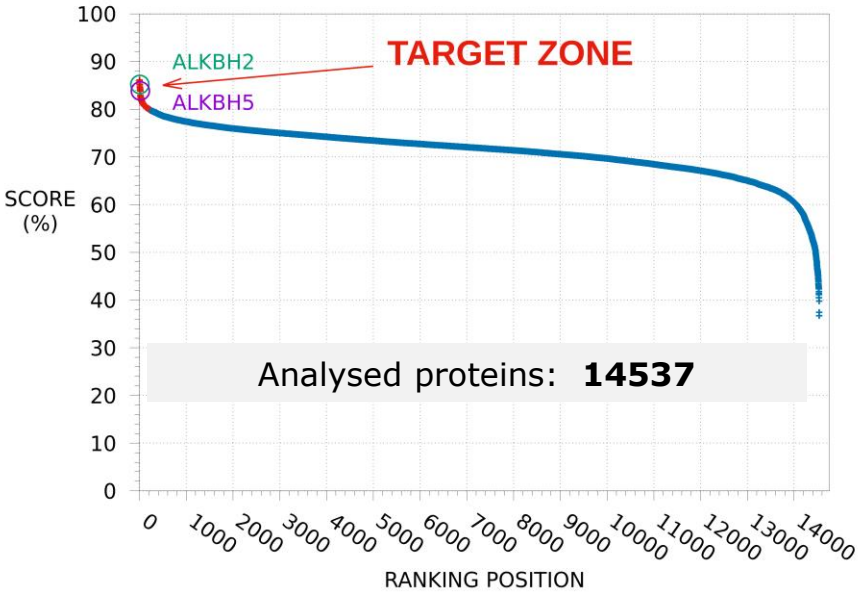
Small molecule: MV1035

OUTPUT

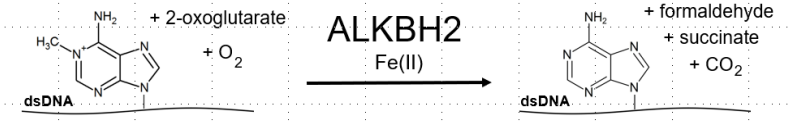
Ranking of the protein database



Among the **top-20** targets of MV1035 identified by SPILLO-PBSS:

- **RNA demethylase ALKBH5**

- **DNA oxidative demethylase ALKBH2**



NOTE: ALKBH5 and ALKBH2 highly expressed in glioblastoma
NOTE: ALKBH2 responsible for temozolomide resistance

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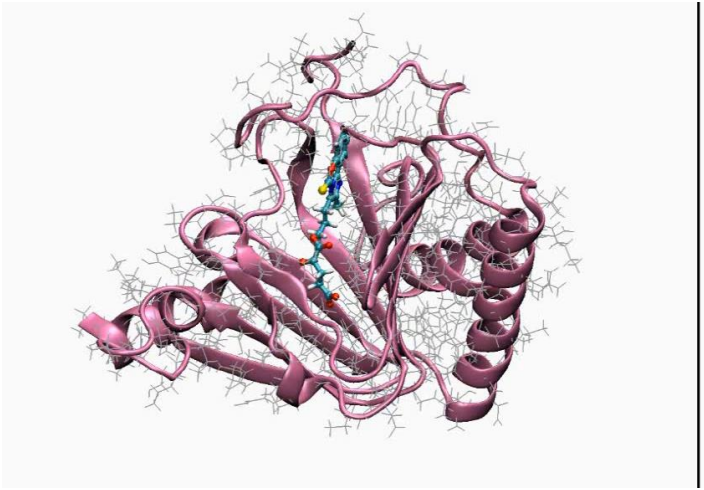
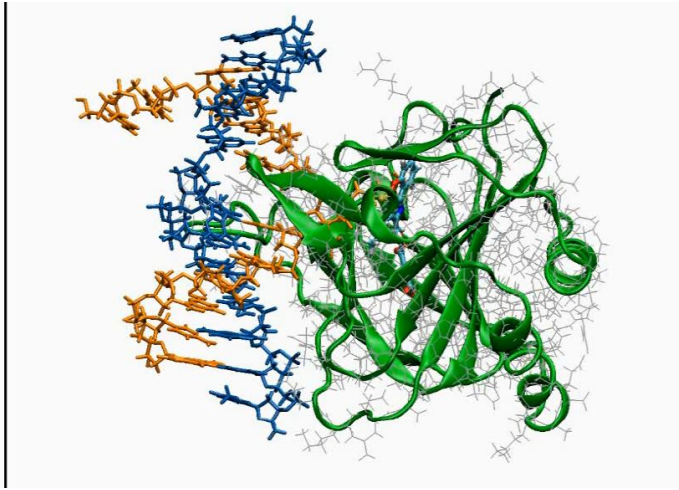
Small molecule: MV1035

In silico hypothesis:

- Reduction of ALKBH5 demethylase activity (competitive inhibition)
- Reduction of ALKBH2 demethylase activity (competitive inhibition)



ALKBH5-MV1035



ALKBH2-MV1035

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Small molecule: MV1035

ALKBH5: inhibitory interaction with MV1035 confirmed *in vitro*

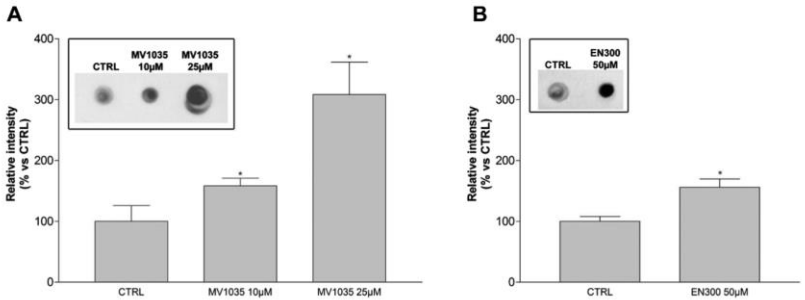

Fig. 7. Dot blot analysis of ALKBH5 demethylase activity - Dot blot analysis for the detection of m6A-RNA after the inhibition of ALKBH5 with MV1035 10 or 25 μM (A) or EN300 50 μM (B). The inserts show a representative image of m6A-RNA dot blot. Data are represented as the average percentage ± SD compared to controls without MV1035 or EN300 (CTRL, 100%). (* $p < 0.05$).

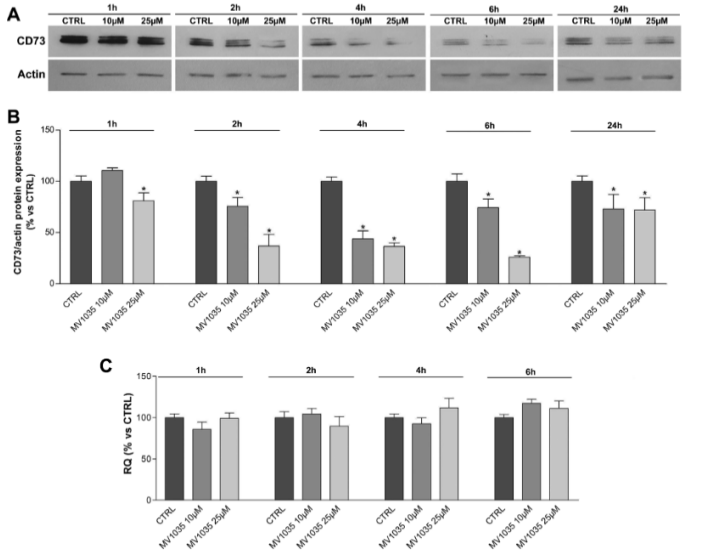

Fig. 8. Western blot and RT-PCR analysis in U87-MG cells. Representative images of CD73 western blotting (A) and respective graphs (B). Cells were treated for 1, 2, 4, 6 and 24 h with MV1035 10 or 25 μM. Graphs represent the average percentage ± SD of CD73 expression, normalized to actin and compared to untreated control cells (CTRL, 100%). (C) RT-PCR analysis of CD73 mRNA in U87-MG cells treated with MV1035 10 and 25 μM for 1, 2, 4 and 6 h. Data are represented as the average percentage ± SD compared to respective controls without MV1035 (CTRL, 100%).

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Small molecule: MV1035

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3D proteome-wide scale screening and activity evaluation of a new ALKBH5 inhibitor in U87 glioblastoma cell line

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ARTICLE INFO

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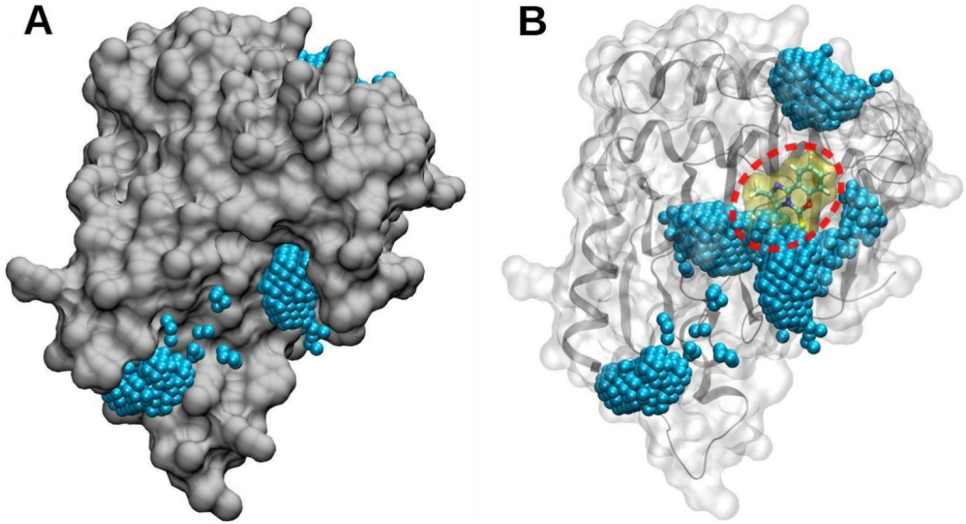
ABSTRACT

The imidazobenzoxazin-5-thione MV1035, synthesized as a new sodium channel blocker, has been tested on tumoral cells that differ for origin and for expressed Na_V pool (U87-MG, H460 and A549). In this paper we focus on the effect of MV1035 in reducing U87 glioblastoma cell line migration and invasiveness. Since the effect of

Small molecule: MV1035



Many steric clashes (in yellow)
Binding site **not identifiable**
by other methods

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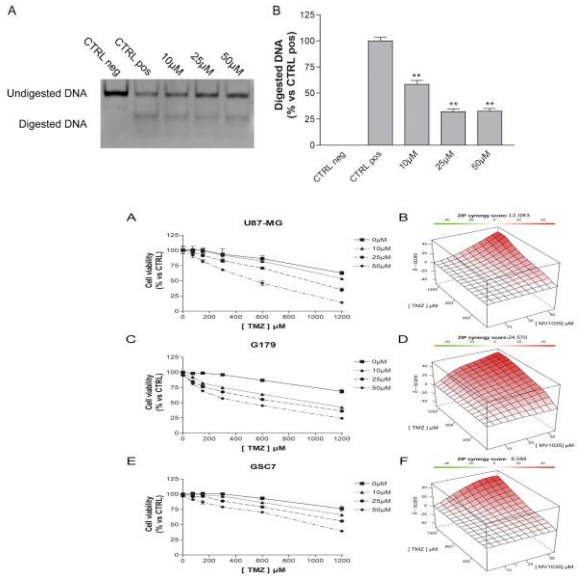
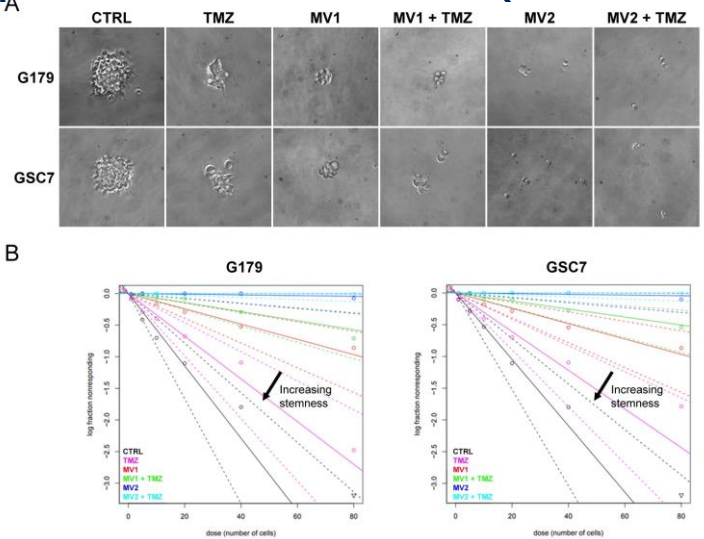
Small molecule: MV1035

ALKBH2:

- **inhibitory interaction** ALKBH2-MV1035 **confirmed *in vitro***
- **synergic effect** of the combination 'Temozolomide and MV1035' **confirmed in patient derived Glioma Stem Cells (GSCs)**



Spheres: Patient-Derived Lines (G179 and GSC7)



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MV1035 OVERCOMES TEMOZOLOMIDE RESISTANCE IN PATIENT-DERIVED GLIOBLASTOMA STEM CELL LINES

Alessio Malacrida[1] *, Alessandro Di Domizio[2] *, Angela Bentivegna [3], Giacomo Cislaghi[2], Eleonora Messuti [4], Carlo Giussani[3,5], Valentina Zuliani [6], Mirko Rivara [6] (a), Gabriella Nicolini[1]

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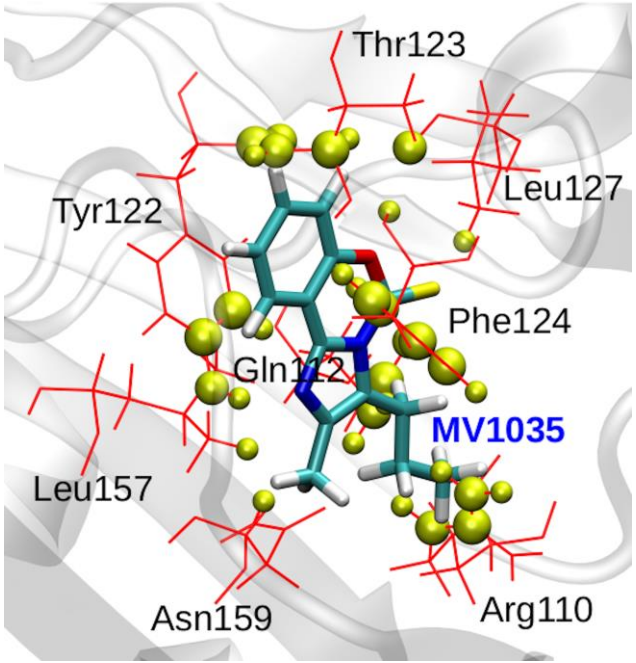
Small molecule: MV1035



Many steric clashes (in yellow)
Binding site **not identifiable**
by other methods

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MOREOVER…

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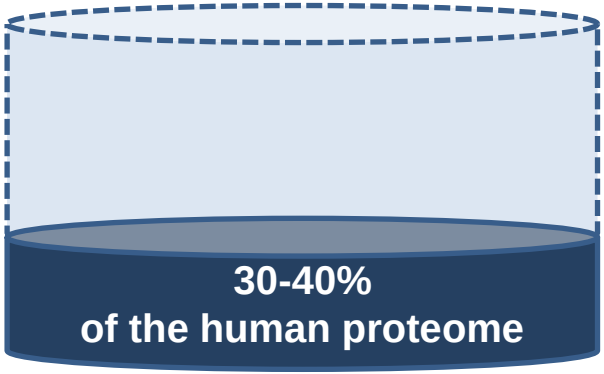
Structural coverage of the human proteome

RCSB Protein Data Bank

Experimental 3D-structures

(X-ray diffraction, solution NMR, cryo-EM, …)

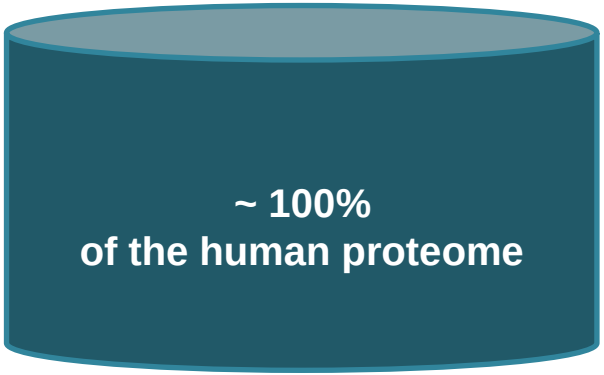


51,480+ 3D structures
with redundancies (May 2021)

AlphaFold Protein Structure Database

Predicted by Artificial Intelligence

(Released in July 2021 by DeepMind)



23,390+ 3D structures
without redundancies (July 2021)

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FOR FURTHER INFORMATION, PLEASE VISIT THE WEBSITE:

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THANK YOU FOR YOUR ATTENTION!

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Comparison with other tools

- **SPILLO-PBSS** performs better than other structure-based software in the identification of protein binding sites (Table 1)

SEARCH METHOD	BINDING SITE CONFORMATIONS			
	SUITABLY OPEN	WIDE-OPEN	WIDE-OPEN and OCCUPIED	COMPLETELY CLOSED
GEOMETRIC POCKET SEARCH	Yes	No	Yes	No
CHEMICAL POCKET SEARCH	Yes	Yes	No	No
BINDING SITE COMPARISON	Yes	No	No	No
BLIND DOCKING	Yes	Yes	No	No
MONTE CARLO SEARCH	Yes	Yes	No	No
SPILLO-PBSS FLEXIBLE SEARCH	Yes	Yes	Yes	Yes

Di Domizio A. et al., *J. Comput. Chem* (2014), doi: 10.1002/jcc.23714

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