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1 Insights into the Interaction Dynamics between 2 Volatile Anesthetics and Tubulin through 3 Computational Molecular Modelling

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8 **Abstract:** General anesthetics, able to reversibly suppress all conscious brain activity, have baffled
9 medical science for decades, and little is known about their exact molecular mechanism of action.
10 Given the recent scientific interest in the exploration of microtubules as putative functional targets
11 of anesthetics, and the involvement thereof in neurodegenerative disorders, the present work
12 focuses on the investigation of the interaction between human tubulin and four volatile anesthetics:
13 ethylene, desflurane, halothane and methoxyflurane. Interaction sites on different tubulin isotypes
14 are predicted through docking, along with an estimate of the binding affinity ranking. The analysis
15 is expanded by Molecular Dynamics simulations, where the dimers are allowed to freely interact
16 with anesthetics in the surrounding medium. This allowed for the determination of interaction
17 hotspots on tubulin dimers, which could be linked to different functional consequences on the
18 microtubule architecture, and confirmed the weak, Van der Waals-type interaction, occurring
19 within hydrophobic pockets on the dimer. Both docking and MD simulations highlighted
20 significantly weaker interactions of ethylene, consistent with its far lower potency as a general
21 anesthetic. Overall, simulations suggest a transient interaction between anesthetics and
22 microtubules in general anesthesia, and contact probability analysis shows interaction strengths
23 consistent with the potencies of the four compounds.

24 **Keywords:** Volatile Anesthetics; Molecular Dynamics; Molecular Docking; Tubulin; Microtubules,
25 Cytoskeleton

26

27 List of Abbreviations:

COM	Center of Mass
MAC	Minimum Alveolar Concentration
MD	Molecular Dynamics
MT	Microtubule
RMSD	Root-Mean-Square Deviation
RMSF	Root-Mean-Square Fluctuation
VA	Volatile Anesthetic

28

29

30 1. Introduction

31 General anesthetics are a unique class of drugs in modern medicine. They are able to reversibly
32 suspend conscious brain activity while sparing most of the other brain functions with extraordinary
33 selectivity. This, along with their analgesic and amnesic properties, has effectively made them a
34 cornerstone of modern surgery, yet little is known about their molecular mechanism of action. This
35 issue encompasses a rather large subset of open questions at many different scales: research failed to
36 determine a single biological site of action capable of explaining not only the clinical manifestation
37 of general anesthetics, but also the lack of any anesthetic effects in certain molecules with similar
38 physicochemical properties[1]. Theories of anesthetic action started from early considerations on
39 their solubility in lipid-like media, pioneered by the Meyer-Overton correlation [2–4] which is shown
40 in Supplementary Fig. S1, to subsequent studies regarding effects on hydrophobic, lipid regions of
41 the brain cells' membranes. Most recently, after further studies highlighted significant involvement
42 of cytoskeletal proteins based on post-anesthetic-exposure proteomic alterations [5–7], investigations
43 focused on possible involvements of the cytoskeleton, specifically microtubules (MTs), in the
44 processes of memory formation, consciousness and side effects of anesthesia.

45 The theory elaborated in the 1980s by Penrose and Hameroff proposed to explain consciousness
46 as the result of quantum resonance in the microtubule bundles extending to a neuron and eventually
47 an entire brain, and is referred to as the 'Orch OR' theory [8], later expanded to what is known as the
48 Quantum Mobility theory [1], based on computational evidence of alterations in the oscillation
49 frequencies of π -electrons in aromatic amino acids of microtubules in the presence of anesthetics,
50 with possible long-term effects also on MT polymerization. While the Orch OR theory has not been
51 confirmed experimentally yet, the binding of anesthetics to tubulin is known to occur experimentally
52 [9], and interactions between the latter and anesthetic agents are of particular interest due to potential
53 implications in (a) Post-operative cognitive dysfunction (POCD), which is associated with
54 microtubule instability and the separation of microtubule-associated protein (MAP) tau from MTs
55 [10,11]; (b) memory formation, a process relying on synaptic plasticity [12–14] which is impaired
56 during general anesthesia and has been linked in previous computational studies to the microtubule
57 lattice [15]; (c) the unique spatial organization of microtubules in neurons [16,17] and their putative
58 ability to create specific conduction pathways, hypothesized to be involved in information processing
59 [15,18–20]; (d) clinical decisions regarding anesthesia in patients undergoing chemotherapy or with
60 neurodegenerative comorbidities, both of which can imply pathological or drug-induced alterations
61 of the microtubule cytoskeleton respectively, which might be influenced by the simultaneous
62 presence of anesthetics [21,22].

63 These considerations support the investigation of microtubules, and their constitutive protein
64 tubulin, as a putative target for anesthetic molecule interactions.

65 Indeed, volatile anesthetics (VAs) exhibit many different chemical structures and cover a wide
66 range of molecular weights, from single atoms such as Xenon to heavier halogen-substituted ethers
67 such as Sevoflurane. Among these, Halothane (2-bromo-2-chloro- 1,1,1-trifluoroethane) is a volatile
68 haloalkane with a MAC of 0.74% [23]. Experimentally, it has been found to alter the genetic
69 expression of tubulin [6] and to directly bind to it [9]. Also, it is known to alter the polymerization
70 rate of tubulin in microtubules *in vivo* [24,25], and thus is of particular interest in the context of VA-
71 tubulin interaction. Conversely, Desflurane (2-(difluoromethoxy)-1,1,1,2-tetrafluoroethane) is a
72 poorly soluble, fluorinated ether with a slightly higher MAC value of 6% in oxygen [26], also causing
73 alterations in tubulin expression after exposure *in vivo* [5]. Methoxyflurane (2,2-dichloro-1,1-difluoro-
74 1-methoxyethane) is a potent anesthetic gas, also belonging to the class of halogenated methyl ethyl
75 ethers, with a MAC value of just 0.16% [27], and now mostly abandoned as a general anesthetic due
76 to the nephrotoxicity of its metabolites [28]. It has been shown, just as Halothane, to influence the
77 polymerization of tubulin *in vitro* and significantly alter the axonal microtubule structure [29]. Lastly,
78 ethylene (or ethene) is the simplest alkene and has a comparably low molecular weight of just 28.054
79 g/mol. It is highly volatile and with a MAC value of as much as 67% [30], but it is not used as a general
80 anesthetic due to its very low potency.

81 The four above-mentioned VAs were chosen for our investigation not only due to experimental
82 evidence of interaction with tubulin, but also to cover a wide range of clinical potencies (MAC values
83 of 0.16% for Methoxyflurane up to 67% for ethylene), and to include molecules belonging to different
84 classes (namely, ethers, alkanes and gases as classified in [26]).

85 To investigate the effect of said anesthetics on the cytoskeleton network, in the present work
86 tools provided by Computational Molecular Modeling, namely Homology Modelling, Molecular
87 Dynamics and Molecular Docking were deployed to provide new insights into their interaction at an
88 atomic scale as those tools have widely demonstrated their value in investigating the molecular basis
89 of biological effects [31–38].

90 2. Materials and Methods

91 2.1. Homology Modelling of human tubulin isotypes

92 Due to the lack of experimentally determined 3D structures for most human tubulin isotypes,
93 $\alpha\beta$ -tubulin dimers were modeled according to previous tubulin modelling protocols based on
94 crystallographic data for bovine and porcine tubulin [39,40]. Following the nomenclature also found
95 in Leandro-García et al. (2010) [41], human isotypes β VI (Beta-1, Class VI, Gene TUBB1), β IIa (Beta
96 2A, Gene TUBB2A) and β IVa (Beta 4A, Gene TUBB4) were chosen for this analysis; β IVa and β IIa
97 were chosen due to their highest reported expression in the brain tissue with respect to other tubulin
98 isotypes (46% and 30%, respectively); β VI was chosen as a non-brain-specific control [41]. Manually
99 annotated and reviewed amino acid sequences for human tubulin isotypes α Ia, β VI, β IIa and β IVa
100 were downloaded from the UniProt database (accession codes Q71U36, Q9H4B7, Q13885 and P04350,
101 respectively). Since the goal was to model human tubulin in its dimeric form, the 3J6F [42] entry from
102 the Protein Data Bank (www.rcsb.org) was selected as a template, consisting of a minimized structure
103 of GDP-bound microtubules with a resolution of 4.9 Å. First, the alpha and beta tubulin isotypes were
104 modeled as single monomers. To do so, sequence alignment to the 3J6F target was carried out using
105 UCSF Chimera software [43] and missing residues were modelled using modeller 9.21 [44]. Then, the
106 homology model was built using modeller 9.21 with the options of building models with hydrogens,
107 using thorough optimization and performing loop refinement. All models were built including their
108 C-terminal domains. The generated models were evaluated based on the GA341 and zDOPE score,
109 inspected manually by visual comparison to the target structure, and further checked using the
110 packages PROCHECK [45], WHATCHECK [46], ERRAT [47] and Verify-3D [48]. A further quality
111 assessment was carried out using the QMEAN score [49,50] implemented in the SWISS-MODEL
112 server [51]. The same general protocol was subsequently used to generate α Ia- β -tubulin dimers for
113 every β isotype mentioned, since data regarding β tubulin isotype expression is readily available in
114 the literature and the present interaction study was also aimed at assessing differences between β
115 tubulin isotypes.

116 2.2. Molecular Docking

117 To evaluate putative binding sites for anesthetic molecules of interest, conformations for each
118 tubulin dimer were extracted every 10 ns from the second halves of each of three 100ns MD
119 simulations of the tubulin dimer with each VA and exported in pdb format, yielding 18 protein
120 snapshots for each of the three dimers, each simulated with one of the four anesthetics, for a total of
121 18 snapshots * 3 isotypes * 4 anesthetics = 216 protein snapshots. AutodockTools [52] was
122 subsequently used to add Gasteiger charges information and export the snapshots in pdbqt format.
123 The 4 anesthetics were obtained in 3D-SDF format from the DrugBank database (www.drugbank.ca)
124 , energy minimized with explicit hydrogens and exported in pdbqt in AutodockTools, again
125 assigning Gasteiger charges. Docking was performed in AutoDock-Vina [53], which accounts for
126 ligand flexibility by continuously rotating rigid parts of the ligands around rotatable bonds and
127 keeping the protein rigid. The search box, centered at the center of mass (COM) of the dimer was
128 built in order to encompass the whole dimer and perform blind docking, and the center of the search
129 box was conserved at the COM of the dimer in all blind docking runs, for all anesthetics. For all

130 docking runs, the exhaustiveness was set to of 64 and the maximum number of binding modes to be
131 generated was left at the program's default setting of 9 poses per run, providing a good compromise
132 between speed and pose sampling. With 9 conformations generated in each docking run, repeated
133 for a total of 216 protein snapshots as discussed above, a total of $9 \times 216 = 1944$ docked conformations
134 were generated, 162 for each anesthetic-isotype pair. Considering the low affinity difference between
135 predicted poses, around tenths of kcal/mol, all of these 162 docking poses were analyzed for each
136 ligand-isotype blind docking run. To facilitate the analysis and comparison of individual docking
137 results, said 162 binding poses obtained from blind docking for each isotype-VA pair were exported
138 in pdb format with the dimer, and residues within 6 Å of the ligand were extracted using GROMACS,
139 and saved along with the corresponding predicted affinity of the pose into a simple text file. A custom
140 MATLAB code was subsequently deployed to count the occurrence of each residue across all the 162
141 poses of each docking run, and the 50 most recurring residues were deemed as involved in binding
142 sites with the highest consensus, given the negligible difference in affinity among different docking
143 poses, below the noise level of $k_B T$, and further analyzed. The predicted binding affinity of each
144 anesthetic for each tubulin dimer is also reported, as mean \pm standard deviation of all output poses.

145 To check and refine the above-described blind docking approach, a local docking validation has
146 been carried out as exhaustively explained in the Supplementary Information.

147 2.3. Molecular Dynamics

148 For molecular dynamics simulations tubulin isotypes βVI , βIIa , βIVa were chosen for
149 investigation, each in its dimeric form with αIa tubulin. Each dimer was completed with GTP, GDP
150 and the Mg^{2+} ion from the 3J6F template, and each was simulated both without anesthetics, and
151 separately with halothane, desflurane, methoxyflurane and ethylene present in the surrounding
152 solvent at a concentration of 10 mM, which experimentally showed polymerization inhibition of
153 microtubules [54] and is at the upper end of the range of clinical concentrations for more potent
154 volatile anesthetics. It is to be noted that data regarding intracellular concentration of volatile
155 anesthetics during general anesthesia is fairly dispersed, reporting concentrations ranging from a few
156 mM [55] up to hundreds of mM [56], possibly due to the large differences in potency between different
157 compounds. Overall, a total of 15 systems for Molecular Dynamics simulations were obtained – 3
158 isotypes times 4 anesthetics + control without anesthetics – and each was simulated in three replicates
159 by re-initializing velocities from the Maxwell distribution at 300K at the beginning of the NVT
160 equilibration. The Visual Molecular Dynamics [57] (VMD) environment was used for visual
161 inspection of the systems and trajectories and for further roto-translational corrections. GROMACS
162 2019.1 [58] was used for MD simulations, specifically with the AMBER ff99SB-ILDN force field [59].
163 Molecular 3D structures for the 4 volatile anesthetics were obtained in 3D-SDF format from
164 DrugBank [60], energy minimized with explicit hydrogens, and their topologies, just as for GTP and
165 GDP, were generated through the ANTECHAMBER [61] package employing the AM1-BCC charge
166 method [62] and the general AMBER force field. The MD system was configured in GROMACS in a
167 dodecahedral box with xyz periodic boundary conditions and a minimum distance between the
168 protein and the box edge of 1.0 nm, to avoid interaction with periodic images. All the systems were
169 solvated with TIP3P explicit water and neutralized with counterions. Moreover, a physiological ionic
170 strength of 0.1 M was imposed by adding appropriate amounts of Na^+ and Cl^- ions. In the case of
171 systems with anesthetics, a custom script was deployed to add an appropriate number of molecules
172 given the target concentration of 10 mM. The minimization was carried out using the steepest descent
173 method, with 1000 kJ/(mol*nm) maximum force and no restriction on maximum steps. All
174 subsequent steps, namely equilibration in NVT and NPT ensembles along with the production NPT
175 simulations were carried out remotely on HPC resources. The NVT and NPT equilibrations were
176 carried out at $T=300K$ and $P=1.0$ bar, respectively, with the protein restrained and a total of 100 ps
177 each. In the case of NVT, the modified Berendsen thermostat [63] was used with τ constant of 0.1,
178 while NPT equilibration was carried out using the Parrinello-Rahman barostat [64] with isotropic
179 coupling and a τ constant of 2.0. For both NVT and NPT equilibrations, PME [65] Electrostatics were
180 used, with an interpolation order of 4 and an FFT grid spacing of 0.16 nm. Production simulation

181 followed in the NPT ensemble, without any restraint, for 100 ns per replica with a 2 fs timestep and
182 coordinate saving set every 1000 steps, i.e. every 2 ps. The stability of the tubulin dimer during the
183 simulation was determined by the RMSD of the protein backbone followed by cluster analysis, both
184 carried out in GROMACS.

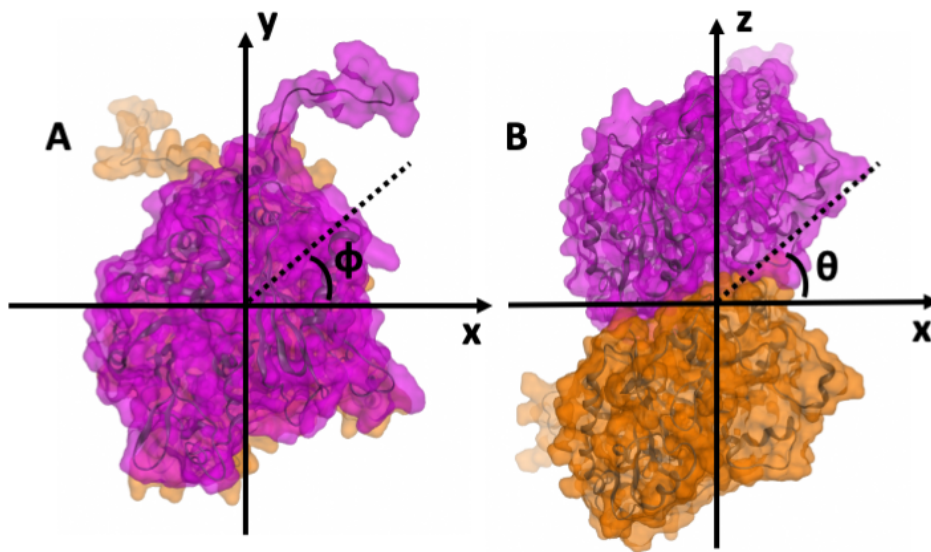
185 2.4. Analysis

186 2.4.1. Structural Effects

187 The structural effects on the dimer in the presence of the VAs were evaluated by analyzing the
188 RMSF with and without anesthetic molecules, as calculated with GROMACS excluding the highly
189 fluctuating C-terminal regions, and separately for the alpha and beta subunit of the dimer. Structural
190 effects were further assessed using cluster analysis on each simulation, as implemented in the gmxc
191 cluster tool of GROMACS, using the single linkage method with 0.15 nm RMSD cutoff. Effects on
192 secondary structure were assessed using DSSP 3.0.0 [66] after extracting snapshots every 100 ps from
193 the last 50 ns of each simulation.

194 2.4.2. Contact Probability

195 To quantitatively assess the interaction between each of the four anesthetics and the tubulin dimer,
196 the raw trajectory was analyzed: for each frame, the minimum distance between each residue and
197 any ligand molecule in the solvent was calculated with GROMACS. A custom script subsequently
198 calculated the per-residue contact probability by averaging interactions in each frame between
199 residues and ligands. Following previous computational work [38], the ligand was considered to be
200 in close contact with a residue whenever its distance to that residue fell below 2.8 Å, corresponding
201 roughly to the diameter of a water molecule, and at the end the overall probability of contact with
202 the anesthetic was obtained for each residue over the whole trajectory consisting of the concatenation
203 of the last 50 ns of each of the 3 replicates. The individual contribution of the second half (50ns) of
204 each replica of a given system to the per-residue contact probability was also calculated and is
205 reported in the Supplementary Information. Residence times of each contact event were calculated
206 by counting the number of consecutive frames in which the ligand stayed within 5 Å of a given
207 residue. This cutoff was chosen to include consecutive frames in which the ligand briefly repositions
208 itself, temporarily increasing its distance to the residue above the 2.8 Å cutoff used for contact
209 probability calculation, but effectively staying in the same binding pocket. To efficiently compare
210 contact areas between different isotopes and different anesthetics, the system was analyzed in a
211 spherical coordinate system, built as follows: the position of each dimer was aligned with a custom
212 VMD script so that the origin of the cartesian coordinate system relocated to the center of mass of the
213 dimer, between the alpha and beta subunit: this way, residues belonging to the alpha subunit had
214 coordinate $z > 0$, residues on the beta subunit had $z < 0$ and residues at the alpha-beta interface had z
215 $\cong 0$. Subsequently, another custom VMD script rotated the dimer so that the x axis was parallel to the
216 vector connecting the C α of residues α 128Q and α 285Q, which are known to be involved in lateral
217 contacts between adjacent protofilaments [67]. This allowed to broadly discriminate between
218 residues located towards the abluminal side of the microtubule, residues located towards the luminal
219 side and residues involved in lateral contacts, based on their y coordinate ($y > 0$ corresponds to
220 residues facing the outer surface of the MT, $y < 0$ residues facing the lumen and $y \cong 0$ residues
221 involved in lateral contacts between adjacent protofilaments), as highlighted in Figure 1.



222

223 **Figure 1.** (a) Schematic representation of the phi angle from a top view of the tubulin dimer, which
 224 represents the azimuthal position in the xy plane, around the z axis: positive values correspond to the
 225 outer side of the protofilament while negative values correspond to the inner side; (b) Schematic
 226 representation of the theta angle from a side view of the tubulin dimer. Elevation theta represents the
 227 angular position between the z axis and the xy plane: negative values correspond to the beta subunit
 228 (shown in orange), while positive angles represent residues on the alpha subunit (shown in pink).

229 To better represent and interpret the data, the geometrical center of each residue was determined
 230 using the MDAnalysis[68,69] toolbox for python, and its (x,y,z) Cartesian coordinates subsequently
 231 transformed into spherical coordinates with an *ad-hoc* python script. In this new system, the elevation
 232 angle theta was calculated starting from the xy-plane, so that $\theta > 0$ corresponds to points with $z > 0$
 233 and vice versa. The φ angle on the other hand represents the azimuth, i.e. the rotation around the
 234 original z axis. A 3D rendering of the tubulin dimer in this new spherical coordinate system is shown
 235 in Supplementary Video S1. Residues were represented in this new coordinate system, with the radial
 236 coordinate ignored. As a matter of fact, since contact in the 100ns trajectories only occurs on the
 237 surface, there is no chance of radial ambiguity and a single couple of (θ, φ) always identifies a single
 238 surface residue in this spherical approximation, except for the highly fluctuating C-terminus, which
 239 was checked for contact by visual inspection of the trajectories. To further enhance this
 240 representation, the surface on the tubulin dimer has been divided into sectors on the theta-phi plane.
 241 Instead of plotting the contact probability of individual residues, the contact probability was
 242 evaluated on a per-sector basis: contact was recorded on a given sector of the dimer surface in a frame
 243 of the MD trajectory if any of the residues within that sector were within 2.8 Å of any anesthetic
 244 molecule. Contact counts were subsequently normalized to the total number of frames to yield the
 245 contact probability. The resulting coordinate system, as previously discussed, implies that the four
 246 quadrants qualitatively represent distinct areas on the dimer, as reported in Table 1.

247

Table 1. Quadrants on the (theta,phi) plane and corresponding gross locations on the dimer.

Quadrant	θ	φ	Subunit	MT surface
I	> 0	> 0	Alpha	Abluminal
II	< 0	> 0	Beta	Abluminal
III	< 0	< 0	Beta	Luminal
IV	> 0	< 0	Alpha	Luminal

248

249 After qualitatively assessing the location of highly interacting residue groups in this
250 representation and checking for recurring interaction patterns across different anesthetics and
251 different tubulin isotypes, each trajectory was further analyzed manually, and the regions of
252 interaction inspected and reported. The main binding clefts where interaction consistently occurred
253 across different replicas (corresponding to dark areas on the contact probability maps) were precisely
254 defined – in terms of residues forming the clefts – and reported. Their location is also reported in the
255 previously mentioned coordinate system to aid their visual localization on the dimer and allow for a
256 direct comparison with overall contact probabilities.

257 To statistically assess whether the differences in per-residue contact probability between the four
258 tested ligands for a given isotype were significant, a one-way ANOVA was used, testing the null
259 hypothesis that the means of the contact probabilities for each group (i.e. with each of the four
260 ligands) are equal, rejected at $p < 0.05$. This allowed to determine if at least one of the ligands had a
261 significantly different probability of interacting with the tubulin dimer. To further compare the
262 contact probabilities of Ethylene, the weakest molecule used as a control reference, versus the contact
263 probabilities of the other three ligands (Desflurane, Halothane and Methoxyflurane), Dunnett's Test
264 was used with a significance threshold of $p < 0.05$.

265

266 2.5. MM/PBSA Binding Energy estimation

267 To provide a further quantitative assessment of the interaction between the four simulated
268 anesthetics and the tubulin dimer, MM/PBSA [70] estimations of binding energies were performed
269 for all four tested anesthetics. More in detail, for each of the four anesthetics, the following workflow
270 was adopted: starting from the contact maps obtained from the MD simulations with anesthetics, the
271 main binding clefts were identified as described above. Subsequently, the concatenated trajectories
272 containing the last 50 ns at equilibrium of each of the three replicas, from which the contact
273 probability maps were built as described previously, were iteratively filtered for frames where the
274 given anesthetic was present in the cleft. This was repeated for all anesthetics and for all clefts, so that
275 sub-trajectories were generated for each ligand and each cleft, representing different snapshots of the
276 bound state. These trajectories were used for MM/PBSA calculations using the *pbsa* tools included in
277 AmberTools 20 [71], after converting GROMACS trajectories and topologies into their respective
278 amber counterparts using ParmEd. Calculations were performed using one every two frames. The
279 final binding affinity and the contributions of the VDWAALS, EEL, ENPOLAR and EDISPER
280 components are also reported on the same, sectorized (θ, φ) plots as the contact probability maps, to
281 provide a direct comparison and a visual localization of the different clefts.

282

283 2.6. Plots and Figures

284 Data plots for RMSD and RMSF distributions were generated using the Grace package. Three-
285 dimensional representations of the tubulin dimers were rendered in VMD and in MOE, while
286 auxiliary figures for the spherical coordinate system were assembled in Microsoft PowerPoint. The
287 animated 3D-view of the tubulin dimer within its spherical coordinate system, with the theta and phi
288 angles highlighted, was generated using a 3D rendering of the dimer in Blender 2.80 and is available
289 in the Supplementary Information. Sectorized contact probability plots, residue count histograms
290 from docking runs and MM/PBSA binding energy maps and their corresponding decomposition
291 maps were generated in MATLAB. Detailed, 3D views of docking pose ensembles and of interesting
292 MD contact sites on the dimer and Ramachandran plots reported in Supplementary Figure S2 were
293 generated in MOE 2019.01 [72].

294 3. Results

295 3.1. Homology Modelling

296 Modeller 9.21 [73] was used to build homology models for human isotypes β VI, β IIa and β IVa
 297 in dimeric form with α 1a tubulin, starting from the 3J6F PDB template. The four modeled sequences
 298 α 1a, β VI, β IIa and β IVa shared an identity with the respective 3J6F templates of 99.32% (sequence
 299 Q71U36), 80.80% (sequence Q9H4B7), 99.06% (sequence Q13885) and 97.19% (sequence P04350)
 300 respectively. The obtained models all showed comparable zDOPE scores fluctuating around $-1.55 \pm$
 301 0.1 for β tubulin and -1.49 for the α 1a isotype. PROCHECK [45] validation reported more than 94%
 302 of residues in most favoured regions and no residue in disallowed regions for every isotype modeled.
 303 QMEAN4 values for the three modelled α - β dimers were -1.56 for $\alpha\beta$ VI, -1.49 for $\alpha\beta$ IIa and -1.67 for
 304 $\alpha\beta$ IVa, respectively, while the 3J6F template dimer had a QMEAN4 value of -0.37 . The quality of the
 305 models was confirmed through the tools Verify-3D [48], WHAT_CHECK [46] and ERRAT [47] as
 306 shown in detail in the Supplementary Material.

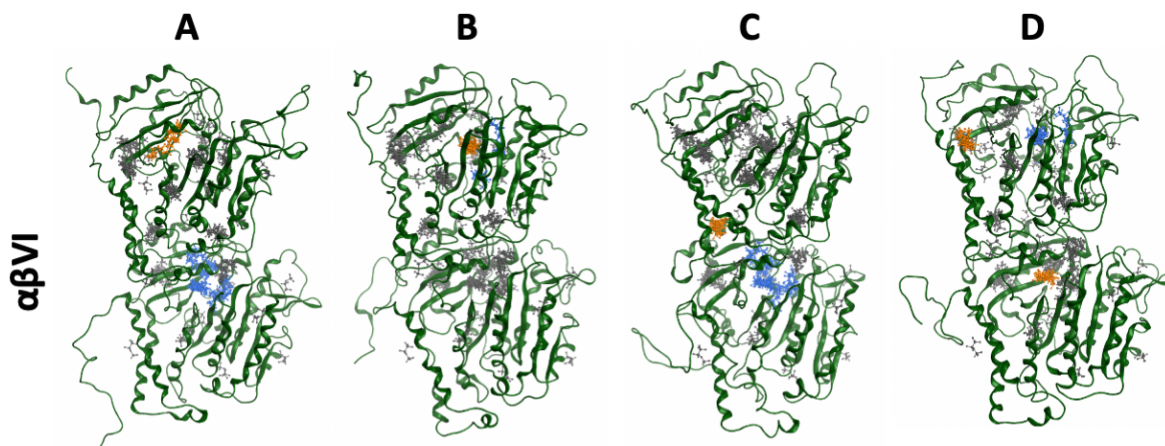
307 3.2. Docking

308 Firstly, blind docking was performed for all isotypes with all anesthetics by setting the grid box
 309 geometry so as to encompass the whole dimer, centered at the COM of the latter. Across all the 162
 310 blind docking poses determined for each anesthetic-isotype pair, the average predicted binding
 311 affinities are shown in Table 2. Data points are reported in kcal/mol, as mean \pm standard deviation.

312 **Table 2.** Predicted binding affinities in kcal/mol with each anesthetic, reported as mean and standard
 313 deviation calculated among the 162 docking poses for each isotype-anesthetic pair.

	Isotype		
	$\alpha\beta$ VI (kcal/mol)	$\alpha\beta$ IIa (kcal/mol)	$\alpha\beta$ IVa (kcal/mol)
Ethylene	-2.00 ± 0.14	-2.00 ± 0.16	-2.01 ± 0.15
Desflurane	-5.15 ± 0.24	-5.02 ± 0.22	-5.02 ± 0.23
Halothane	-4.45 ± 0.26	-4.14 ± 0.17	-4.45 ± 0.34
Methoxyflurane	-4.22 ± 0.18	-4.26 ± 0.30	-4.25 ± 0.26

314 Overall, blind docking yielded binding affinity ranges consistent with combinations of
 315 hydrophobic Van der Waals-type interactions. Ethylene consistently showed very low affinity values
 316 hovering around -2.00 kcal/mol with little differences among different poses on the same snapshot
 317 and among different snapshots of the same isotype (as seen by the comparatively low std. dev.), along
 318 with little differences between isotypes. The differences in affinity between the other three ligands
 319 were more subdued, and with a slightly higher std. dev., highlighting how some docking poses and
 320 sites were predicted to be more energetically favorable for hosting these molecules with respect to
 321 others. Overall, Desflurane reported the best predicted affinity across all three isotypes, while the
 322 predicted affinities for Halothane and Methoxyflurane were comparable.
 323



324 **Figure 2.** Ensemble of docking poses on the $\alpha\beta$ VI dimer, with individual poses shown in grey: (a)
 325 Desflurane; (b) Halothane; (c) Methoxyflurane and (d) Ethylene. The largest clusters of recurring
 326

327 poses are highlighted in blue, clusters of poses with highest average predicted binding affinity (most
328 negative) are highlighted in orange.

329 In terms of binding sites, isotype $\alpha\beta VI$ (shown in Figure 2) featured with Desflurane a large
330 number of docking poses around $\beta ILE368$, $\beta LEU250$, $\beta ALA314$, $\beta ILE236$, $\beta LEU253$ and $\beta ASP249$,
331 while the binding site with highest predicted affinity was located around $\alpha PHE202$, $\alpha ALA201$ and
332 $\alpha MET203$. In the case of Halothane, the most frequently involved residues were $\alpha TRP21$, $\alpha ALA65$,
333 $\alpha PRO63$, $\alpha GLY17$, $\alpha PHE67$, with the best overall affinity at the sites around $\alpha GLU168$, $\alpha VAL137$,
334 $\alpha LEU167$ and $\alpha PHE202$. Methoxyflurane consistently docked as Desflurane near $\beta LYS252$, $\beta LEU253$,
335 $\beta ALA254$, $\beta ALA314$, $\beta ASP249$, $\beta MET257$, $\beta ILE316$, $\beta ASN247$, an area delimited at the top by
336 $\alpha ASN101$ at the inter-monomer interface, with peaks of highest affinity around $\beta ARG251$, $\alpha ALA99$
337 and $\alpha SER178$. Ethylene did not show specific preferred binding sites, but docked with slightly more
338 frequency around $\alpha PHE138$, $\alpha VAL235$ and $\alpha PHE169$, with best affinities at the sites around $\beta ILE368$
339 and $\alpha TRP388$. In the case of isotype $\alpha\beta IIIa$ with Desflurane, the most recurring sites were two, located
340 between $\alpha TYR172$, $\alpha SER187$, $\alpha HIS139$, $\alpha SER140$, $\alpha SER170$, and $\alpha PHE141$, with one of them,
341 involving also $\alpha THR190$ and $\alpha THR191$, $\alpha GLU168$, and $\alpha THR194$ also showing the lowest average
342 affinity across the poses. With Halothane, the frequent locations were at $\beta ARG251$, $\alpha PHE141$,
343 $\alpha SER187$ and $\alpha PRO173$, with low-energy sites around $\alpha LEU92$, $\alpha ILE122$, $\alpha ALA65$, $\alpha ARG121$,
344 $\alpha GLU90$, $\alpha GLN91$, $\alpha PRO63$, $\alpha VAL62$ and $\alpha VAL66$. Methoxyflurane frequently docked at $\alpha PHE141$,
345 $\alpha SER147$, $\alpha THR194$, $\alpha GLY142$, $\alpha THR190$ and $\alpha THR191$, with the highest affinities predicted around
346 $\beta PHE294$, $\beta TYR310$ and $\beta VAL313$, $\beta MET293$. Ethylene again was not predicted to have preferred
347 sites, with a slightly higher count of poses around $\alpha TRP388$, $\alpha MET203$, $\alpha PHE267$, $\alpha ALA201$ and
348 $\alpha PHE202$, $\alpha PRO173$. A lower-energy binding site was predicted around $\beta PHE294$, also found in the
349 previous case. Lastly, docking of Desflurane to isotype $\alpha\beta IVa$ predicted three recurring binding sites
350 around $\alpha ILE384$, between $\beta VAL236$, $\beta THR237$ and $\beta GLU198$, and near $\alpha THR239$ and $\alpha LEU136$
351 respectively, with the latter also with comparatively high affinity across the different sampled poses,
352 especially in poses in close contact with $\alpha PHE138$ or $\alpha PHE135$. Halothane frequently docked into a
353 cleft lined by $\alpha LEU92$, $\alpha PHE67$, $\alpha GLN91$, $\alpha VAL14$, $\alpha VAL78$, $\alpha PHE87$ and $\alpha ASN18$. The binding site
354 with lowest average binding energy was instead located between $\beta VAL333$, $\beta GLN334$, $\beta MET330$ and
355 $\beta VAL349$. Docking of Methoxyflurane frequently accommodated the ligand in a cleft lined by
356 $\beta ALA248$, $\beta ASP249$, $\beta LEU240$, $\beta LEU253$, $\beta LYS252$, $\beta ASN247$, $\beta LEU250$ and $\beta ILE368$, which was also
357 predicted in [54], with high-affinity sites located instead surrounded by $\alpha GLY81$, $\alpha PHE67$, $\alpha PHE87$,
358 $\alpha THR82$, $\alpha TYR83$, $\alpha VAL78$ and $\alpha ARG84$. Lastly, ethylene frequently docked into a broader area
359 delimited by $\alpha TRP21$, $\alpha HIS8$, $\alpha PHE67$, $\alpha TYR24$, $\alpha GLY17$ and $\alpha VAL235$. Poses near $\alpha HIS8$ were also
360 the ones with lowest mean energy, along with poses docked to a second site near $\alpha THR150$. For a
361 graphical summary of frequently found residues across all docking poses, along with the mean
362 predicted affinity associated with the respective poses, see supplementary information.

363 To check and further refine the ligand-protein binding estimation, a second set of local docking
364 runs was performed, which confirmed data and trends reported from blind docking (see
365 Supplementary Information and Supplementary Table ST1).

366 Overall, the binding sites found by docking were generally lined by a majority of hydrophobic
367 residues, which again suggests a predominantly hydrophobic interaction, consistent with the
368 predicted affinity estimates. In blind docking, isotype $\alpha\beta VI$ showed putative interactions on both
369 subunits, isotype $\alpha\beta IIIa$ had more overall binding poses on the alpha subunit, and isotype βIVa
370 showed a similar number of binding poses on both subunits. The results from blind docking, and
371 their subsequent validation through local docking refinement, confirmed the main drawbacks of
372 docking techniques in the context of ligands interacting with low affinity, possibly within multiple,
373 energetically equivalent binding sites on the dimer simultaneously. This level of investigation is not
374 accessible to plain docking. Overall, the lack of an experimentally known set of binding sites against
375 which to perform docking, along with the mentioned methodological drawbacks such as the
376 inaccuracy of predicted affinity[74], especially in the context of the systems under investigation in
377 the present work, justified a more detailed analysis of the sites of interaction through the use of
378 Molecular Dynamics, in the light of the low interaction strength and absence of evidence for a specific,

379 'lock-and-key'-type binding site for anesthetics. Indeed, the adopted molecular dynamics approach
380 allows for a more efficient sampling of multiple, simultaneous, low-affinity binding sites on the dimer
381 surface, by simulating the dimer with a fixed anesthetic concentration in the solvent. This enables the
382 statistical investigation of the most explored areas, which is not feasible through docking alone.

383 3.3. Protein Ligand binding dynamics

384 3.3.1 Molecular Dynamics stability analysis

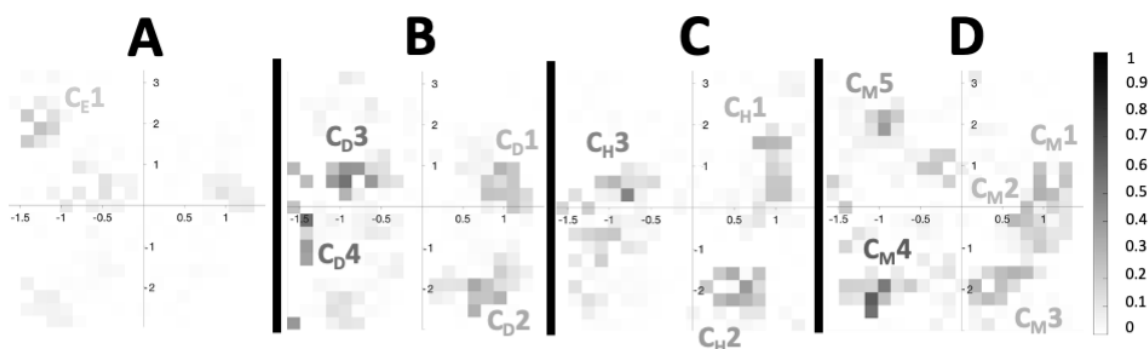
385 First, the stability of all the simulations was checked by examining the RMSD over each 100ns
386 trajectory. All three isotypes had reached a plateau after about 50 ns, with the $\alpha\beta\text{IVa}$ dimer in the
387 presence of Desflurane being the only case with slightly more accentuated fluctuations up to about
388 70 ns. See Supplementary Figure S3 for the complete RMSD details. Potential energy plots for all
389 simulated systems are reported in Supplementary Figure S4.
390

391 3.3.2 Protein-Ligand interaction dynamics

392 Molecular Dynamics simulations allow to overcome the limitations of the docking approach in the
393 context of low-affinity ligands, such as the volatile anesthetics investigated in the present work,
394 which interact simultaneously in multiple sites on the tubulin dimer. Simulating the dimers in the
395 presence of VAs in the surrounding solvent at a fixed concentration enables a more significant
396 sampling of frequent interaction clefts, including simultaneous interaction in multiple, low-affinity
397 binding sites, and provides a quantitative assessment thereof, which is precluded to single-ligand
398 docking in the case of weak binding events.

399 Per-residue contact probabilities extracted from MD simulations show preferential interaction
400 with specific residues for each anesthetic and for each isotype. The contact probability plots, reported
401 in the Supplementary Information, visually highlight how the different anesthetics interact with
402 partially different strengths and in specific locations, both on the same tubulin isotype and across
403 different isotypes, albeit some commonly involved residues emerge. Ethylene clearly displays lower
404 overall contact probabilities, in a manner consistent both with its considerably lower potency in clinic
405 and with the significantly lower predicted affinities in blind and local docking runs. To better
406 highlight the actual location where binding occurs, both longitudinally along the major axis of the
407 dimer (i.e. *on which subunit and how far from the inter-monomer interface*), and circumferentially around
408 said axis (i.e. *where around the dimer*), the contact probabilities are reported on a sectorized spherical
409 coordinate system in terms of theta and phi angles, where elevation theta discriminates between
410 subunits and azimuth phi locates residues around the dimer (see details described in the Methods
411 section). Such contact maps highlight the patterns of interaction between each anesthetic and specific
412 tubulin isotypes and provide qualitative information about preferential binding location both around
413 the dimer (luminal vs. abluminal side in the MT) and longitudinally along the major axis (top α -
414 subunit vs. α - β interface vs. bottom β -subunit). The resulting high-probability contact areas are
415 shown in Figure 4 for the most highly interacting isotype, namely $\alpha\beta\text{IVa}$, which is also the most
416 highly expressed in the brain.

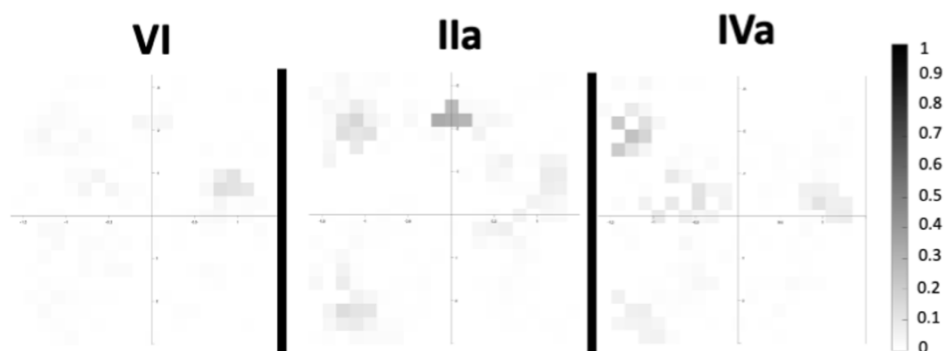
417 To visualize the proximity of tryptophan residues, which is a key requirement of the Quantum
418 Mobility theory of anesthetic action [18], also in comparison to previous computational work [1]
419 predicting aromatic amino acids as functional targets of anesthetics on tubulin, the location of Trp
420 residues on the tubulin dimer in this spherical coordinate representation is shown as orange crosses
421 on the heatmaps in Supplementary Figure S6.



422

423 **Figure 3.** Per-area contact probability for isotype $\alpha\beta IVa$, the one with the most overall interactions,
 424 with all the tested ligands. (a) Ethylene; (b) Desflurane; (c) Halothane and (d) Methoxyflurane. Color
 425 scale is 0 to 1, i.e. 0% to 100% probability of interaction with any residue within a given area. The main
 426 recurring interaction clefts are also highlighted, with the subscript differentiating the four ligands.

427 Briefly, Desflurane interacts substantially on the β subunit of isotype $\alpha\beta VI$, both on the luminal
 428 and abluminal side of the dimer, along with different inter-monomer transient contact clefts. A
 429 distinct interaction zone also emerges on subunit alpha laterally (where φ is close to 0), enclosed by
 430 residues $\alpha 293ASN$ on helix $\alpha H9$ and $\alpha 334THR$ on helix $\alpha H10$. On isotype $\alpha\beta IIIa$, interaction was
 431 recurring more markedly on the α subunit mainly at the same lateral contact area already seen in the
 432 previous case between H9 and H10. Minor contact probabilities were also recorded on the luminal
 433 side of the alpha subunit, and on the abluminal and lateral area of subunit beta. The interaction is
 434 similar on isotype $\alpha\beta IVa$ (Figure 3B), with a high probability of residence laterally between H9 and
 435 H10 subunit alpha, with the addition of recurring interaction on the β subunit, laterally in close
 436 proximity to the exchangeable GTP binding site, between helix H6 and the start of H7, near residues
 437 $\beta 208TYR$, $\beta 221THR$ and $\beta 225LEU$, and transiently on the rest of the surface. Halothane showed
 438 slightly lower overall contact probabilities, especially on isotype $\alpha\beta IIIa$. A high probability of
 439 residence is again visible on the α subunit's H9-H10 lateral contact zone on all three simulated
 440 isotypes. Interaction sites were more abundant on isotype βIVa (Figure 3C), a substantial fraction of
 441 which on the luminal side of the dimer, with two distinct clusters on the α and β subunit and the
 442 addition of an interaction site on the lateral contact area of the β subunit, a semi-closed cleft
 443 defined by residues $\beta 231ALA$ and $\beta 227HIS$ belonging to helix H7 and capped by the sidechain of $\beta 276ARG$
 444 of loop S7-H9 which folds over the ligand molecules (up to two halothane molecules at the same time
 445 seen during the simulation). Methoxyflurane, the most potent anesthetic – i.e. the one with the lowest
 446 MAC – showed the highest number of high-probability contact sites for all three simulated isotypes,
 447 with the addition of the highest overall residence times (and thus contact probabilities). Interaction
 448 was ubiquitous on isotype $\alpha\beta VI$, with contacting residues localized on both subunits and on either
 449 side, luminal and abluminal. Isotype $\alpha\beta IIIa$ showed mostly overlapping areas of preferential
 450 residence. The interaction with isotype $\alpha\beta IVa$ (Figure 4D) was recorded both on the luminal and
 451 abluminal sides of both the α and β subunit. Notably, this isotype showed a unique interaction area
 452 located on the luminal side of the beta subunit, in a binding site near $\beta GLY79$ at the end of helix H2.
 453 The lateral contact area towards the top of the α subunit was again involved in numerous contacts
 454 with methoxyflurane on all three isotypes.



455

456 **Figure 4.** Contact probabilities of Ethylene with all three simulated isotypes (left to right: $\alpha\beta$ VI, $\alpha\beta$ IIa,
457 $\alpha\beta$ IVa), which is visibly lower than the amount of contact of the other three anesthetics.

458 Ethylene (Figure 4) showed a much weaker overall interaction with the tubulin dimer, visible
459 both during the simulations themselves and on the contact probability analysis. With the exception
460 of a more frequent interaction on isotype $\alpha\beta$ IIa, on the abluminal site of the α - β inter-monomer
461 interface (a cleft between H11 and H12, on H11', lined by residues β 413MET, β 415GLU, β 409VAL,
462 β 418PHE and β 408TYR), residence on the surface of the dimer was transient and with very low
463 overall probability, demonstrating how the ligand spends most of the simulation time floating freely
464 in the solvent. This is consistent not only with the significantly lower predicted affinities through
465 docking and low molecular weight of the molecule, but also with the much lower efficacy as a volatile
466 anesthetic if compared to the other compounds.

467 In summary, the analysis of the described interaction hotspots, i.e. the areas with highest contact
468 probabilities visible as dark zones in Figures 3 and 4, pinpoints the location of specific transient
469 binding clefts. These were characterized in detail, and are marked in Figure 3, numbered
470 progressively and with the subscript indicating the involved anesthetic. The detailed description of
471 the main binding clefts, with their adopted nomenclature and the list of involved residues, is
472 provided in the following Table 3 for isotype $\alpha\beta$ IVa. The corresponding table for isotypes $\alpha\beta$ VI and
473 $\alpha\beta$ IIa is provided in the Supplementary Information.

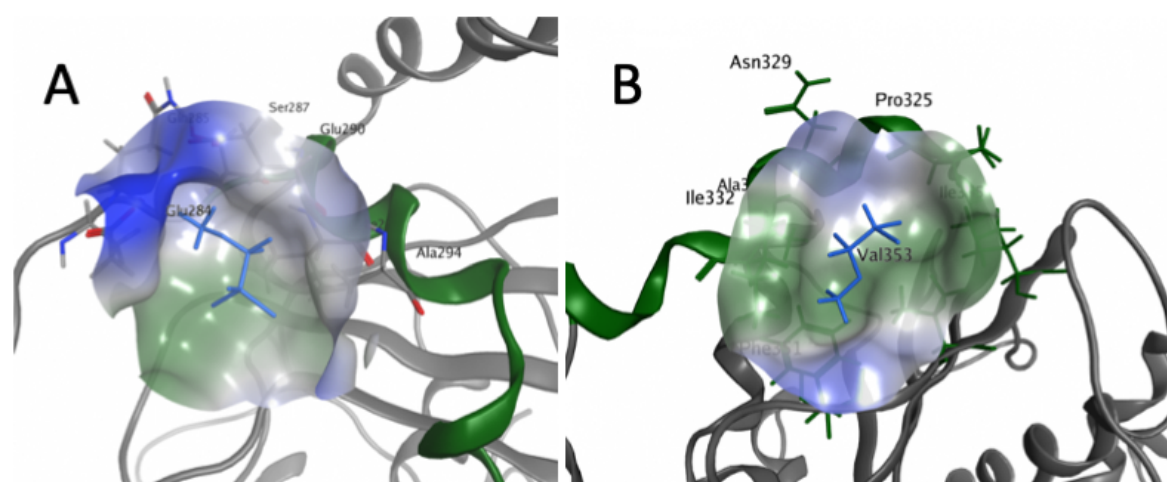
474

475 **Table 3.** Binding clefts for isotype $\alpha\beta$ IVa for all the simulated anesthetics.

Ligand	Cleft	Residues
Desflurane	C _{D1}	α ILE335, α LYS336, α ILE332, α ASN329, α VAL328, α PRO325, α GLY350, α PHE351, α VAL353, α ILE355
	C _{D2}	α GLU90, α GLN91, α PRO89, α LEU125, α LYS124
	C _{D3}	β ARG306, β PRO305, β PHE294, β ASN295, β ALA296, β ASN337, β SER339
	C _{D4}	β ASN204, β GLU205, β TYR208, β VAL175, β THR221, β TYR222, β PRO220
Halothane	C _{H1}	α ILE335, α LYS336, α ILE332, α ASN329, α VAL328, α PRO325, α GLY350, α PHE351, α VAL353, α ILE355
	C _{H2}	α GLU90, α GLN91, α PRO89, α LEU125, α LYS124, α ILE75, α VAL78, α ARG79, α ARG84
	C _{H3}	β ARG306, β PRO305, β PHE294, β ASN295, β ALA296, β ASN337, β SER339
Methoxy-flurane	C _{M1}	α ILE335, α LYS336, α ILE332, α ASN329, α VAL328, α PRO325, α GLY350, α PHE351, α VAL353, α ILE355
	C _{M2}	α SER287, α GLU290, α ALA294, α ILE276, α LYS280, α ALA281, α GLU284
	C _{M3}	α THR82, α TYR83, α ARG84, α ARG79, α PHE87
	C _{M4}	β ASP74, β GLY71, β PRO70, β PRO87, β ASN89, β PHE90, β VAL91, β MET73, β VAL76
	C _{M5}	β ARG390, β PHE389, β MET415, β ASN414, β ASP417
Ethylene	C _{E1}	β PRO182, β ALA185, β VAL170, β SER168, β SER188, β VAL189

476

477 The interaction sites are distinct with each anesthetic but with notable overlaps, the most
478 important of which is a vast binding area located on the upper part of the α subunit in the lateral PF-
479 PF contact zone, and comprising clefts C_{D1} , C_{H1} and C_{M1} in all isotypes and C_{M2} (isotype $\alpha\beta IVa$ and
480 $\alpha\beta VI$) and C_{D2} (isotype $\alpha\beta VI$). Every anesthetic, except ethylene, stuck to a cleft in this area for a
481 significant portion of the simulation, on all three isotypes. As discussed, this cleft is in fact formed by
482 two distinct hydrophobic patches located at the proximity of helices H9 and H10, respectively, the
483 latter located towards the top of the alpha subunit, thus actually on the longitudinal dimer-dimer
484 interface. The first area, corresponding to clefts C1 and shown in Figure 5A, largely consists of a
485 hydrophobic patch delimited on the lower part by the start of the S7-H9 loop and the final residues
486 of S7, and on the upper part by helix H9 and the last residues of the S7-H9 loop, able to accommodate
487 lipophilic ligands.



488

489 **Figure 5.** Rendering of the two sites on the alpha subunit. (a) cleft C_{M2} near helix H9 (shown in green
490 ribbons); (b) cleft C_{M1} near helix H10 (shown in green ribbons), at the top of the dimer. The ligand
491 shown in purple is Methoxyflurane, as extracted from a snapshot of the simulation with isotype $\alpha\beta VI$.
492 Rendering includes molecular surface in transparency, with lipophilic areas shown in green and
493 hydrophilic areas shown in blue. Labels indicate nearby residues forming the cleft.

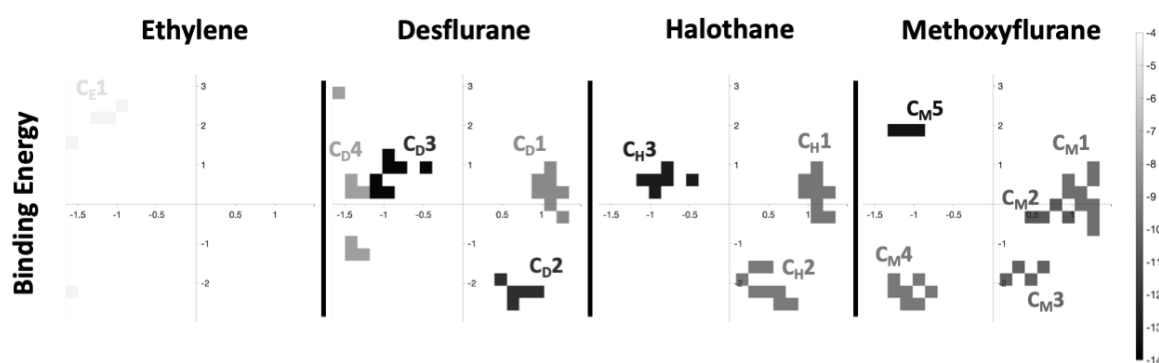
494 The second cleft, which corresponds to cleft C_{M1} shown in Figure 5B, is a nearby, mostly
495 lipophilic patch located at the top of the alpha subunit and formed by helix H10 at the top and
496 delimited by sheet S9 at the bottom

497 The broader picture of the binding patterns highlights how contact probabilities for each residue
498 change with different ligands (ANOVA $p < 0.0001$ for all three isotypes), more specifically pointing
499 towards a clearly weaker interaction of Ethylene with all three isotypes, with mean contact
500 probabilities significantly lower than the other three anesthetics (Dunnett's multiple comparisons
501 test: $p < 0.0001$ for all isotypes and ligands, except $\alpha\beta IIa$ with Halothane vs. Ethylene $p = 0.0015$), and a
502 global contact probability peak of 0.27 only with isotype $\alpha\beta IIa$. Ethylene also showed a substantially
503 lower predicted binding energy, coherently with the previous finding. Also, Methoxyflurane shows
504 the most ubiquitous interactions with high overall contact probabilities during the simulation,
505 peaking at 0.48 with $\alpha ASN329$ on isotype $\alpha\beta IVa$, located in cleft C_{M1} (also visible as a darker area in
506 Figure 3). Desflurane and Halothane show similar interaction patterns, both in terms of locations and
507 probabilities, consistent with interactions in sterically compatible lipophilic patches located around
508 the dimer. Notably, some interaction occurs on the side of the dimer facing the MT lumen
509 (corresponding to the lower part of the graphs in Figures 3, 4 and 6 where $\varphi < 0$), especially on isotype
510 $\alpha\beta IVa$ with methoxyflurane. Whether the MT lumen remains actually accessible to these ligands
511 when the MT is assembled requires further investigations, but given the dimensions of these
512 anesthetics, this appears to be feasible.

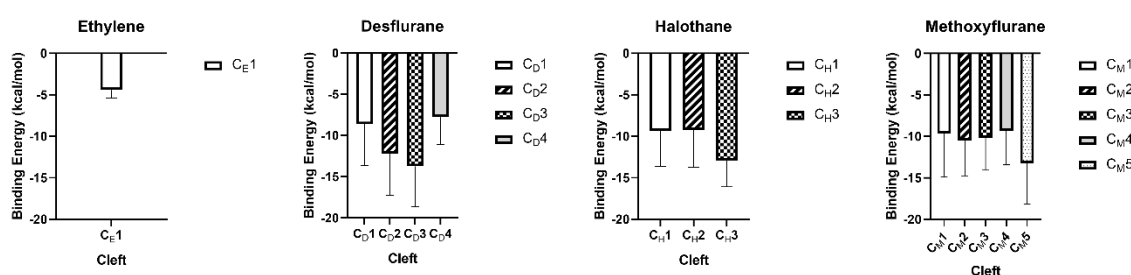
513

514 3.3.3 Protein-Ligand interaction energies

515 Binding energies for each anesthetic were predicted using the MM/PBSA method implemented in
 516 AmberTools, separately for each of the clefts reported in Table 3. Overall, predicted binding energies
 517 for Desflurane ranged from -7.10 ± 3.58 kcal/mol (isotype $\alpha\beta\text{VI}$, cleft $\text{C}_{\text{D}1}$) to -14.89 ± 6.83 kcal/mol
 518 (isotype $\alpha\beta\text{VI}$, cleft $\text{C}_{\text{D}4}$). In the case of Halothane, energies ranged from -6.31 ± 3.16 kcal/mol (isotype
 519 $\alpha\beta\text{IIa}$, cleft $\text{C}_{\text{H}1}$) to -12.91 ± 3.17 kcal/mol (isotype $\alpha\beta\text{IVa}$, cleft $\text{C}_{\text{H}3}$). Methoxyflurane featured binding
 520 energies from -7.13 ± 3.80 kcal/mol (isotype $\alpha\beta\text{VI}$, cleft $\text{C}_{\text{M}1}$) to -14.55 ± 4.39 kcal/mol (isotype $\alpha\beta\text{IIa}$,
 521 cleft $\text{C}_{\text{M}2}$). Lastly, in comparison, the predicted energies of Ethylene ranged from a minimum of -4.43
 522 ± 0.97 kcal/mol (isotype $\alpha\beta\text{IVa}$, cleft $\text{C}_{\text{E}1}$) to a maximum of -6.72 ± 1.74 kcal/mol (isotype $\alpha\beta\text{IIa}$, cleft
 523 $\text{C}_{\text{E}1}$). The detailed map containing only the interaction clefts for all four ligands on isotype $\alpha\beta\text{IVa}$ is
 524 reported in Figure 6, where color intensity represents the predicted binding energy. The latter are
 525 also reported for all clefts and all ligands in Figure 7 as means with standard deviations. The
 526 decomposition of binding energies into VDWAAALS, EEL, ENPOLAR and EDISPER components is
 527 reported for isotype $\alpha\beta\text{IVa}$ in Supplementary Figure S8 and shows the relative contribution of each
 528 term to the overall calculated binding energy, separately for each anesthetic and each binding site.
 529



530
 531 **Figure 6.** Per-area MM/PBSA binding energy estimate for isotype $\alpha\beta\text{IVa}$, the one with the most overall
 532 interactions, with all the tested ligands. Left to right: Ethylene; Desflurane; Halothane;
 533 Methoxyflurane. Color scale is -4 kcal/mol to -14 kcal/mol. The different clefts are highlighted in the
 534 figure.



535
 536 **Figure 7.** MM/PBSA binding energy estimates for isotype $\alpha\beta\text{IVa}$ with all the simulated ligands. Left
 537 to right: Ethylene; Desflurane; Halothane; Methoxyflurane. Data reported as mean with standard
 538 deviation bars for all clefts determined for each ligand.

539
 540 Rather than representing definitive estimations for binding affinities, the collected data do
 541 however allow for a quantitative comparison between clefts and between different ligands, and show
 542 how the predicted binding energy is dependent of the specific surface cleft the ligand interacts with;
 543 the energy ranges and standard deviations yielded by MM/PBSA calculations are explainable with
 544 the transient nature of surface contacts inside the reported clefts, which do not allow, in the time
 545 scales investigated in the present work, for the formation of a protein-ligand complex which remains

546 stable throughout the simulation. Rather, local alterations of sidechain arrangements permit the
547 temporary accommodation of dissolved ligand in specific clefts, with average residence times as
548 reported below. Interestingly, while all anesthetics tend to preferentially sample specific locations of
549 the dimer surface, as discussed in the contact probability analysis and shown in figures 4, 5 and S7,
550 the actual clefts where they eventually accommodate into are not always exactly the same, as detailed
551 in Table 3 and visible in figure 6. In the case of isotype $\alpha\beta IVa$, all ligands interact in the same cleft C1,
552 whereas cleft C2 is largely the same for Desflurane and Halothane, but slightly shifted for
553 Methoxyflurane, where it corresponds to cleft C3. The latter also featured three additional clefts C2
554 (near C1), C4 and C5 where no consistent binding emerged for the other anesthetics. Desflurane also
555 showed a specific binding cleft, C4, located at the bottom of subunit beta towards the polymerization
556 interface. This data confirms at the same time both the consistency of some binding sites, able to
557 accommodate different ligands, and the existence of interaction areas which are selective towards
558 some of the anesthetics. Also, as visible in Figures 6 and 7, Ethylene consistently showed the weakest
559 predicted binding energy – and with lowest standard deviation – and the existence of only one weak
560 binding site, in line with the previous contact probability analysis highlighting only negligible
561 interaction.
562

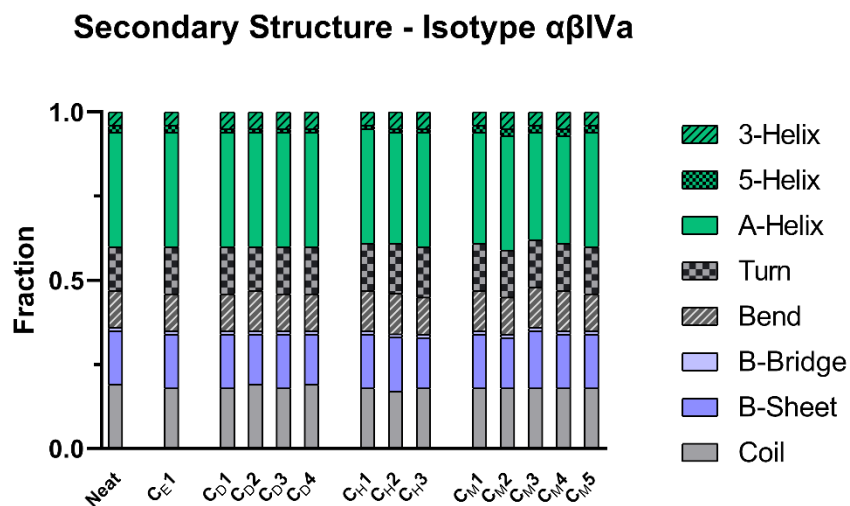
563 3.3.4 Residence Times

564 Residence times were generally consistent with the reported contact probabilities: on isotype
565 $\alpha\beta VI$, Desflurane (Figure S22A) showed residence times in high-probability contact areas between 8
566 and 25 ns. Interestingly, the area with highest reported contact probability featured at the same time
567 short average residence times, which is indicative of frequent short contacts, as opposed to stable
568 binding. Halothane (Figure S22D) featured residence times between 3 and 25 ns near highly
569 interacting residues, again with the area of peak probability showing frequent contacts of 7.5 ns on
570 average. Methoxyflurane (Figure S22G) consistently showed average residence times between 6 and
571 24 ns near residues with high reported contact probabilities, most of them being above 15 ns where
572 contact probability was the highest. As for the previous ligands, it showed an area on the alpha chain
573 with frequent and short contacts (between 2 and 7 ns on average), as seen by the high reported per-
574 residue contact probabilities, up to 0.37. Ethylene on the other hand (Figure S22L) featured residence
575 times consistently below 5 ns, in good agreement with the low reported contact probabilities. On
576 isotype $\alpha\beta IIIa$ the average residence times of Desflurane (Figure S22B) spanned from 5 to 12 ns in
577 areas with high contact probability. Again, high-probability contact residues on the alpha chain
578 featured short mean residence times, below 10 ns, despite contact probabilities up to 0.35. Residence
579 times for Halothane (Figure S22E) were between 7 and 22 ns on average around highly interacting
580 residues, with the same area of short contacts below 10 ns on the alpha chain, with contact
581 probabilities up to 0.22. Methoxyflurane (Figure S22H) featured the highest mean residence times,
582 up to 23 ns and consistently above 12 ns in areas with high contact probability of up to 0.41. Ethylene
583 (Figure S22M) confirmed the short residence times seen in the previous isotype, below 7 ns in most
584 of the interacting residues, and with a maximum below 8 ns in the area with the highest contact
585 probability (0.27). Lastly on isotype $\alpha\beta IVa$, Desflurane (Figure S22C) interaction lasted on average
586 up to 25 ns on chain alpha, and spanning from 4 to 19 ns in most other areas with high contact
587 probability. Interestingly, the residue group with highest contact probability (0.56) also showed low
588 average residence times, below 5 ns, indicative again of frequent short contacts, i.e. lower stability
589 inside the cleft. Halothane (Figure S22F) residence times were higher on average, between 10 and 25
590 ns in most interaction areas. Also, contact probability peaks correspond to higher average residence
591 times in all cases except one, near $\beta 295ASP$, where a contact probability of 0.48 corresponded to mean
592 residence times of 12 ns. In the case of Methoxyflurane (Figure S22I), areas with high contact
593 probability corresponded to average residence times of the ligand between 4 and 25 ns, while the
594 highest-probability cleft interacted on average for 14 ns. Finally, average residence times of ethylene
595 (Figure S22N) never topped 5 ns, in agreement with the comparably low contact probability (0.21 at

596 most). Interestingly, most areas with high contact probability showed particularly low residence
597 times, below 1 ns, indicative of the lack of stable binding clefts.
598

599 3.3.5 Conformational Analysis

600 The RMSF analysis in the presence and absence of anesthetic molecules, respectively, focused
601 on differences in C-alpha backbone fluctuations, for each of the three dimers. It is reported in detail
602 in the Supplementary Information. Overall, different isotypes show slightly different behaviors in
603 the presence of different anesthetics: The β M loop was destabilized on isotype $\alpha\beta$ VI with Desflurane
604 and Methoxyflurane, while it showed decreased mobility in isotype $\alpha\beta$ IIa with Halothane,
605 Methoxyflurane and Ethylene and in isotype $\alpha\beta$ IVa with halothane. A visible increase in fluctuations
606 is reported in the area of residues 235-245 on the beta chain of isotype $\alpha\beta$ IIa in the presence of
607 Methoxyflurane and Desflurane. The same isotype showed a similar spike in RMSF around residue
608 320 in the presence of Desflurane and Ethylene. Isotype $\alpha\beta$ IVa showed increased fluctuations on the
609 β subunit at residues 325 to 340 with anesthetics compared to the control condition, which was not
610 evident for the $\alpha\beta$ VI and $\alpha\beta$ IIa dimers. Overall, no major conformational changes were observed over
611 the course of the simulations: cluster analysis of the trajectories, performed with a 0.15 nm cutoff,
612 both with and without anesthetics, yielded a single dominant conformation for each run. In terms of
613 secondary structure, possible alterations were assessed quantitatively using DSSP, comparing the
614 secondary structure in the control simulations with the secondary structure in the ligand-bound state,
615 separately for each binding site. As shown in Figure 8 for isotype $\alpha\beta$ IVa, no significant secondary
616 structure alteration emerges throughout the dimer upon ligand binding in the different clefts.
617 Differential interaction with different anesthetics might thus not be directly related to major
618 conformational changes of the tubulin dimer.
619



620 **Figure 8.** DSSP average Secondary Structure of the dimer in the control simulation without any
621 ligands (“Neat”) vs. in the ligand-bound states, differentiated between different binding clefts. No
622 significant alterations emerge.
623
624

625 4. Discussion

626 Blind docking of anesthetics to tubulin dimers $\alpha\beta$ VI, $\alpha\beta$ IIa and $\alpha\beta$ IVa highlighted low binding
627 affinities compatible with a combination of hydrophobic interactions with surrounding residues.
628 What emerges is a substantially indistinguishable predicted affinity between Halothane and
629 Methoxyflurane, at a thermal noise level of $k_B T$, while affinity of Desflurane was predicted to be
630 minimally better with respect to all three isotypes. What is consistently predicted is a much weaker
631 affinity of Ethylene to all three isotypes, barely completing docking runs successfully and averaging

632 at around -2.00 kcal/mol in every run. The important hint provided by blind docking experiments
633 points toward the lack of a precise binding site, rather a preference for specific hydrophobic pockets
634 of the tubulin dimers, able to transiently accommodate the anesthetic molecules. This underlined the
635 weakness of the docking approach alone in the case of weak binders which interact at multiple sites
636 simultaneously, and warranted a more thorough investigation of the interaction, in its dynamic
637 nature, by simulating the dimer in the presence of anesthetic agents in the surrounding medium at
638 fixed concentration.

639 Analysis of residue groups on the dimer surface with high probability of contact with each
640 anesthetic confirmed that the tested compounds do not seem to have a single, specific binding site on
641 the target, but they do, however, stay in contact with the dimer for prolonged times in specific clefts.
642 These areas are partially overlapping for all isotypes and all tested VAs, with the notable exception
643 of Ethylene, the weakest among the four, which showed a tendency to remain floating in the solvent
644 rather than sticking to the dimer surface. The transient residence of volatile anesthetics may alter local
645 mobility of residue sidechains with functional consequences on the MT, especially in the light of the
646 high amount of predicted contact both in the luminal and lateral side of the tubulin dimers, where
647 adjacent protofilaments assemble, as well as on the dimer-dimer polymerization interface. Contact
648 probability, directly correlated to residence time at specific locations, confirmed similar interactions
649 of Desflurane and Halothane, and a slightly increased interaction of Methoxyflurane with all
650 isotypes. Moreover, the significantly weaker interactions of Ethylene emerged, consistent both with
651 blind docking affinity estimates and with clinical potencies. Above all, the existence of different
652 binding clefts, some of which shared between different anesthetics, some specific to a particular
653 ligand, was confirmed. MM/PBSA predicted binding energies that were comparable, within error,
654 between Desflurane, Halothane and Methoxyflurane, but again visibly lower for Ethylene, in
655 agreement with the much lower contact probability.

656 Most notably, the three VAs Desflurane, Halothane and Methoxyflurane interacted in all the
657 simulations of all three isotypes on the upper portion of the alpha subunit, predominantly in two
658 lipophilic patches located near helices H9 and H10, an area corresponding in spherical coordinates
659 to values of φ close to 0 and θ between 0.75 and 1.25. The patch around H9 is located on the lateral
660 PF-PF contact area of the dimer, and might alter PF assembly in the presence of ligands, while the
661 lipophilic pocket at helix H10, at the top of subunit alpha, might have functional consequences on
662 tubulin polymerization in the process of dimer-dimer assembly. Ethylene did not show any
663 interaction within these pockets.

664 Contact probability heatmaps also hint at how some high probability interaction zones were
665 located near tryptophan residues, especially in the case of Halothane and Methoxyflurane on the
666 alpha subunit in the area where α TRP21 is localized, i.e. $0.7 < \theta < 1.1$ and $0 < \varphi < 1$ in the spherical
667 coordinate system (Supplementary Figure S7). The quantitative assessment of the involvement of
668 Tryptophans in the interaction with anesthetic molecules requires further, more detailed work,
669 possibly with higher-resolution methodologies. A direct role of tryptophan residues in the analyzed
670 binding clefts was not confirmed with the methodologies used in the present work.

671 The interaction between volatile anesthetics and tubulin has been evaluated synergistically both
672 through blind docking and Molecular Dynamics. The former approach confirmed the weak and
673 transitory nature of putative binding sites suggested by previous research [54], by failing to highlight
674 a single specific region of interaction and consistently reporting low predicted affinities across the
675 different binding pockets. This consideration, along with the known limitations of blind docking [74],
676 and the lack of single, high-affinity binding site following the more traditional lock-and-key
677 paradigm for the investigated ligands, justified a more in depth analysis of the interaction through
678 the use of molecular dynamics. In this last approach, the three different $\alpha\beta$ -tubulin dimers have been
679 simulated in the presence of a fixed concentration of anesthetics in the surrounding medium. To
680 account for the dynamic nature of the interaction, *hotspots* of interaction have been determined on the
681 dimer by sampling the contact probability between tubulin and anesthetic molecules on different
682 portions of the dimer surface. Subsequently, precise binding clefts were determined from the contact
683 map for further binding energy estimation. First, this clearly showed that interaction does feature

684 preferential areas on the dimer surface and does not occur randomly. Rather, it appears to be driven
685 mostly by the lipophilicity of the tested VAs. Secondly, it highlighted differences in interaction mostly
686 between different anesthetics rather than between different tubulin isotypes: a given anesthetic tends
687 to interact with specific areas of the dimer for tens of nanoseconds, and interaction may occur in close
688 proximity of key functional residues of the microtubule. The areas of interaction were reproduced
689 consistently, although with different residence times, in the different replicas, despite the low affinity
690 of VAs for tubulin and the lack of a single, high-affinity binding site. There is no predicted preference
691 of the simulated anesthetic agents for a specific tubulin isotype. More interestingly, a consistent
692 amount of interaction is predicted to occur on the luminal surface of the assembled microtubule.
693 Whether this area is accessible to volatile anesthetics and under which conditions, along with the
694 functional and structural consequences of this on the microtubule structure, warrants further
695 computational and experimental research. Since larger molecules, such as paclitaxel or epothilone,
696 are known to bind on the luminal surface of microtubules due to the diffusion through the nanopores
697 formed between neighboring tubulin dimers, a similar ability to reach the microtubule lumen appears
698 entirely possible.

699 5. Conclusions

700 The present work computationally investigated the interaction between four distinct Volatile
701 Anesthetics with different clinical potencies with human tubulin dimers, through Molecular Docking
702 and Molecular Dynamics. The simulated isotypes are highly homologous, but each features a unique
703 distribution across different organs and tissues, and the interaction of VAs with each of them
704 appeared to be similar, but not identical. Results confirmed the absence of a lock-and-key type of
705 interaction, and highlighted transient interactions on specific hotspots of the tubulin dimer, i.e.
706 hydrophobic patches able to transiently accommodate the ligands. Methoxyflurane, the most potent
707 among the tested VAs, showed the highest contact probability on all three simulated isotypes, while
708 Ethylene, the weakest VA, had the lowest predicted binding affinity in Docking, the lowest overall
709 contact probability in molecular dynamics simulations, and the lowest predicted binding energy in
710 MM/PBSA calculations. These findings are consistent with previous works exploring the weak
711 interaction between tubulin and anesthetics [9,24,29,75,76]. No distinct preference for a specific
712 isotype emerges, while different anesthetics did show different interaction hotspots on the dimer
713 surface, with only partial overlaps between them, the most notable of which is composed of two
714 hydrophobic patches at the top of the alpha subunit, which interacted with all VAs except ethylene
715 for a significant fraction of the simulations. Whether VAs can actually disrupt or alter microtubule
716 assembly and dynamics, and how this process may occur, demands further investigations. While this
717 process may not be directly involved in the primary mode of action of General Anesthetics, several
718 considerations underline the importance of possible VA-tubulin interactions in the clinical context,
719 including the abundance and peculiar anisotropic spatial organization of tubulin and microtubules
720 in the brain; the role of microtubules in disorders such as POCD; putative cross-interactions with MT-
721 targeting chemotherapies in oncological patients; side-effects in the presence of neurodegenerative
722 diseases involving an altered MT cytoskeleton. In this context, effects of anesthetics could be of
723 significance in the clinical setting and are worth exploring further.
724

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728

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731 interpreted the results and proof-read the paper. MC provided supervision about general anesthetics and their
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