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Research paper

Biophysical interaction of temozolomide and its active metabolite with biomembrane models: The relevance of drug-membrane interaction for Glioblastoma Multiforme therapy

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ABSTRACT

Temozolomide (TMZ) is the first-line treatment for Glioblastoma Multiforme (GBM). After administration, TMZ is rapidly converted into its active metabolite (MTIC). However, its pharmacological activity is reduced due MTIC low bioavailability in the brain. Since drugs' permeability through biological barriers and tumor cell membranes affects its bioavailability, the ability of MTIC to interact with the biological membranes presents a major contribution on its pharmacological properties and activity. Biomembrane models mimic the physiological conditions, allowing to predict the drug's behavior at biological membranes and its effects on drug biodistribution profiles. In this work, lipid bilayer models using liposomes were applied for the drug-membrane interaction studies. The zwitterionic phospholipid, 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC), and cholesterol were chosen for the composition of the model, since they represent the major components of the membranes of GBM cells and brain capillary endothelial cell. Thus, the molecular interactions between MTIC and these models were studied by the evaluation of the partition of the drug into the phospholipid's membrane, its location within the bilayer and its effect on the fluidity of the membrane. The attained results suggest that the composition of membranes affects drugs partition, showing that drug biodistribution depends not only on its physicochemical features, but also depends on the characteristics of the membrane such as the packing of the lipid molecules. Also, MTIC exhibited low affinity to biological membranes, explaining its low bioavailability on the target cells.

1. Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive type of brain tumor, presenting high mortality rates. Patients with GBM present high resistance to therapy, due the high proliferation rate of this type of tumor. The first-line treatment combines surgery, radiation and chemotherapy. Though, is not effective in treating the disease and patients die within one year of initial diagnosis [1].

Temozolomide (TMZ) is the most commonly used drug for the treatment of GBM. This alkylating drug leads to cell death by inducing DNA degradation by its methylation. TMZ is a pro-drug and at physiologic pH is rapidly hydrolyzed into the active form of the drug, the 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide metabolite (MTIC) (Fig. 1) [2]. After oral administration, TMZ is rapidly absorbed and is completely converted to MTIC in within approximately 2 h. TMZ conversion into MTIC is irreversible and occurs mainly by pH-dependent hydrolysis and the hepatic metabolism does not show a significative contribution [3]. Nonetheless, the pharmacological efficiency of this drug is reduced due its low bioavailability.

This low bioavailability may occur due to a low permeability through the blood-brain barrier (BBB) and tumor cell membranes. Also a multidrug resistance mechanism by the p-glycoprotein pump present in GBM tumor cellular membranes is reported [4]. MTIC must interact with the biological membranes to cross them to reach the target tissues,

Abbreviations: BBB, blood-brain barrier; DLS, dynamic light scattering; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-1,3,5-hexatriene; Kp, partition coefficient; LUVs, large unilamellar liposomes; MLVs, multilamellar vesicles; MTIC, 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide; TMA-DPH, N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenyl ammonium p toluenesulfonate; TMZ, Temozolomide

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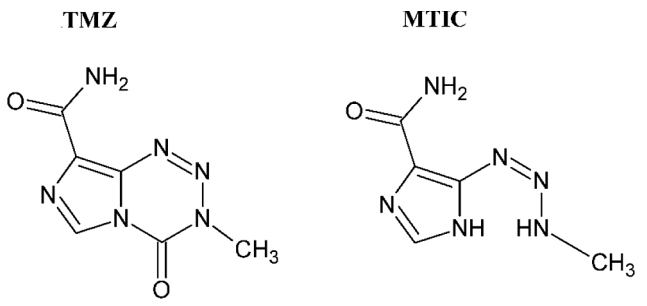

Fig. 1. Chemical structures of TMZ and MTIC.

and, several receptors and proteins are located at the cellular membrane. Therefore, cell membranes can act as a barrier or a target for a therapeutic drug [5]. Then the ability of MTIC to interact with the biological membranes and barriers presents a major contribution on its pharmacological properties and activity [6]. However, no studies concerning the interaction of both TMZ and MTIC molecules with biological membranes have been reported so far.

Several drugs with great therapeutic potential fail to be effective *in vivo* due to their poor bioavailability. Therefore, several strategies have been studied to overcome this issue [7–10]. However, understanding the interaction of a drug with biological membranes is essential. As *in vitro* biomembrane models mimic the physiological environment allowing to better understand the drug's pharmacokinetics and biodistribution profiles [11], a lipid bilayer model using liposomes was applied for the drug-membrane interaction study. Liposomes are composed of phospholipids that self-assemble into several bilayers forming small spherical vesicles in aqueous buffers [12]. These nanosystems have been widely studied as *in vitro* membrane models due to their structure and composition that are very similar to the cell membranes [13]. 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) is a zwitterionic phospholipid and was chosen for this work, because neutral phosphatidylcholine (PC) was identified as the lipid component in higher amount in eukaryotic cell membranes [14], as in the membrane of GBM cells [15] and in brain capillary endothelial cells [16]. In this work, liposomes composed solely of DMPC phospholipids or a liposomal system combining DMPC with cholesterol (DMPC:chol) molecules are proposed. Cholesterol is a major component of cellular membranes accounting for up to 20% of lipid content in eukaryotic cells. This molecule provides mechanical strength and its interactions with membranal phospholipids affect the structural organization of the membranes, as well as their fluidity and packing. Cholesterol molecules also regulate the phase behavior of membranes, promoting ordering and rigidity at the physiologically fluid state, while in the gel state exhibits opposite effects. Therefore, cholesterol displays a crucial role in many membrane processes, as drug transport [17].

Liposomes as biomembrane models present some advantages for the assessment of drug partition coefficients (*Kp*) comparatively with classical methods. *Kp* is an important indicator of the lipophilicity of a drug, that is one of the most crucial parameters that influence the biodistribution and pharmacokinetics of a drug [18]. Several methodologies have been proposed for the determination of *Kp* values involving or not physical separation of the different phases. After drug partition and the equilibrium is reached, the phases are separated and the amount of drug in each phase is determined. Physical separation can be achieved by centrifugation, dialysis, chromatography or filtration. Besides being time-consuming, these separative methods also yield results with low reproducibility. Derivative spectrophotometry offers an advantage of being a non-separative method and allows to remove the background originated by the scattered light from the DMPC liposomes by the use of the second or third derivative [19]. The proposed liposomal model also acknowledge the electrostatic interactions between the membrane phospholipids and the drug [20], that the classic two-phase octanol/water system fail to account [21]. Electrostatic interactions play a major role in several membrane binding phenomena such the transport of macromolecules [22].

Thus, the main goal of this experimental work was to study the molecular interactions between the active metabolite – MTIC - and mimetic biomembrane model composed of DMPC and cholesterol, focusing in the lipidic components of the membrane. TMZ was used for comparison. Different biophysical parameters were evaluated such as the partition of the drug metabolites into the lipid bilayers, their location within the membrane and its effect on the fluidity of the membrane.

2. Materials and methods

2.1. Reagents

1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, MW 677.9) and cholesterol (MW 386.65) were acquired from Avanti polar lipids (Alabama, USA). 1,6-diphenyl-1,3,5–1,3,5-hexatriene (DPH, MW 232.32) and N,N,N-Trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl)phenyl ammonium p-toluene sulfonate (TMA-DPH, MW 461.60), phosphate buffered saline (PBS), chloroform and methanol were acquired from Sigma-Aldrich (Germany).

TMZ (MW 194.15, purity ≥ 99%) was obtained from Selleck Chemicals (Munich, Germany). TMZ was dissolved in PBS (0.01 M, pH 7.4) for total conversion to MTIC (MW 182.18) and stored at room temperature. For the preparation of TMZ solution, the drug was dissolved in PBS (0.01 M, pH 5.5) and stored at room temperature. Acidic PBS was used to ensure that TMZ does not undergoes hydrolysis into conversion to MTIC.

All PBS solutions were prepared using filtered and deionized ultrapure water (Milli-Q Academic, Millipore, France).

2.2. Preparation of in vitro membrane models

Large unilamellar vesicles (LUVs) were used as *in vitro* model for biological membranes and were prepared using the lipid film hydration method [23]. For the establishment of our membrane model, liposomes containing solely DMPC in its composition, and DMPC and cholesterol (molar ratio of 85:15) liposomes were prepared. The final lipid concentration was 5 mM. Briefly, a chloroform solution composed of DMPC or DMPC and cholesterol was poured into a test-tube, and a lipid film was formed by evaporating the chloroform through manual rotation under a nitrogen atmosphere. The dried lipid film was then hydrated with PBS buffer. After the lipid film was peeled off completely through vigorous shaking in a vortex (Genius 3, ika®vortex, Germany) and ultrasounds in a sonicator bath (ultrasonic frequency of 45 kHz, Ultrasonic cleaner, VWRTM, Malasya) for 15 min, to produce large multilamellar vesicles (MLVs). To obtain LUVs, MLVs were submitted to an extrusion procedure using different Nuclepore™ track-etched polycarbonate membranes (Maidstone, UK) with a specific pore size through an extruder pressurized with nitrogen gas (Thermobarrel Extruder, Lipex Biomembrane, B.C., Canada). All steps were performed above the lipids phase transition temperature, at 37 °C [24], to ensure maximal membrane fluidity.

For fluorescence measurements, DPH and TMA-DPH were previously dissolved in a chloroform/methanol (volume ratio of 3:1) and added to the lipid mixture at a lipid/probe molar ratio of 100:1.

The psychochemical properties (size, polydispersity, and zeta potential) of the prepared LUVs were confirmed by dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments, UK).

2.3. Evaluation of in vitro membrane models' stability

The LUVs prepared at pH 7.4 and 5.5 were stored at room temperature. Stability was assessed by DLS measurements.

2.4. Determination of partition coefficient of TMZ and MTIC by derivative spectrophotometry

Derivative spectrophotometry was used to assess the partition coefficients (K_p) of TMZ and MTIC between lipid vesicles and the aqueous medium. DMPC and DMPC:chol LUvs were applied. TMZ and MTIC were dissolved in PBS with different pH values, 5.5 and 7.4 respectively, at a final concentration of 150 μM. Acidic PBS (pH 5.5) was used to prevent TMZ conversion into MTIC, and pH 7.4 to mimic physiological conditions for the active metabolite. The prepared solutions were then added to liposomes suspensions with lipidic concentrations ranging from 0 to 4000 μM, in a 96-well plate. Controls were prepared without addition of drugs. The samples were incubated at 37 °C for 30 min with agitation, to allow the drugs to reach the partition equilibrium between the lipid and the water phases. The absorption spectra (200–360 nm range) of samples and control solutions were obtained at 37 °C to mimic physiological conditions (HT Microplate Spectrophotometer, BioTek). The attained data were treated as previously described [19]. Briefly, (i) to each sample, the correspondent control spectrum is subtracted to each absorption spectrum giving the corrected spectrum;; (ii) the spectra of second and third derivative are obtained to improve the method's resolution since it allows to remove the interferences originated due to the light scattered by the LUVs; (iii) the most suitable wavelength value, where scattering is negligible, is chosen and its second or third derivative spectra versus LUVs concentration graph is obtained; and (iv) finally the K_p value is determined fitting a non-linear regression curve using the following equation:

$$D_T = D_W + \frac{(D_m - D_W)K_P[L]V_m}{1 + K_P[L]V_m} \quad (1)$$

with D representing the second or third derivative of the absorbance values of: the initial quantity drug (D_T), quantity of drug in the lipid membrane phase (D_m), and in the aqueous phase (D_w), respectively. *[L]* refers to the lipid molar concentration and V_m to the lipid molar volume. The V_m values for DMPC and DMPC:Chol are 0.663 and 0.623 L mol^{-1}, respectively [25,26].

Predicted octanol/buffer partition coefficients were determined for comparison purposes by the Marvin Sketch Calculator software (Chemaxon™).

2.5. Membrane location studies of TMZ and MTIC by fluorescence quenching

Steady-state fluorescence quenching studies were used to assess the preferential location of both TMZ and MTIC molecules within the membrane. DMPC and DMPC:chol LUVs labelled with DPH or TMA-DPH fluorescence probes were used. This fluorescence probes have a well-known membrane position and depth [27–29] allowing to easily identify the location of a drug within the bilayer. Increasing concentrations of MTIC or TMZ (0 to 150 μM) were added to fluorescent-vesicles at a fixed concentration of 500 μM in PBS. TMZ and MTIC were dissolved in PBS with different pH values, 5.5 and 7.4, respectively. The samples were incubated in the dark at 37 °C for 30 min with continuous agitation, to allow the drugs to reach the partition equilibrium between the lipid and the water phases. Measurements were then performed at 37 °C (microplate spectrophotometer, Thermo Scientific Varioskan Flash), at excitation/emission wavelengths of 357/427 nm and 361/427 nm for DPH and TMA-DPH, respectively [30,31]. Attained data was plotted as obtained (I_0/I) versus the quencher concentration ($[Q]_m$), and the quenching ability of the molecules was determined by calculation of the Stern–Volmer constant (K_{SV}) by fitting a linear regression model to the plot [32]:

$$\frac{I_0}{I} = 1 + K_{SV}[\mathrm{Q}]_m \quad (2)$$

where I and I_0 are the fluorescence intensities with and without the drug, respectively. Q_m is drugs' concentration that is able to partition the membrane and is calculated using the equation below [33]:

$$[Q]_m = \frac{K_P[Q]_T}{(K_P\alpha_m) + (1 - \alpha_m)} \quad (3)$$

where α_m represents the volume fraction of the membrane phase ($\alpha_m = V_m/V_T$); Q_T is the used drug concentration; V_m and V_T are the volumes of the membrane and aqueous phases, respectively [33].

2.6. Determination of phase transition temperature of TMZ and MTIC by dynamic light scattering

The effects of both molecules on the membrane fluidity were evaluated by assessment of changes in the phase transition temperature (T_m) and cooperativity (B). T_m and B values were determined by DLS measurements. Briefly, DMPC or DMPC:chol vesicles (4000 μM) were incubated with TMZ or MTIC (150 μM) for 30 min at 37 °C with continuous agitation, to allow the drugs to reach the partition equilibrium between the lipid and the water phases. TMZ and MTIC were dissolved in PBS with different pH values, 5.5 and 7.4 respectively. Samples were then analyzed at a temperature interval ranging from 10.0 to 55.0 °C (Zetasizer Nano ZS, Malvern Instruments, UK), and data were plotted as normalized count rate versus temperature and fitted with a non-linear regression using the following equation [34]:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{B(\frac{1}{T} - \frac{1}{Tm})}} \quad (4)$$

with A_1 representing initial count rate of lipids in phase 1 (y_{initial}); A_2 is the final count rate of lipids in phase 2 (y_{final}) and T is the temperature.

Measurements without addition of drugs were also performed as control.

2.7. Statistical analysis

All results are presented as mean and standard deviation (SD), for at least three independent experiments. Statistical analysis was performed using Student's *t*-test, with a 95% confidence interval. Data with p-values below 0.05 were considered significant.

3. Results and discussion

3.1. Partition coefficient of TMZ and MTIC

Kp value is an important indicator of the lipophilicity of a drug, that is one of the most important parameters that influences drug biodistribution and pharmacokinetics [18]. Lipophilicity can also be expressed by the logarithm of the partition coefficient (log *P*) or distribution coefficient (log *D*) for ionized molecular species. *Kp* and log *D* values of TMZ and MTIC were determined in mimetic biomembrane model by derivative spectrophotometry. This methodology evaluates changes in the absorbance properties of a drug due to the partition of the molecule from the aqueous medium to the lipid membrane. Derivative spectrophotometry also offers an advantage of being a non-separative method and allows to remove the background caused by the scattered light from the DMPC liposomes by the use of the second or third derivative [19]. The prepared DMPC and DMPC:chol LUVs proved to be stable for at least 3 days (data not shown).

Fig. 2 shows an example of the steps for the determination of *Kp* value for MTIC in DMPC vesicles. Fig. 2A shows the absorption spectra of MTIC with increasing concentrations of vesicles. The second-derivative of experimental data was obtained (Fig. 2B) allowing to eliminate the background of the light scattered by the lipids. The isosbestic points (arrows in Fig. 2B) indicate that the residual background signal of lipids is eliminated by the use of the second derivative [19]. Thus, a

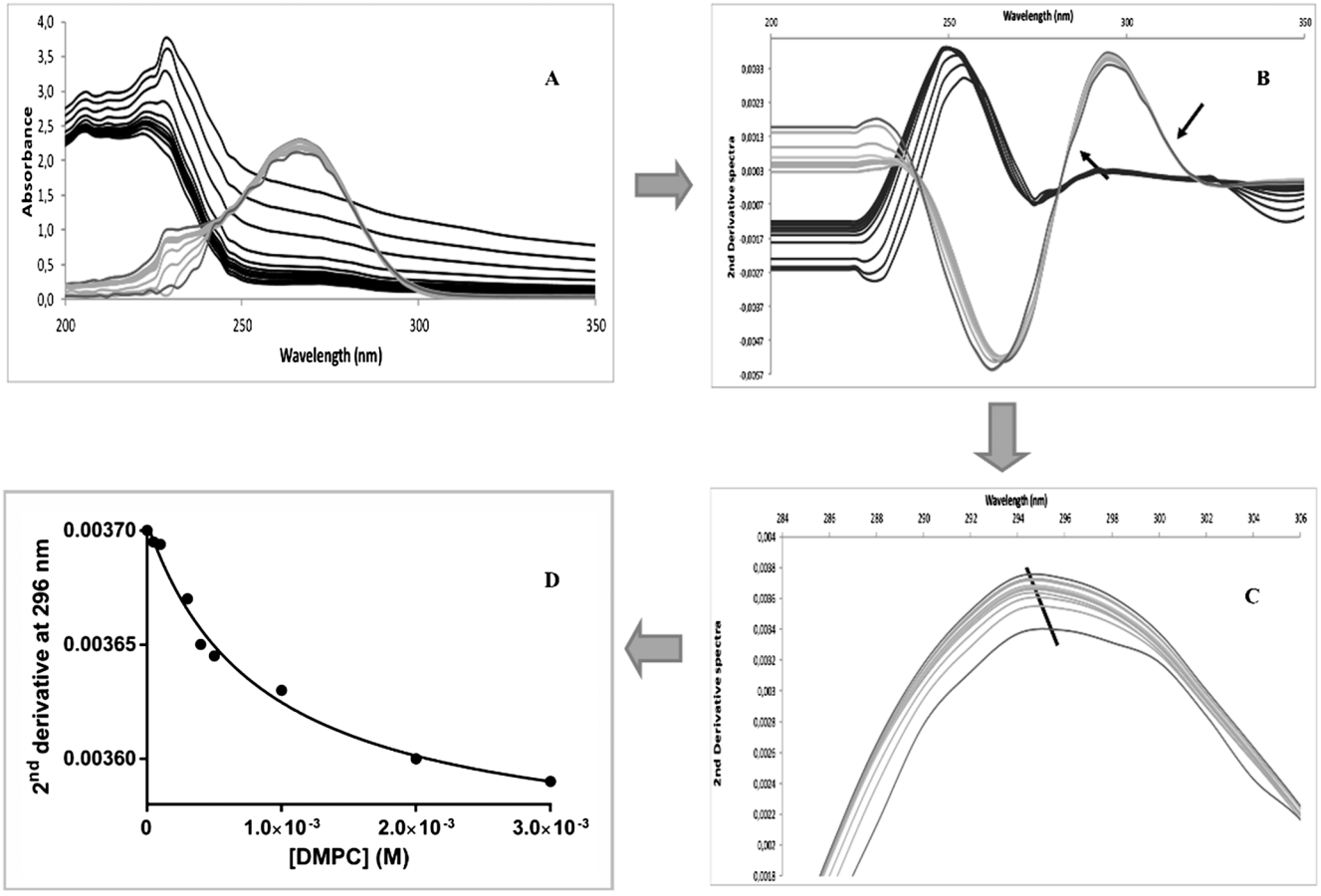

Fig. 2. Illustration of the steps for *Kp* value determination. (A) Absorption spectrum and (B, C) second-derivative spectrum of MTIC at 150 μM incubated in DMPC vesicles at 37 °C (gray lines) and DMPC vesicles without drug (black lines) at increasing lipid concentrations. Black arrows point to isosbestic points, and black line indicates a bathochromic shift. (D) Non-linear regression fitting by Eq. (1) to experimental second-derivative spectrophotometric data at wavelength 296 nm where the scattering is eliminated.

wavelength corresponding to a maximum of the second derivative between these two points was chosen, in this case 296 nm. Then, the K_P value was obtained by fitting Eq. (1) to the experimental second-derivative spectrophotometric data (Fig. 2D). The same steps were applied for the other model and for TMZ (data not shown)..

In Fig. 2C, a change of the spectral band position in the absorption spectrum of the drug with increasing lipid concentrations can be observed. This bathochromic shift occurs due to a decrease on the polarity of the medium, which indicates that the drug molecules are partitioning from the polar aqueous phase to the non-polar DMPC vesicles [18].

The calculated *Kp* and respective log *D* values are presented in Table 1. Predicted octanol/buffer log *P* were determined for comparison using the Marvin Sketch Calculator software (Chemaxon™) (Table 1). Pka values were also calculated using this software.

As observed in Table 1 predicted log *P* values are significantly different from the calculated experimental log *D* values for both molecules. This occurs because the proposed liposomal model acknowledge the electrostatic interactions between the membrane phospholipids and the drug [20], that the classic two-phase octanol/water system fails to account since it only considers the hydrophobic interactions between the studied compounds and octanol [21]. However, electrostatic interactions play a major role in several membrane binding phenomena such as the transport of macromolecules across membranes and barriers [22], and must be considered. From the calculated pKa value for MTIC (6.55 for nitrogen atom) it was predicted that the majority of the MTIC molecules are negatively charged at physiological pH. This indicates that not only hydrophobic interactions are causing the drug partition, but in fact MTIC-membrane interactions are mainly due to the electrostatic and ion-dipole forces between the negatively charged MTIC species and the polar heads of the phospholipids. While for TMZ neutral species are predominant at pH 5.5, but a contribution of anionic species still exists (pKa is 10.27 for nitrogen atom) justifying the higher experimental log *D* value. These results show that the ionization state of the molecules regulates their drug distribution between the aqueous solution and the lipids membrane. Both drugs are ionizable molecules and it is well established that ionizable drugs significantly partition into the lipid membrane model due to electrostatic interactions and formation of hydrogen bonds with polar groups of the phospholipids heads [35]. In fact, several studies report different experimental log *D* values from the predicted log *P* values that are calculated only for the neutral microspecies of the drugs [36].

Also, it was observed that drug's partition is dependent on the lipid composition of the biomembrane model, since *Kp* and log *D* values

Table 1
Partition coefficient (*Kp*) and distribution coefficient (log *D*) values of TMZ and MTIC between DMPC and DMPC:chol vesicles and the aqueous medium PBS. The aqueous medium is PBS (0.01 M) at pH 5.5 or 7.4 for TMZ and MTIC, respectively. Data represented as mean ± SD (n = 3).

Drug	DMPC		DMPC:Chol		
	K_P	log *D*	K_P	log *D*	Theoretical log *P*
TMZ	3662 ± 127	3.56 ± 0.02	2527 ± 174	3.40 ± 0.03	0.36
MTIC	1772 ± 11	3.25 ± 0.03	890 ± 243	2.95 ± 0.12	−0.65

significantly decreased in DMPC:chol LUVs for both molecules ($p < 0.05$). Cholesterol regulates the phase behavior of membranes, promoting their ordering and rigidity over the region in which there is contact between the cholesterol and the hydrophobic lipid tails [34]. Therefore, the lipid bilayer presents a more organized and packed structure in DMPC:chol LUVs, slowing the diffusion of the molecules into the biomembrane model [37]. DMPC:chol LUVs are a more realistic and comparable model for the biological membranes.

As expected *Kp* and log *D* values are significantly higher for TMZ than for MTIC in both LUVs, DMPC and DMPC:chol vesicles ($p < 0.05$). TMZ molecules are more lipophilic that MTIC molecules, therefore TMZ is more likely to exhibit a higher partition into the lipid phase. Although TMZ molecules have showed higher affinity to the biomembrane model, after oral administration, TMZ is completely converted to MTIC in within approximately 2 h [2]. The lower K*p* and log *D* values for MTIC can explain its low bioavailability on the target tissues, since this molecule proved in this experiment to have low affinity to biological membranes. In fact, it has been reported that only negligible amounts of MTIC are detected in tumor tissues [3].Hence, drug distribution depends not only on its physicochemical features as structure and degree of ionization, but also depends on the characteristics of the membrane such as the packing of the lipid molecules [38]. Thus, is of the outmost importance to employ a suitable LUVs/water system to obtain a realistic and accurate information about TMZ and MTIC lipophilicity's and their *in vivo* membrane partition.

However, *Kp* values do not allow to conclude about the location of the drug molecules within the biomembrane, since the changes in absorbance values can occur due to the drug internalization in the bilayer or to the drug adsorption at the membrane interface [39]. Thus, location studies to assess this issue were conducted and are discussed in the next section.

3.2. Membrane location of TMZ and MTIC

The location of TMZ and MTIC within the LUVs models, DMPC and DMPC:chol, was assessed by steady-state fluorescence quenching assays. These assays evaluate the accessibility of the drug molecules to the chosen probes, providing information on the location of the drug within the membrane. For that, two fluorescent probes with a well-known location, DPH and TMA-DPH, were used. DPH molecules are located deeply in the bilayer, parallelly aligned to the phospholipids carbon chains. On the other hand, TMA-DPH probe possesses a charged group and consequently is located at the region of the polar head groups of the phospholipids closer to the lipid/water interface. A higher proximity of the drugs to the probes decreases their fluorescence intensity, yielding a higher Stern-Volmer constant (K_{SV}) value [40].

Stern-Volmer graphs with increasing concentrations of TMZ were plotted and the attained plots are presented as an example in Fig. 3.

Table 2
Stern-Volmer constants (K_{SV}) values of TMZ and MTIC between DMPC and DMPC:chol vesicles and the aqueous medium PBS. The aqueous medium is PBS (0.01 M) at pH 5.5 or 7.4 for TMZ and MTIC, respectively. Data represented as mean ± SD (n = 3).

Drug	DPH		TMA-DPH	
	K_{SV} (M^{-1})		K_{SV} (M^{-1})	
	DMPC	DMPC:Chol	DMPC	DMPC:Chol
TMZ	3.5 ± 0.6	2.2 ± 0.2	4.4 ± 0.9	3.5 ± 0.4
MTIC	0.0 ± 0.1	−0.1 ± 0.1	0.0 ± 0.5	−0.1 ± 0.2

Equivalent plots were obtained for MTIC (data not shown). K_{SV} values were calculated from these plots and are presented in Table 2.

K_{SV} values correspond to the slope of the linear regression. Thus, as shown in Fig. 3, the higher the quenching effect, the higher the slope, and consequently the higher the constant value. The obtained K_{SV} values for TMZ show that the quenching effect was more pronounced for TMA-DPH in both DMPC and DMPC:chol vesicles reporting a more superficial location of TMZ near to the phospholipid head groups. However, the attained K_{SV} values for DPH probe suggest that this molecule is also able to penetrate the bilayer. These results can be explained by the significant contribution of anionic species of TMZ mentioned in the previous section.

The attained results also suggest that the quenching phenomenon depends on the organization and fluidity of the membrane. In fact, K_{sv} values for DMPC:chol are lower than for DMPC vesicles. As already discussed in the previous section, cholesterol molecules create a more packed and rigid membrane, hampering the diffusion of TMZ molecules into the bilayer, resulting in a decreased fluorescence deactivation of both probes.

The obtained K_{SV} values for MTIC indicate that this molecule does not interact with both probes. Fluorescence quenching can occur by two main processes, static quenching and collisional or dynamic quenching. Static quenching occurs when a complex between the probe and the quencher is formed, and dynamic quenching occurs when the probe collides with the quencher. Either way, both quenching processes require close proximity between the quenching and the fluorophore molecules [41]. Thus, the method used for these location studies is based in "contact quenching" phenomenon, so it depends on the probe-quencher distance [42]. The attained K_{SV} results for MTIC may be explained by the insufficient proximity between the MTIC molecules and the probes, since large distances invalidate the measurements. Although MTIC exhibited some affinity for the lipid membrane as shown by its *Kp* and log *D* values, MTIC has been described as an amphipathic molecule, exhibiting high affinity to the water phase [43]. Due to its small size

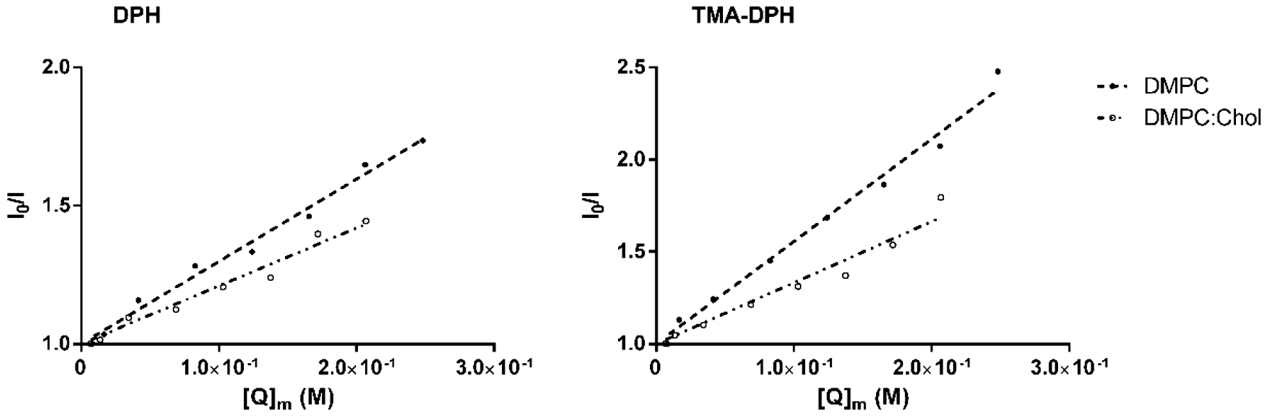

Fig. 3. Stern-Volmer plots for DPH and TMA-DPH probes in LUVs of DMPC and DMPC:chol at pH 5.5 at 37 °C by increasing concentrations of TMZ.

and high affinity to water, free energy regulates MTIC diffusion. It has been reported that for small molecules, the free energy increases with their partition from the outer aqueous medium into the lipid membrane, due to the increased density of the lipid bilayer [44]. Therefore, a resistance to the diffusion of MTIC molecules through the membrane is created. MTIC partition and diffusion are less favorable in the membrane than in the water phase. Thus, the MTIC molecules may be spontaneously driven to diffuse out of the lipid bilayer to minimize the free energy [44]. So MTIC molecules may diffuse continually in a cycle from the outer aqueous medium to the lipid bilayer, and then from the membrane back to the external aqueous phase. This cyclic in-out-in diffusion may not allow to achieve sufficient proximity between the probe and MTIC molecules for the quenching process occur.

Also, the observed linear plotting of I_0/I versus $[Q]_m$ suggests that quenching by TMZ occurs by collisional quenching [45].

3.3. The effect of TMZ and MTIC on membrane fluidity

To reach the intracellular environment, drugs must cross several biological membranes. Since the intracellular environment constitute the pharmacological target in most cases, it is of outmost importance to evaluate the drugs' effects on the biophysical state of the phospholipid membranes. The membrane fluidity plays a major role in several cellular functions, so its study allows to understand the therapeutic and toxic effects of the drug. Changes in the T_m from the gel to the fluid state are suitable indicators for membrane fluidity alterations [46]. So, T_m and cooperativity (B) values were determined in mimetic biomembrane model before and after incubation with TMZ or MTIC by DLS analysis to study the effect of the drugs on the membrane's fluidity.

The effect of temperature on the normalized count rate (number of photons detected per second) of the biomembranes models in the absence and presence of TMZ or MTIC is presented in Fig. 4. Changes in the count rate with variation of temperature suggest the occurrence of a macroscopic alteration in the membrane. The measured variations in the scattering intensity occur due to changes in the optical features of the studied sample due to the gel-to-fluid phase transition or *vice versa* [47].

Table 3
Phase-transition temperature (T_m) and cooperativity (B) values of DMPC and DMPC:chol vesicles with and without TMZ or MTIC. The aqueous medium is PBS (0.01 M) at pH 5.5 or 7.4 for TMZ and MTIC, respectively. Data represented as mean ± SD (n = 3).

Drug	pH	T_m (°C)		Cooperativity (B)	
		DMPC	DMPC:Chol	DMPC	DMPC:Chol
–	5.5	25.2 ± 0.2	22.1 ± 0.8	282 ± 45	337 ± 38
TMZ	5.5	23.6 ± 0.1	20.6 ± 0.3	109 ± 36	130 ± 39
–	7.4	24.3 ± 0.1	21.5 ± 0.2	326 ± 78	333 ± 36
MTIC	7.4	23.6 ± 0.1	20.6 ± 0.1	201 ± 18	191 ± 13

T_m and B values were determined by fitting the non-linear regression curve (Eq. (4)) to the plotted graphs, and the attained values are presented in table 3. The obtained T_m value for DMPC vesicles at pH 7.4, 24.3 ± 0.1 °C, is accordingly with previously reported [41]. It was observed that this value significantly decreased to 21.5 ± 0.2 °C for the DMPC:chol LUVs model ($p < 0.05$). As already mentioned, the cholesterol molecules embedded in the lipid bilayer increase the membrane's rigidity, therefore decreasing its fluidity altering the T_m value. The same decrease was verified for measurements conducted at pH 5.5 as control. Although no significant changes occurred to cooperativity values.

The attained T_m values indicate that both molecules produce a perturbation in membrane's fluidity, although in a higher extent for TMZ. In fact, TMZ significantly decreased T_m by 1.8 and 1.5 °C in DMPC and DMPC:chol models, respectively ($p < 0.05$), while MTIC only decreased T_m by 1.1 and 0.9 °C in DMPC and DMPC:chol models, respectively ($p < 0.05$). These results are consistent with the determined Kp and log D values that indicate that TMZ has a higher affinity for the membrane than MTIC, and therefore will penetrate the bilayer in a greater extent, creating a higher perturbation in the fluidity of the membrane. Also, these results support that drug depth diffusion into the membrane is less noticeable when cholesterol molecules are embedded in the membrane, as expected to the increased rigidity and organization

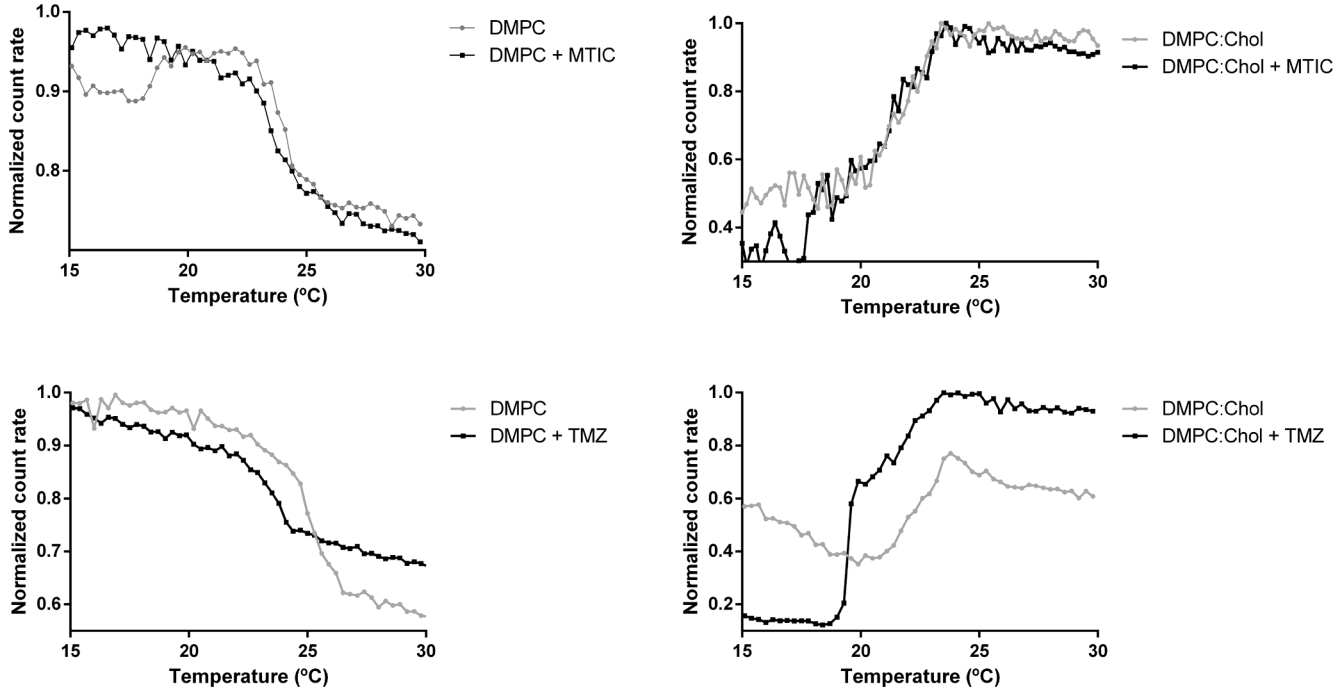

Fig. 4. Normalized count rate of DMPC and DMPC:chol vesicles with and without TMZ and MTIC (150 μM) as a function of temperature. The aqueous medium is PBS (0.01 M) at pH 5.5 or 7.4 for TMZ and MTIC, respectively.

of the bilayer.

In addition, it was observed that TMZ significant decreased the cooperativity of both DMPC and DMPC:chol models ($p < 0.05$). Changes in the lipids cooperativity (B), due to acyl chains undergoing changes simultaneously during phase transition, indicate the presence and interaction of the compounds with the lipid bilayer [48]. Charged microspecies of the drug interact with charged groups at the phospholipid polar head, reducing the electrostatic repulsions between DMPC molecules, and consequently modifying the cooperativity of the lipids [49]. Drug molecules reduce the cooperativity of the phase transition because they intercalate into the phospholipid bilayers affecting the number of phospholipid molecules that a single phospholipid molecule can influence [50].

Also, these changes in the properties of the membrane corroborate our conclusions on the location of TMZ molecules within the lipid bilayer. It is well-reported that lipid bilayers are characterized by a fluidity gradient with more rigid and ordered outer region near the polar head groups (C1 to C9 of the acyl chains), and a more fluid and disordered deeper region (C10 to C14). Hence, if the drug induces changes in the fluidity of the bilayer, the drug is more likely to be located in the outer regions of the membrane, near to the polar head groups [49]. The presented T_m and B values suggest that TMZ should be located near the head groups of the phospholipids, as also indicated by the calculated K_{SV} values. Besides, due to its ionization state, TMZ and MTIC establish electrostatic and ion-dipole interactions with the phospholipids polar head groups. A more superficial location of TMZ is expected (shown by higher K_{SV} values for TMA-DPH). Thus, changes in the lipid organization of such region occur resulting in alterations on the membrane's fluidity as shown by T_m measurements. Still, TMZ molecules also interact through hydrophobic forces with the acyl chains of the phospholipid (shown by high K_{SV} values for DPH) and alters the fluidity of deeper regions of the membrane as proved by T_m measurements.

MTIC also decreased the B values, but in a smaller extent ($p > 0.05$). This is in agreement with all the previously presented results. As location studies suggested, MTIC molecules may constantly partitioning in and out of the lipid bilayer due to its low affinity to the lipid membranes. As MTIC has low affinity to the lipid membranes, it creates a smaller influence on the lipid cooperativity as expected.

4. Conclusions

Biomimetic model to study the drug-membrane interactions at physiological conditions were used to evaluate the penetration of the active metabolite – MTIC - into biological membranes and its ability to reach the target cells to efficiently exert the pharmacological activity. Although its therapeutics effects are well-established, the drug fails to efficiently treat GBM tumors. It is reported that MTIC is not able to reach the target cells at effective concentrations. Therefore, this work presented an approach to study its molecular interactions with biological barriers and membranes, to understand their role in the low bioavailability of the drug. The inactive form - TMZ - was used as comparison.

In this context, different experiments were conducted to evaluate the drugs' partition coefficient, their preferential location within the membrane and their effects on membrane properties such as fluidity. TMZ molecules have shown higher affinity to membranes, however this pro-drug is rapidly converted into the active form MTIC after administration. Since TMZ conversion into MTIC is irreversible, it was also crucial to evaluate the interactions with membranes of the active metabolite. The partition, location and fluidity studies suggest that MTIC have low affinity to biological membranes, explaining its low bioavailability on the target cells.

Also, membrane models were used with different lipid composition. Membrane compositions proved to influence drugs partition. Hence, drug biodistribution depends not only on its physicochemical features as its structure and degree of ionization, as also depends on the characteristics of the membrane such as the packing of the lipid molecules. DMPC:chol LUVs are a more realistic and comparable model for the biological membranes. Thus, is of the outmost importance to use a suitable the LUVs/water system to obtain a more realistic prediction about the active metabolite' lipophilicity and its *in vivo* membrane partition and interactions. These predictions can be used for the design of new therapeutic molecules or to envisage new strategies to improve drug's bioavailability to enhance its efficacy.

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Declarations of interest

None.

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