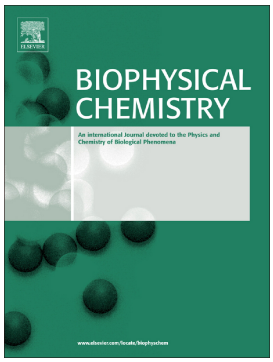

New antineoplastic agent based on a dibenzoylmethane derivative: Cytotoxic effect and direct interaction with DNA

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New Antineoplastic Agent Based on a Dibenzoylmethane Derivative: Cytotoxic Effect and Direct Interaction with DNA

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ABSTRACT

Melanoma accounts for only 4% of all skin cancers but is among the most lethal cutaneous neoplasms. Dacarbazine is the drug of choice for the treatment of melanoma in Brazil through the public health system mainly because of its low cost. However, it is an alkylating agent of low specificity and elicits a therapeutic response in only 20% of cases. Other drugs available for the treatment of melanoma are expensive, and tumor cells commonly develop resistance to these drugs. The fight against melanoma demands novel, more specific drugs that are effective in killing drug-resistant tumor cells. Dibenzoylmethane (1,3-diphenylpropane-1,3-dione) derivatives are promising antitumor agents. In this study, we investigated the cytotoxic effect of 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP) on B16F10 melanoma cells as well as its direct interaction with the DNA molecule using optical tweezers. DPBP showed promising results against tumor cells and had a selectivity index of 41.94. Also, we demonstrated the ability of DPBP to interact directly with the DNA molecule. The fact that DPBP can interact with DNA *in vitro* allows us to hypothesize that such an interaction may also occur *in vivo* and, therefore, that DPBP may be an alternative to treat patients with drug-resistant melanomas. These findings can guide the development of new and more effective drugs.

Keywords: dibenzoylmethane; dibenzoylmethane derivative; melanoma; optical tweezers; DNA.

1. Introduction

Melanoma is a cancer of low incidence but high lethality. It accounts for only 4% of all cancers in Brazil. About six thousand new cases are expected for the biennium 2016–2017, of which 1500 will probably result in death. The southern region of Brazil has the highest incidence rates of the country, 8.02 and 7.06 cases per 100,000 men and women, respectively. The lowest rates occur in the northern region, 0.20 cases per 100,000 men and 0 cases among women [1].

The main causes of melanoma are genetic predisposition and excessive sun exposure. The use of sunscreen is recommended as a preventive measure. The disease in its initial stages is treated by surgical removal of the tumor and sentinel lymph nodes. This procedure aims to eliminate all cancer cells before they reach the bloodstream and lymphatic vessels and thereby avoid metastasis. For patients with more advanced stages of the disease, treatment consists in the administration of antineoplastic drugs, which have low effectiveness and high cost. In addition, the more advanced the disease, the lower the chances of patient survival, even with treatment.

Drugs that intercalate or interact with DNA have been widely used as chemotherapeutic agents, such as cisplatin, doxorubicin, and dacarbazine. However, despite their therapeutic success, these drugs cause serious side effects, and tumor cells can become resistant to their cytotoxic mechanisms. Dacarbazine (Figure 1) is the most used drug to treat melanoma. This alkylating agent binds to the DNA, preventing DNA strands from separating and disrupting protein and DNA synthesis [2]. Dacarbazine is a non-specific agent, as it can act at any stage of the cell cycle. This drug elicits a therapeutic response in only 20% of cases for approximately five months and a complete response in 5% of cases [3].

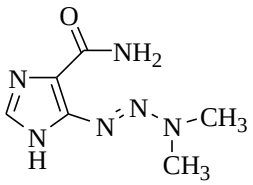


Figure 1. Chemical structure of dacarbazine, a drug used for the treatment of melanoma.

Dibenzoylmethane (DBM, Figure 2) is a β-diketone and natural flavonoid found in families such as Annonaceae, Asteraceae, Menispermaceae, Rosaceae, Salicaceae, and especially Fabaceae (genera *Glycyrrhiza* and *Lonchocarpus*) [4, 5]. DBM is widely used in sunscreens as an ultraviolet blocking agent [6]. A variety of biological properties have been attributed to DBM, including anti-inflammatory [7], anti-mutagenesis [8], and anti-estrogenic activities [9]. In addition, DBM exerts many anticancer effects. DBM has been shown to induce apoptosis and cell cycle arrest in human prostate cancer cells [10] and in colon cancer cells [11] and to prevent the formation of DNA-adducts induced by carcinogens, both *in vitro* and *in vivo*, in mammary glands [12] and in the lungs by activation of the Nrf2 detoxification pathway [13].

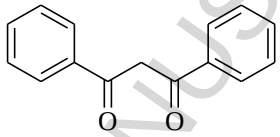


Figure 2. Chemical structure of dibenzoylmethane (DBM).

As an extension of the pioneering work of Nogueira et al. (2003) [5, 14] reporting the photoprotective properties and promising results of a series of dibenzoylmethane derivatives against human melanoma cell lines, in the present study, we evaluate the anti-melanoma activity of 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP, Figure 3) against the B16F10 cell line. DPBP was one of the best exponents of the series; its complete mechanism of action, however, is still unknown. Here we advance the understanding of DPBP's mechanism of action, studying its efficiency at a cellular level as well as at a single-molecule level by optical tweezers analysis, a technique used to tether DNA and measure its mechanical properties. This technique allows the elucidation of the binding modes and the physical chemistry of drug–DNA interactions [15].

In order to evaluate the performance of DPBP as a potential anticancer agent, first, we investigated the cytotoxic effect of DPBP on melanoma cells. DPBP gave promising results against B16F10 cells, showing a high selectivity index (SI) of 41.91. By comparing the effect of DPBP on normal melanocytes with the effects obtained using other classical chemotherapeutic agents, we concluded that this molecule is a potential chemotherapeutic agent for the treatment of melanoma.

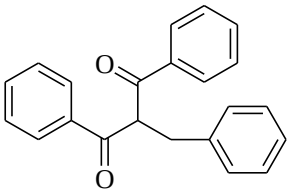


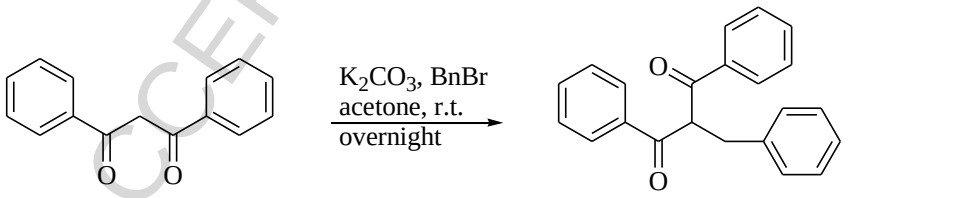
Figure 3. Chemical structure of 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP).

Finally, we investigated the direct interaction of DPBP with DNA using optical tweezers. DNA is the therapeutic target of many antineoplastic agents, such as cisplatin and doxorubicin, and it directs the search for new antineoplastics [16]. The fight against melanoma demands new and more specific drugs that are effective in cases of drug resistance.

2. Results and discussion

2.1. Chemistry

DPBP was synthesized by the reaction of dibenzoylmethane and benzyl bromide in acetone in the presence of anhydrous potassium carbonate (Scheme 1). DPBP was characterized by IR, ^{1}H and ^{13}C NMR, and mass spectra. The spectral data of DPBP were in agreement with the described in the literature [5].



dibenzoylmethane — 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP)

Scheme 1. Synthesis of 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP).

2.2. Biological activity

2.2.1. Cell cytotoxicity

Data presented in Figure 4 show that DPBP was more effective against B16F10 cells than against melan-A cells. The selectivity indices (SI = IC_{50} melan-A/IC_{50}

B16F10) obtained were 41.94 and 1.00 for DPBP and dacarbazine, respectively, which indicates the high selectivity of DPBP for B16F10 tumor cells (detailed data are shown in Table 1).

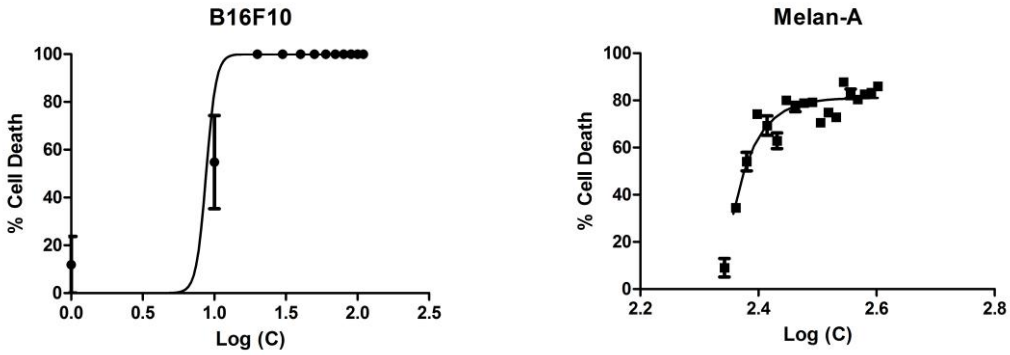


Figure 4. Plots of the percentage of cell death induced by DPBP at different concentrations on B16F10 and melan-A cells. Results are the mean ± standard deviation of three independent experiments.

Table 1. IC_{50} values and selectivity indices (SI) of dacarbazine, DBM, and DPBP.

Compound	IC_{50} B16F10 (μg/mL)	IC_{50} melan-A (μg/mL)	SI (melan-A/B16F10)
Dacarbazine	253.97	253.91	1.00
DBM	11.59	5.68	0.50
DPBP	6.25	262.13	41.94

The results presented in Figure 4 and Table 1 indicate that DPBP is a promising agent for the treatment of melanoma, as its SI (41.94) is considerably high and its IC_{50} is considerably low (6.25 μg/mL = 1.97 μM) when compared with the SI (0.50) and IC_{50} (11.59 μg/mL = 51.7 μM) of DBM. Moreover, cisplatin and doxorubicin, two well-known DNA-intercalating drugs, exhibited IC_{50} values of approximately 6 μM and SI of 1.0 (data not shown). These results demonstrate that DPBP is a molecule of high efficiency, suggesting that it has a great potential as a chemotherapeutic agent in the treatment of melanoma.

2.2.2. Optical tweezers experiments

In Figure 5, we show the persistence length A of DPBP–DNA complexes as a function of DPBP concentration in solution (C_T). Black dots represent the experimental data, and the red solid line is the fit to the model, discussed in Section 4.7. Observe that the persistence length presents a non-monotonic behavior: it initially decreases (for C_T < 40 μM) from the bare DNA value (~47 nm) until reaching ~34 nm and then increases (for C_T > 40 μM). The model returned the characteristic binding parameters of the DPBP–DNA interaction: $K = (2.7 \pm 0.2) \times 10^4$ M^{-1} and $n = 5.1 \pm 1.5$. The local persistence lengths were also determined: $A_1 = (22 \pm 3)$ nm, corresponding to the binding of DPBP to a single binding site; and $A_2 = (68 \pm 9)$ nm, corresponding to two bound molecules becoming nearest neighbors.

In Figure 6, we show the contour length (L) of the same DPBP–DNA complexes as a function of DPBP concentration (C_T). This parameter exhibits a monotonic behavior, increasing from the bare DNA value (~16.5 μm) until reaching the saturation value of ~19.1 μm.

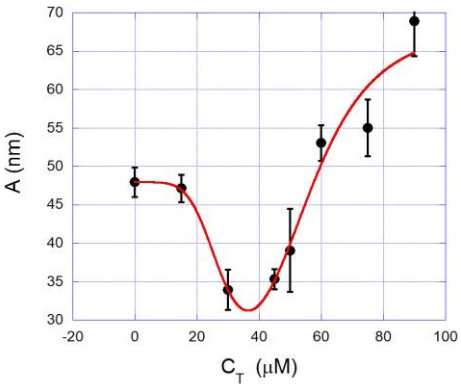


Figure 5. Persistence length (A) as a function of DPBP concentration in solution (C_T) (black dots). The red solid line is the fit to the model (discussed in Section 4.7).

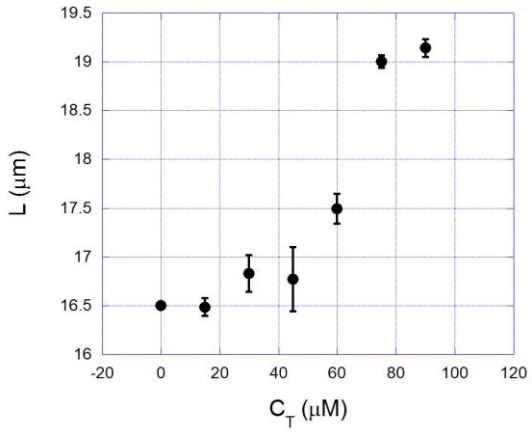


Figure 6. Contour length (*L*) of the same DPBP–DNA complexes as a function of DPBP concentration (C_T).

DNA is one of the main targets in the discovery of new antineoplastic drugs. Drugs that interact with DNA can prevent transcription and/or replication. A drug can interact with DNA molecules in many ways, such as by intercalation, groove binding, covalent binding, and electrostatic binding [15].

Single-molecule stretching experiments such as those performed using optical and/or magnetic tweezers have become the state of the art in the past years for characterizing interactions between DNA and small molecules. The basic idea of these experiments is to monitor changes in the mechanical properties of DNA induced by the drug upon binding [15, 17]. The binding parameters of the interaction can be further deduced from the changes in mechanical properties. By using this type of approach, it is possible to promptly verify if a ligand interacts with DNA and deduce the possible binding mechanisms [15, 17].

In Figures 5 and 6, we presented the behavior of the two basic mechanical properties of DNA, persistence and contour lengths, as a function of DPBP concentration. Both properties changed as DPBP concentration increased, indicating that DPBP binds to the double helix and alters the mechanical behavior of the formed complexes.

The behavior of persistence length was non-monotonic, suggesting a complex binding mechanism between DPBP and DNA [15]. Note that DPBP has three aromatic rings in its structure (see Figure 3). Antineoplastic drugs are mostly polyaromatic and interact with DNA mainly by two binding mechanisms: groove binding and intercalation [18].

For ligands that interact with the minor groove of the DNA, there is usually a correlation between the resulting persistence length and changes in the minor groove width. A narrowing of the minor groove cavity of the double helix is in general accompanied by a decrease in the bending rigidity of the biopolymer, thus decreasing the effective persistence length [19]. Olsen et al. (2003) [20], for example, observed the narrowing of the minor groove cavity after the interaction of DNA with the ligand distamycin-A, which resulted in a decrease in persistence length. On the other hand, some ligands, such as netropsin, lengthen the minor groove cavity, increasing the effective persistence length [21]. The effect of groove binders on the DNA contour length also depends on the drug. Some molecules, such as diminazene aceturate, increase contour length by unwinding the DNA double helix [22], whereas other molecules, such as Hoechst 33258, decrease the apparent value of this parameter by promoting DNA condensation [23].

Intercalators, on the other hand, are well recognized for increasing DNA contour and persistence lengths upon binding [24, 25].

Thus, on the basis of data of Figures 5 and 6, we propose the following mechanism of interaction between DPBP and DNA. For C_T < 40 μM, there was a strong decrease in persistence length, whereas contour length had only a slight increase. These observations strongly suggest that DPBP binds to the DNA minor groove at these concentrations. In general, ligands with a molecular weight of less than 1000 g/mol bind to the DNA minor groove floor instead of the major groove, as is the case of DPBP (314.38 g/mol) [26]. At higher concentrations, C_T > 40 μM, there was a considerable increase in persistence and contour lengths. This increase in mechanical parameters may be related to structural changes in the double helix as a result of the accommodation of DPBP molecules. Alternatively, there is a possibility of partial intercalation of DPBP molecules into the double helix by insertion of an aromatic ring between DNA base pairs. In any case, the sigmoidal shape of the contour length curve strongly suggests that groove binding is the dominant mechanism and that there is a relatively high cooperativity between DPBP molecules upon binding, a feature incompatible with simple intercalative binding. Thus, intercalation, if it takes place here, is only a secondary binding mechanism. In fact, strong intercalators such as ethidium bromide and anthracycline antibiotics typically increase DNA contour length by 27–45%. In the present case, DPBP promoted an increase of only 16% in this mechanical parameter, corroborating the conclusions drawn above.

The binding parameters obtained from the model fitting were $K = (2.7 \pm 0.2) \times 10^4\ M^{-1}$ and $n = 5.1 \pm 1.5$. The value of the equilibrium constant (K) is compatible with the results obtained for some antineoplastic drugs, such as cisplatin [27], under similar experimental conditions (PBS, [Na] = 150 mM). Typical intercalating antineoplastic drugs, such as doxorubicin, usually present high binding constants, in the order of $10^5\ M^{-1}$ [25].

The relatively high value found for the Hill exponent ($n = 5.1$) confirms the high positive cooperativity of the present interaction, indicating that DPBP binding increases the affinity of the DNA molecule for subsequent binding, which can result in bound clusters along the double helix, as $n > 1$ [15]. Cisplatin and doxorubicin also exhibit positive cooperativity upon binding to DNA under similar experimental conditions, having Hill exponents of approximately 3.5 [25, 27]. This property is important in chemotherapies because it indicates that DPBP has a narrow concentration range between the initial effective dose and the saturation dose when binding to the DNA molecule.

These results suggest that the molecular mechanism of action of DPBP within cells involves interaction with DNA. Upon binding to the biopolymer, DPBP changes the double-helix structure locally, which probably hinders fundamental processes such as transcription and DNA replication.

3. Conclusion

In the present study, we tested the antineoplastic efficiency of a novel dibenzoylmethane derivative, DPBP, on B16F10 melanoma cells. The selectivity results show that DPBP is a promising agent for the treatment of melanoma, as it presents a considerably high SI and a considerably low IC_{50}. In addition, we proposed that its molecular mechanism of action within cells involves binding to DNA. In fact, a direct interaction between DNA and DPBP was verified using optical tweezers. Minor groove binding was determined as the main binding mode, although the possibility of a second binding mechanism (partial intercalation) could not be discarded. Important binding parameters, such as the equilibrium binding constant and the cooperativity degree, were determined, characterizing the physical chemistry of DPBP–DNA interaction. Thus, we characterized the efficiency of DPBP at a cellular level as well as at a molecular level.

4. Experimental section

^{1}H and ^{13}C NMR (nuclear magnetic resonance) spectra were recorded on a Bruker Avance spectrometer at 400 and 100 MHz, respectively. The chemical shifts (δ) are reported in ppm using TMS (δ 0) and residual $CHCl_3$ (δ 77.00) signals as internal references for ^{1}H and ^{13}C NMR spectra, respectively. Mass spectra (HRMS) were recorded on a VG Auto Spec-10000 instrument using a 70 eV ionizing potential. IR spectra were recorded on an FTIR spectrometer with a diamond ATR (attenuated total reflectance) accessory as a thin film. Purification by preparative thin layer chromatography (TLC) was performed by using 80:20 *n*-hexane/AcOEt as eluent. TLC visualization was achieved by spraying with 5% ethanolic phosphomolybdic acid and heating.

4.1. Synthesis of 1,3-diphenyl-2-benzyl-1,3-propanedione (DPBP)

Anhydrous potassium carbonate (0.56 g, 4.0 mmol) and DBM (0.3 g, 1.34 mmol) were suspended in acetone (20 mL) and stirred for 30 min at room temperature. Benzyl bromide (0.16 mL, 1.34 mmol) was added to this mixture and stirred overnight at the same temperature. The mixture was filtered, and the solvent was evaporated. The crude product was purified by TLC by using 80:20 *n*-hexane/AcOEt as eluent to afford DPBP as a colorless solid in 85% yield. m.p.: 58.3–58.7 °C; R_f = 0.74 (80:20 *n*-hexane/AcOEt); IR (ν/cm^{-1}): 3031, 1694, 1664, 999; ^{1}H NMR: 7.88 (dd, 4H, *J* = 8.0 and 1.3 Hz, H-2' and H-6'), 7.53 (tt, 2H, *J* = 7.5 and 1.2 Hz, H-4'), 7.40 (tt, 4H, *J* = 7.9 and 1.3 Hz, H-3' and H-5'), 7.26–7.20 (m, 4H, H-2", H-3", H-5", and H-6"), 7.18–7.15 (m, 1H, H-4"), 5.51 (t, 1H, *J* = 6.7 Hz, H-2), 3.45 (d, 2H, *J* = 6.7 Hz, $C\underline{H}_2Ph$); ^{13}C NMR: 195.6 (2 C=O), 139.3, 136.3, 133.7, 129.2, 129.0, 128.8, 126.8, 59.3, 35.4 ($\underline{C}H_2Ph$); HRMS: calc. for $C_{22}H_{18}O_2$ 314.1307, observed 314.1306.

4.2. Cytotoxicity test

The melan-A and B16F10 cell lines have been established from normal murine melanoblasts and murine melanoma cells, respectively. Melan-A cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum and 1% antibiotics (amphotericin B, penicillin, and streptomycin) in 25

cm^2 culture flasks, incubated at 37 °C, 5% CO_2, and 95% relative humidity. B16F10 cells were cultured in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum and 1% antibiotics (amphotericin B, penicillin, and streptomycin) and maintained under the same conditions as described above.

4.3. Cytotoxic activity measured by the MTT assay

B16F10 cells were plated at 1×10^4 cells/mL; and melan-A cells, at 1×10^5 cells/mL. Each well received 100 μL of culture medium. Plates were incubated for 24 h at 37 °C, 5% CO_2, and 95% humidity. Then, different concentrations of dacarbazine (positive control, 1–12 μM) and DPBP (0.39–25 μM) were added to the cells, which were further incubated for 60 h at 37 °C, 5% CO_2, and 95% humidity. After this period, the MTT assay was performed. Absorbances were read at 570 nm, and the 50% inhibitory concentration (IC_{50}) for each cell line was calculated.

4.4. Selectivity index

The SI indicates the potential therapeutic use of compounds in clinical trials. In this study, SI corresponds to the ratio of the IC_{50} for normal cells (melan-A) to the IC_{50} for neoplastic cells (B16F10).

4.5. Statistical analysis

The IC_{50} values for tumor cells were analyzed using the GraphPad Prism® 5.0 software, according to the methods indicated in the manual. Statistical differences between experimental groups were evaluated by one-way ANOVA ($p < 0.05$).

4.6. Optical tweezers experiments

The DNA samples consisted of λ-DNA molecules (48,502 base-pairs, ~16.5 μm contour length) (New England Biolabs) end-labeled with biotin attached by one end to a streptavidin-coated polystyrene bead of 3 μm in diameter (Bangs Labs) and by the other end to a previously prepared streptavidin-coated coverslip. All measurements were performed in phosphate buffered saline (PBS) pH 7.4 and 140 mM sodium chloride. The DPBP concentration in the sample was varied during the experiments by changing the buffer solution.

The optical tweezers setup was composed of a 1064 nm ytterbium-doped fiber laser (IPG Photonics) mounted on a Nikon Ti-S inverted microscope with a 100X

N.A. 1.4 objective. The DNA molecules were stretched by moving the microscope stage, and consequently the coverslip, under controlled velocity using a piezoelectric device (PINano P-545, Physik Instrument).

We started the experiments with bare DNA molecules (0.0 μM DPBP). First, we randomly chose a bead and tested if there was a DNA attached to it by moving the microscope stage. If the measured contour length agreed with the expected value for λ-DNA (16–17 μm), six more stretching measurements were taken, and the mean values of persistence and contour lengths were obtained. These two mechanical parameters were determined by fitting the experimental force–extension curves, measured in the low-force entropic regime (F < 3 pN), to the Marko–Siggia worm-like chain (WLC) model [28]. Next, we changed the surrounding buffer solution, introducing DPBP at the chosen concentration. We waited for about 1 h before repeating the stretching experiments in order to reach the chemical equilibrium of the binding reaction. A new set of six measurements were then taken, and the mean values and standard errors of the mechanical properties at each concentration were calculated. The complete details of these procedures have been previously described [25].

4.7. A model to determine the DPBP–DNA binding parameters

In 2012, a quenched-disorder statistical model was formulated to determine the binding parameters of a ligand–DNA interaction from persistence length [29]. Here, we will briefly expose this approach. The complete details can be found in another study [15].

Essentially, it can be rigorously demonstrated [29] that the effective persistence length (A_E) of a ligand–DNA complex can be expressed as follows:

$$r = \frac{r_{max}(K_i C_f)^n}{1+(K_i C_f)^n} \qquad (1)$$

where A_0 is the persistence length of the bare DNA molecule, A_1 is the local persistence length induced by the ligand upon binding to a site, A_2 is the local persistence length when two bound sites become nearest neighbors, and x is the ratio of the bound site fraction r to its saturation value r_{max}.

The effective persistence length (A_E) can be connected to the binding parameters of the interaction by choosing an appropriate binding isotherm that captures the physical chemistry of the system. In the case of the DPBP–DNA interaction, an

appropriate binding isotherm is the Hill model, as this molecule exhibits positive cooperativity upon binding to DNA. The Hill model is the simplest binding isotherm capable of providing an estimation of the cooperativity degree of a binding reaction. It reads

$$r = \frac{r_{\max}(K_i C_f)^n}{1+(K_i C_f)^n} \qquad (2)$$

where C_f is the free-ligand (not bound to DNA) concentration; K is the equilibrium association binding constant; and n is the Hill exponent, a parameter generally used to measure the degree of cooperativity of binding reactions.

Equation 2 can be plugged into Equation 1 to fit the experimental data of persistence length. The binding parameters are left as adjustable parameters and can be determined from the fitting process. Complete details of this fitting procedure can be found in a previous work [15].

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Appendix A. **Supplementary data**

Supplementary data related to this article can be found at https://doi.org/.....

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Graphical Abstract

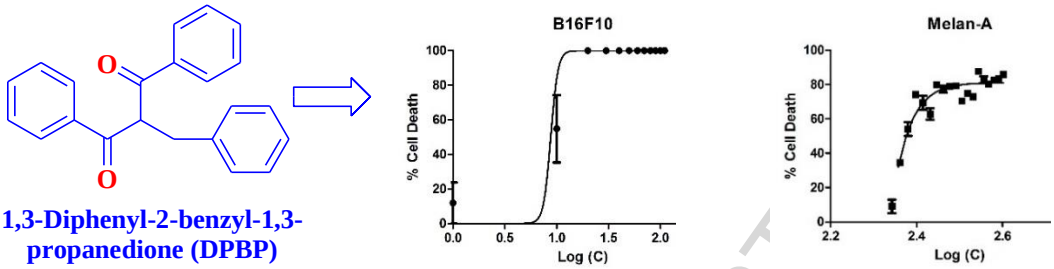
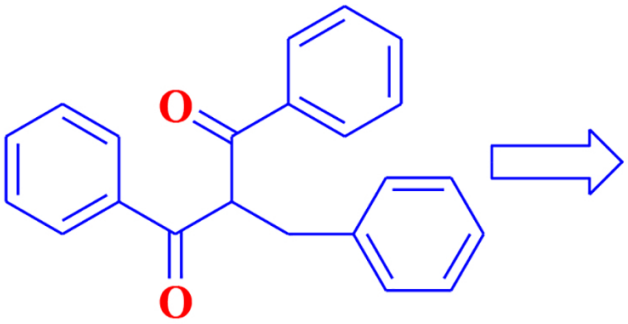


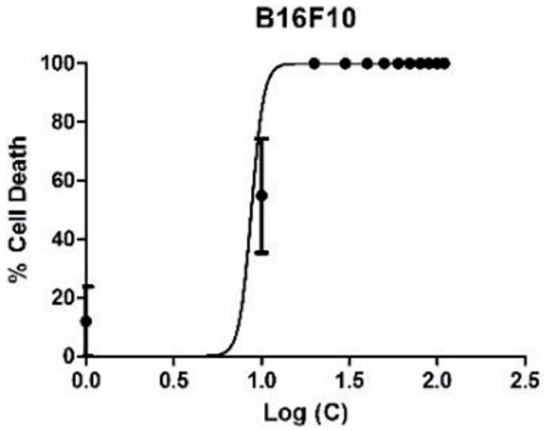
Figure. Plot of the percentage of cell death obtained for DPBP compound against melan-A and B16F10 lineages in different concentrations. The selectivity indices (SI = IC_{50} melan-A/IC_{50} B16F10) was 41.94.

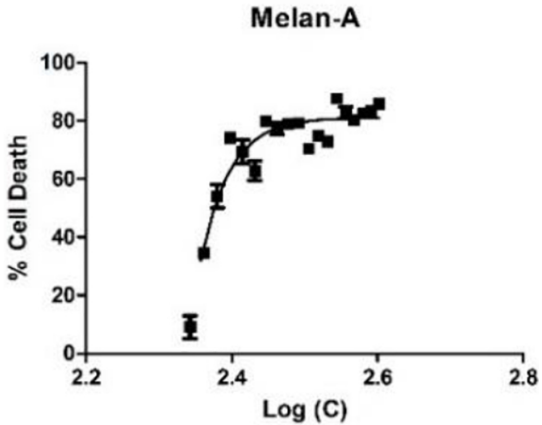
Highlights

- 1,3-Diphenyl-2-benzyl-1,3-propanedione (DPBP), a dibenzoylmethane derivative, is a potential chemotherapeutic agent.
- DPBP had a high selectivity for B16F10 melanoma cells over melan-A cells.
- Its mechanism of interaction with DNA was elucidated using optical tweezers.



1,3-Diphenyl-2-benzyl-1,3-propanedione (DPBP)





Graphics Abstract

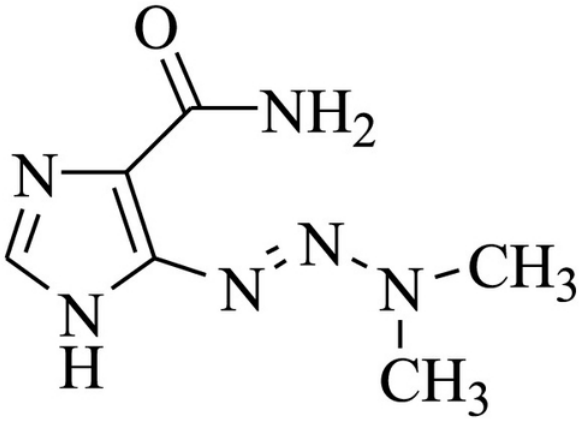


Figure 1

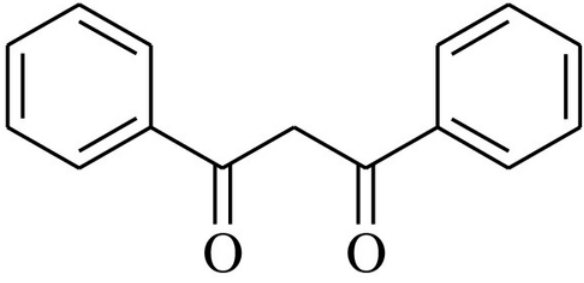


Figure 2

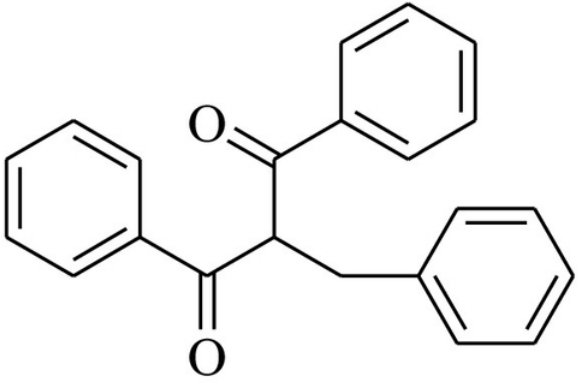
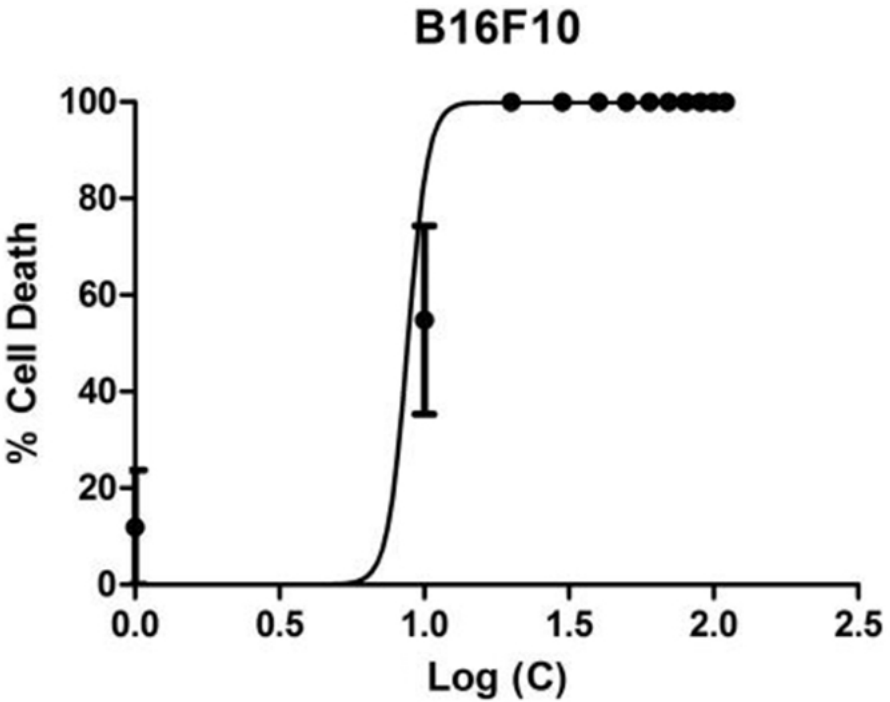


Figure 3


B16F10
% Cell Death
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
Log (C)

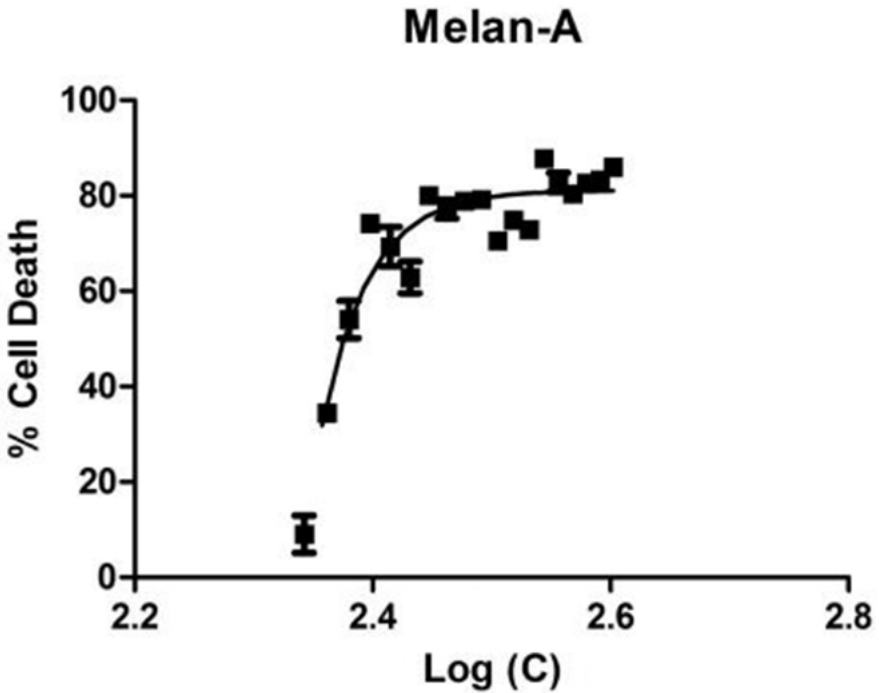

Melan-A
% Cell Death
100
80
60
40
20
0
2.2
2.4
2.6
2.8
Log (C)

Figure 4

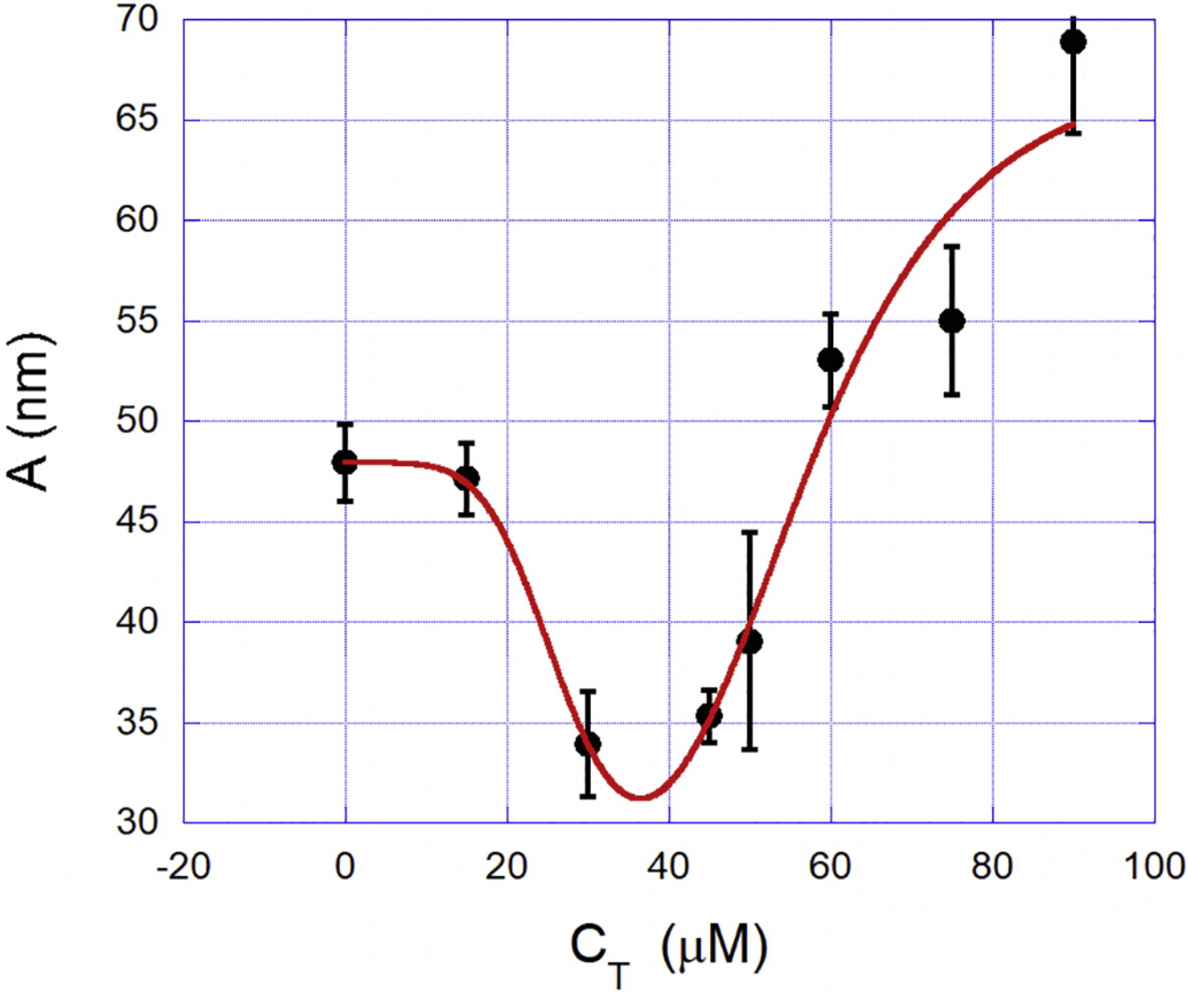


Figure 5

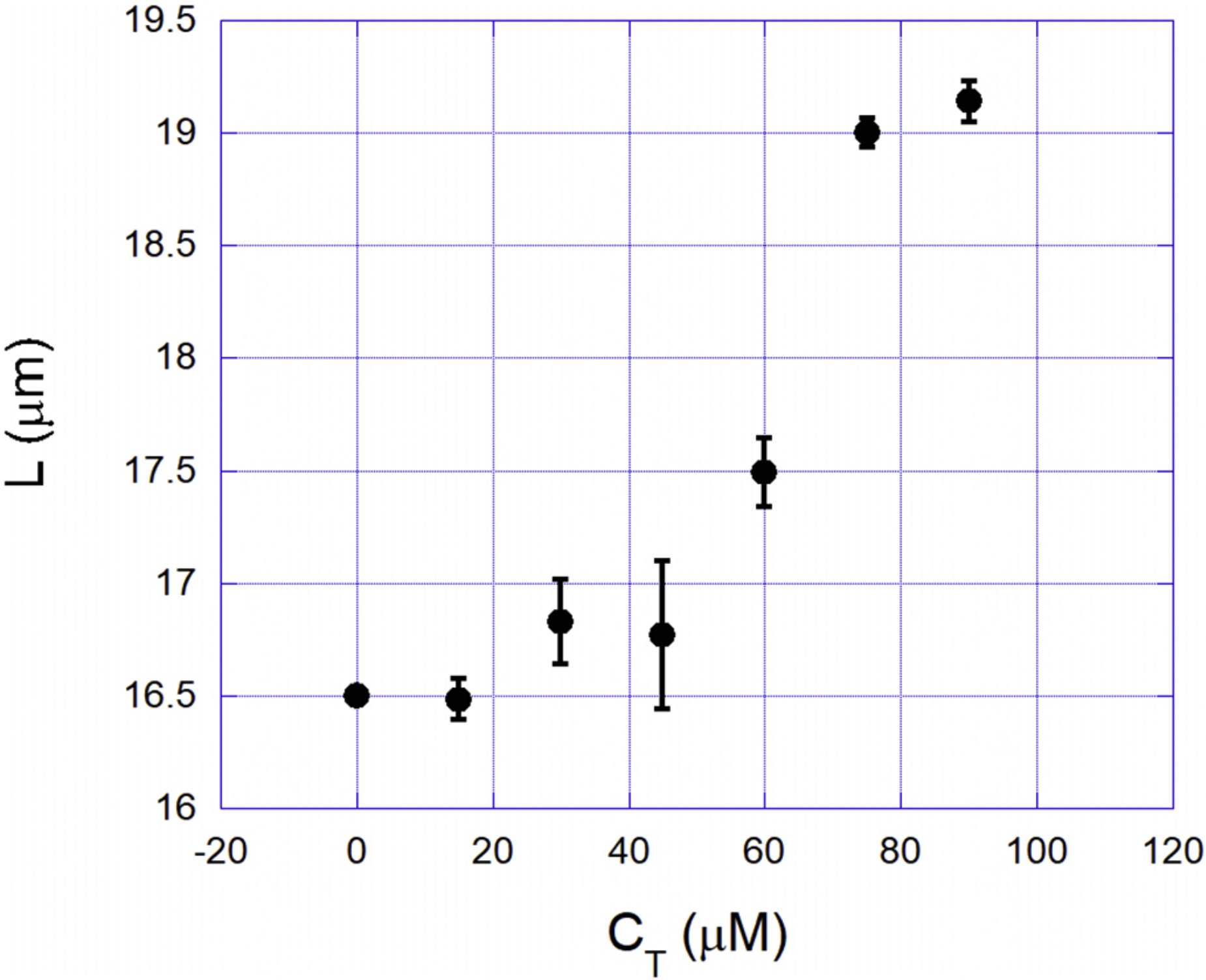


Figure 6