A study on the physicochemical properties and cytotoxic activity of \( p \)-sulfocalix[4]arene-nedaplatin complex

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Abstract. Macromolecules including macrocyclic species have been reported to have the potential to encapsulate biologically active compounds such as drugs through host-guest complexation to increase their solubility, stability and bioavailability. Here we investigate the complexation between nedaplatin, a second generation antineoplastic drug, and \( p \)-4-sulfocalix[4]arene, a macromolecule possessing a bipolar amphiphilic structure with good biocompatibility and relatively low haemolytic toxicity for potential use as a drug delivery system. Data from \(^1\)H NMR, UV-Vis spectroscopy, Job's plot analysis, HPLC, DSC and DFT calculations are detailed and suggest the formation of a 1:1 complex. The stability constant of the complex was experimentally estimated to be \( 3.6 \times 10^4 \) M\(^{-1}\) and \( 2.1 \times 10^4 \) M\(^{-1}\) which correspond to values of \(-6.2\) and \(-5.9\) kcal mol\(^{-1}\), respectively for the free energy of complexation while the interaction free energy is calculated to be \(-4.9\) kcal mol\(^{-1}\). The formed species is shown to be stabilised in solution through hydrogen bonding between the host and the guest. The complex displayed enhanced antitumor activity against MDA-MB-231 cells compared to nedaplatin which may allow for its application in cancer therapy.
1. Introduction
Nedaplatin is a second-generation platinum-based anticancer drug, which has been developed to overcome the drawbacks of cisplatin, the most widely used platinum-based anticancer drug. Nedaplatin, which has been approved in Japan since 1995, shows a much more enhanced anticancer activity and lower systemic side effects in comparison to cisplatin [3-5]. However, the use of nedaplatin in cancer therapy suffers many challenges such as cancer cells resistance and the potential risk to cause renal toxicity and hematotoxicity [5]. These challenges present significant obstacles for its global approval. Recently, efforts focused on designing delivery systems for more effective delivery of platinum-based drugs to their intended sites of action. Full or partial encapsulation of Pt based anti-cancer drugs including liposomes, polymer-based micelles, dendrimers, niosomes, and nanotubes are among the delivery systems reported [4–10]. Macromolecules are playing an increasingly important role in drug delivery, for instance, oxaliplatin-cyclodextrins inclusion complexes have shown to have much more antineoplastic activities against human colon cancer cells (HCT116) and human breast cancer cells (MCF-7) [11]. Macromolecules, such as cyclodextrins, calix[n]arenes and cucurbit[n]urils, have been reported to have the potential to encapsulate biologically active compounds such as drugs through host-guest complexation to increase their solubility, stability and bioavailability [12, 13]. Calix[n]arenes (n = 4, 6 and 8) are a third generation class of synthetic macrocyclic molecules. They are cone shaped, produced through the chemical synthesis of phenols and formaldehyde to yield phenol units linked by methylene bridges producing hydrophilic upper and lower rims of the truncated cone structures with hydrophobic π-electron rich mid-region cavity. Functionalization of these macrocycles allows them to possess different properties at their phenolic faces and their para-aromatic positions, thus the range of calix[n]arene derivatives is very large [13]. Among these many derivatives, p-Sulfonato-calix[4]arene (SC4), possesses a bipolar amphiphilic structure allowing for significant water solubility. More importantly SC4 exhibits good biocompatibility and has been reported to be relatively innocuous demonstrating no haemolytic toxicity in-vitro at concentrations up to 5 mM and is non-toxic in-vivo at doses up to 100 mg/kg [14, 15]. In this paper we investigate host-guest complexation between SC4 and nedaplatin in aqueous media by means of UV–Vis, and 1H NMR spectroscopy for potential use as a drug delivery system for nedaplatin. The stoichiometry and binding constant were determined by means of the continuous variation method (Job's plot) and by HPLC. Structural characterization of the host-guest complexes was carried out theoretically employing quantum mechanical calculations at Density Functional Theory (DFT) level whose outcomes are reported. The intermolecular hydrogen bonds of the nedaplatin-SC4 adducts were investigated by means of the Bader theory of Atoms In Molecules (AIM) [16]. The anticancer activity of the complex and the free drug, against human breast adenocarcinoma cells (MDA-MB-231), were examined, at different concentrations, employing MTT assay.

2. Experimental details

2.1 Chemicals and reagents
Nedaplatin was obtained from Shandong Boyuan Pharmaceutical Co. Ltd. and BIOZOL Diagnostica Vertrieb GmbH, Germany; Para-Sulfonato-calix[4]arene, SC4, Deuterium Oxide and HPLC-grade water were purchased from Sigma-Aldrich, Germany.

2.2 Investigation of the SC4-nedaplatin complex
2.2.1 1H NMR spectroscopy
1H NMR measurements were conducted in D2O employing Bruker Ascend™-400/R–1 MHz spectrometer [16].

2.2.2 UV–Vis Spectroscopy
UV spectrophotometric measurements were carried out on a CARY 500 UV–Vis-NIR Scan dual beam spectrophotometer (Varian, USA) [16].
2.2.3 HPLC

HPLC measurements were performed on a Thermo Fisher Scientific DIONEX ultimate 3000 series HPLC equipped with a RP BDS HYPERSIL C18, 250 × 4.6 mm, 5 μm column [16].

2.2.4 Cell culture

Human mammary gland/breast adenocarcinoma cells (MDA-MB-231), derived from metastatic site, were obtained from American Type Culture Collection (ATCC, Manassas, USA). MDA-MB-231 cells were cultivated at 37 °C and 7% CO₂ under humid conditions in DMEM (Dulbecco’s Modified Eagle Medium) medium supplemented with 10% fetal bovine serum, Gamma irradiated (Capricorn Scientific, Ebsdorfergrund, Germany). Cells were grown as monolayers and passaged upon reaching about 80 - 90% confluency.

2.2.5 In vitro cell viability assay

To investigate the in vitro antineoplastic activity of the SC4-nedaplatin complex, MDA-MB-231 cells were exposed to varying concentrations of SC4, nedaplatin and the SC4-nedaplatin complex. The viability of MDA-MB-231 cells was evaluated via MTT assay. In brief, MDA-MB-231 cells were seeded in a 96-well plate (Nunclon Delta, Thermo Fischer Scientific GmbH, Dreieich, Germany) with a seeding density of 10⁴ cells per well and were incubated with medium for 24 h at 37 °C and 7% CO₂. Solutions of SC4, nedaplatin and SC4-nedaplatin complex at 12.5, 16.5, 20.5, 24.5, 28.5, 32.5 and 36.5 µg ml⁻¹ were made in the culture media and incubated with the cells for 4 h. After incubation, the media were replaced by fresh ones and incubated for 24 h. On the following day, media were removed and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent dissolved with medium in a ratio of 1:10 was added to each well and incubated for 4 h. The resulting purple formazan crystals were solubilized using dimethyl sulfoxide (DMSO) (Sigma–Aldrich, Taufkirchen, Germany). The absorbance of each well was determined at 570nm using FluoStar® Optima plate reader (BMG Labtech, Offenburg, Germany). Cells treated only with media were considered as control.

3. Results and discussion

3.1 ¹H NMR spectroscopy

¹H NMR spectra of nedaplatin, SC4 and 1:1 molar ratio mixture of SC4 and nedaplatin are presented in Figure S1 (supporting information). ¹H NMR measurements indicated no significant complexation-induced shifts between SC4 and nedaplatin [16]. However, significant broadening of the signal due to the protons of the methylene bridges of the 1:1 M ratio mixture of SC4 and nedaplatin relative to the same signal in SC4 alone suggests complexation-induced conformational rigidity of the macrocyclic structure [17-19].

3.2 UV–Vis Spectroscopy

The method of continuous variation was adopted to study the stoichiometry and extract a stability constant for the SC4-nedaplatin supramolecular complex. In this method several solutions of varying molar ratios of SC4 and nedaplatin within the limits of 0 and 1 were prepared while keeping the total concentration of both species constant at 0.1 mM. The UV absorbance of each of these solutions was subsequently measured (Figure S2, supporting information). The derivative ratio method was adopted to determine the absorbance of SC4-nedaplatin complex without being affected with the significant overlap with the absorption of SC4 [16, 20, 21]. Another advantage of the derivative ratio method is the fact that the entire spectrum of the interfering signal, in this case due to SC4, is cancelled making the method much less sensitive to the choice of wavelength used for calibration relative to simple derivative methods [22]. The amplitudes of the first derivative ratio spectra peak at 265 nm which are proportional to the complex concentration were subsequently measured. A Job’s plot where the amplitudes of the first derivative ratio spectra peaks are plotted against the mole fraction of the
The corresponding SC4 molar fraction was constructed. The maximum amplitude of this plot was observed at a molar fraction of 0.5 indicating a complex stoichiometry of 1:1. A normalized version of this Job's plot where each of the amplitude values, $S$, were divided by the maximum amplitude, $S_{\text{max}}$, is shown in Figure 1. The stability constant of the complex was estimated using methods described elsewhere to be $3.6 \times 10^4 \text{M}^{-1}$ [22, 23]. This value lies within the range of the stability constants ($0.01 \times 10^3$–$1.7 \times 10^5$) $\text{M}^{-1}$ previously reported for complexes, many of which are intended for drug delivery [16, 24-27].

3.3 HPLC

The stability constant of the SC4-nedaplatin complex was determined from the HPLC data obtained to be $2.1 \times 10^4 \text{M}^{-1}$ which is in line with the stability constant obtained from Job's plot, suggesting a weakly bound complex [16]. These results agree with theoretical calculations which provide an interaction free energy of $-4.9 \text{ kcal mol}^{-1}$ for the association of nedaplatin with its ammonia ligands pointing inwards toward the wider upper rim of the cavity of SC4 (Figure S3, supporting information). The formed complex is shown to not involve the penetration of nedaplatin within the cavity of the SC4, however, it is mainly stabilized due hydrogen bonding between the hydrogen atoms of the nedaplatin ammonia ligands with the oxygen atoms of the calixarene sulfonato moiety [16].

3.4 In vitro cell viability assay

The antineoplastic activities of SC4, nedaplatin, and the SC4-nedaplatin complex were evaluated individually against MDA-MB-231 cells using MTT assay. Figure 2, shows the complex having a pronounced anticancer activity in comparison to nedaplatin alone. The cytotoxicity of the SC4-nedaplatin complex at 12.5 $\mu$g/ml, being the lowest concentration tested, was nearly twice that of equivalent concentration of the free drug. The cell viabilities decreased as the concentrations of either the complex or free drug increased reaching 30.1 and 32.5%, respectively, at the maximum
concentration used of 36.5µg/ml. The increased anticancer activity of the SC4-nedaplatin complex compared to free nedaplatin may be partially attributed to the improvement of the water solubility of nedaplatin, upon complexation with SC4, and hence improving its bioavailability [11]. The observed antineoplastic activity of SC4-nedaplatin at concentration of 12.5 µg/ml being nearly the same as that of the free drug at concentration of 36.5 µg/ml suggests the use of the developed complex for a possible reduction of the therapeutic dose of nedaplatin which in turn can contribute to the reduction of systemic adverse effects.

![Figure 2](image-url)

**Figure 2.** The % cell viability of nedaplatin (ND), SC4 and SC4-nedaplatin complex at different concentrations ranging from 12.5 to 36.5 µg/ml.

**Conclusion**

Data from ¹H NMR, UV-Vis spectroscopy, Job's plot analysis, HPLC and DFT calculations suggests that complexation between nedaplatin and SC4 in a 1:1 molar ratio under the conditions investigated does exist. The stability constant of the complex was estimated to be $3.6 \times 10^4$ M⁻¹ and $2.1 \times 10^4$ M⁻¹ which correspond to values of −6.2 and −5.9 kcal mol⁻¹ respectively for the free energy of complexation. These results agree with calculations which provide an interaction free energy of −4.9 kcal mol⁻¹ for the association of nedaplatin with its ammonia ligands pointing inwards toward the wider upper rim of the cavity of SC4. The formed complex is shown to not involve the penetration of nedaplatin within the cavity of the SC4, however, it is mainly stabilised due to hydrogen bonding between the hydrogen atoms of the nedaplatin ammonia ligands with the O atoms of the calixarene sulfonato moiety. The stability of the complex in solution may allow for its potential use as a drug delivery system. Data from MTT assay showed that the anticancer activity of SC4-nedaplatin at concentration of 12.5 µg/ml being nearly the same as that of the free nedaplatin at concentration of 36.5 µg/ml. This suggests the use of the developed complex for a possible reduction of the therapeutic dose of nedaplatin which in turn can contribute to the reduction of systemic adverse effects.

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References
[1] Kawai Y, Taniuchi S and Okahara S 2005 Biol. Pharm. Bull. 28 1385–88.
[2] Alberts D, Fanta P, Running K, Adair Jr L, Garcia D and Liu-Stevens R 1997 Cancer Chemother. Pharmacol. 39 493–497.
[3] Koshiyama M, Kinezaki M, Uchida T and Sumitomo M 2005 Anticancer Res. 25 4499–4502.
[4] Dragovich T, Mendelson D, Kurtin S, Richardson K, Von Hoff D and Hoos A 2006 Cancer Chemother. Pharmacol. 58 759–764.
[5] Lu C, Perez-Soler R, Walsh GL, Swisher SG, Smythe WR, Shin HJ, Ro JY, Feng L, Truong M, Yalamanchili A, Lopez-Berestein G, Hong WK, Khokhar AR and Shin DM 2005 J. Clin. Oncol. 23 3495–3501.
[6] Harper BW, Krause-Heuer AM, Grant MP, Manohar M, Garbutcheon-Singh KB and Aldrich-Wright JR 2010 Chem. Eur. J. 16 (24) 7064–77.
[7] Galanski MA, Keppler BK, Kratz F, Senter P, Steinhagen H 2012 (Eds.) (Wiley-VCH Verlag) p 1605–29.
[8] Boulikas T, Pantos A, Bellis E and Christofis P 2007 Structures and Mechanisms 5 537–583.
[9] Koshkaryev A, Sawant R, Deshpande M and Torchilin V 2013 Adv. Drug Deliv. Rev. 65 (1) 24–35.
[10] Alexis F, Pridgen EM, Langer R, Farokhzad OC and Schara-Korting M (Eds.) 2010 (Heidelberg: Springer-Verlag) Vol. 197 p 55–86.
[11] Zhang D, Zhang J, Jiang K, Li K, Cong Y, Pu S, Jin Y and Lin J 2016 Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 152 501–508.
[12] Dibama HM, Clarot I, Fontanay S, Ben Salem A, Mourer M, Finance C, Duval RE and Regnouf-de-Vains JB 2009 Bioorg. Med. Chem. Lett. 19 2679–82.
[13] Ma X, Zhao Y 2015 Chem. Rev. 115 7794–7839.
[14] Guo DS and Liu Y 2014 J. Chem. Res. 47 1925–34.
[15] Coleman AW, Jebors S, Cecillon S, Perret P, Garin D, Marti-Battle D and Moulin M 2008 New J. Chem. 32 780–782.
[16] Fahmy SA, Ponte F, Abd El-Rahman MK, Russo N, Sicilia E and Shoeib T. 2018 Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy 193 528–536.
[17] Abd El-Rahman MK and Mahmoud K 2015 RSC Adv. 5 62469–476.
[18] Guo D, Uzunova V, Su X, Liu Y and Nau W 2011 Chem. Sci. 2 1722.
[19] Shinkai S, Araki K, Matsuda T, Manabe and Bull O 1989 Chem. Soc. Jpn. 62 3856–62.
[20] Salem MY, El-Kosasy AM, El-Bardicy MG and Abd El-Rahman MK 2010 Drug Test. Anal. 2 (5) 225–233.
[21] Karpińska J 2004 Talanta 64 (4) 801–822.
[22] Nebsen M, Abd El-Rahman MK, Salem MY and El-Kosasy AM 2011 Drug Test. Anal. 3 (4) 221–227.
[23] Bosque-Sendra JM, Almansa-Lopez E, Garci-Campana AM and Cuadros-Rodriguez L. 2003Anal. Sci. 19 1431.
[24] Wheate N, Abbott G, Tate R, Clements C, Edrada-Ebel R and Johnston B 2009 J. Inorg. Biochem. 103 448–454.
[25] Bakirci H, Koner AL, Schwarzlose T and Nau WM 2006 Chem. Eur. J. 12 4799–4807.
[26] Wang G, Zhang H, Ding F and Liu Y 2011 J. Incl. Phenom. Macrocycl. Chem. 69 85–89.
[27] Yang W and M. de Villiers M 2005 AAPS J. 7 (1) (Article 23 (http://www.aapsj.org)).