

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Heteronanoparticles by Self-Assembly of Ecdysteroid and Doxorubicin Conjugates to Overcome Cancer Resistance

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1669312> since 2018-06-03T18:12:49Z

Published version:

DOI:10.1021/acsmmedchemlett.8b00078

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This document is confidential and is proprietary to the American Chemical Society and its authors. Do not copy or disclose without written permission. If you have received this item in error, notify the sender and delete all copies.

Hetero-Nanoparticles by self-assembly of ecdysteroid and doxorubicin conjugates as promising approach to overcome cancer resistance

Journal:	<i>ACS Medicinal Chemistry Letters</i>
Manuscript ID	ml-2018-00078v
Manuscript Type:	Letter
Date Submitted by the Author:	15-Feb-2018
Complete List of Authors:	<p>Fumagalli, Gaia; Università degli Studi di Milano, Chimica Giorgi, Giulia; Università degli Studi di Milano, Chimica Vágvölgyi, Máté; Szegedi Tudományegyetem (SZTE) Colombo, Eleonora; Università degli Studi di Milano, Chimica Christodoulou, Michael; Università degli Studi di Milano, Collico, Veronica; Università Milano Bicocca, Dipartimento di Biotecnologie e Bioscienze Prosperi, Davide; Università degli Studi di Milano-Bicocca, Department of Biotechnology and Bioscience Dosio, Franco; University Torino, Scienza e Tecnologia del Farmaco Hunyadi, Attila; Szegedi Tudományegyetem (SZTE) Montopoli, Monica; Università di Padova, Dipartimento di Farmacologia ed Anestesiologia Hyraci, Mariafrancesca; Università di Padova, Pharmaceutical Sciences Silvani, Alessandra; University of Milan, Dipartimento di Chimica Lesma, Giordano; Università degli Studi di Milano, Chimica Dalla Via, Lisa; Università di Padova, Pharmaceutical Sciences Passarella, Daniele; Università degli Studi di Milano, Chemistry</p>

SCHOLARONE™
Manuscripts

Hetero-Nanoparticles by self-assembly of ecdysteroid and doxorubicin conjugates as promising approach to overcome cancer resistance

Gaia Fumagalli,[¥] Giulia Giorgi,[¥] Máté Vágvolgyi,[‡] Eleonora Colombo,[¥] Michael S. Christodoulou,¹ Veronica Collico,[#] Davide Prosperi,[#] Franco Dosio,[‡] Attila Hunyadi,[‡] Monica Montopoli,[□] Mariafrancesca Hyeraci,[□] Alessandra Silvani,[¥] Giordano Lesma,[¥] Lisa Dalla Via,^{*□} Daniele Passarella,^{*,¥}

[¥] Dipartimento di Chimica, Università degli Studi di Milano, Milano 20133, Italy

[‡] Szegedi Tudományegyetem (SZTE), Institute of Pharmacognosy, Szeged, Hungary

¹ DISFARM, Sezione "A. Marchesini", Università degli Studi di Milano, Via Venezian 21, 20133 Milano, Italy

[#] Dipartimento di Biotecnologie e Bioscienze – Università Milano Bicocca – P.zza della Scienza 2, 20126 Milano, Italy

[□] Dipartimento di Scienze del Farmaco - Università degli Studi di Padova - Via F. Marzolo 5, 35131 Padova, Italy

[‡] Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Torino 10125, Italy

KEYWORDS Self-assembled-Nanoparticles, Ecdysteroids, Doxorubicin, Drug toxicity, Cancer

ABSTRACT: Hetero-nanoparticles (H-NPs) consisting of conjugates characterized by a squalene tail linked to doxorubicin and ecdysteroid derivatives are presented. Biological evaluation on A2780ADR cell line confirms not only the maintenance of the activity of the parental drug but also the ability to overcome cancer resistance. The *in vitro* cell uptake was demonstrated and the involvement of an endosomal-mediated pathway was suggested.

The formation of self-assembled nanoparticles (NPs) using anticancer drug conjugates could be a useful and smart approach to face cancer.¹⁻³ In the last few years, we developed a step-by-step project that moved from the preparation of a novel class of squalene-conjugates with paclitaxel, podophyllotoxin, camptothecin and epothilone A⁴ and reached, as highest evidence of efficacy, the preparation of hetero-nanoparticles with doxorubicin and cyclopamine conjugates that were able to reduce *in vivo* the tumour growth and toxicity due to the use of the single drugs.⁵⁻⁸ The conjugates were characterized by a squalene tail that makes them able to self-assemble in water, and by a drug unit connected *via* a disulfide-containing linker to secure the release inside the cell.

Our interest in facing the resistance of different kind of tumor cells,⁹ drove us to consider the formation of self-assembled hetero nanoparticles as a promising approach. Martins *et al.* recently reported the chemo-sensitizing effect of apolar ecdysteroids on MDR cancer cells.¹⁰ The antiproliferative activity of these compounds was very low, and they exerted a mild activity in modulating the ABCB1-mediated efflux of rhodamine 123. Further studies revealed that the chemo-sensitizing activity can be independent of efflux inhibition, in particular, ketals of poststerone, a known *in vivo* metabolite of 20-hydroxyecdysone, sensitized ABCB1-transfected cancer cells

to doxorubicin in a highly MDR selective manner without significantly interfering with the efflux function.

In this scenario we planned the preparation of some ecdysone conjugates containing the squalene tail to be combined with the known squalenoylated doxorubicin¹² (Chart 1) and investigate their ability to form hetero-nanoparticles (H-NPs) towards the treatment of doxorubicin resistant cell lines. In the light of the synergistic effect of ecdysteroid acetonides with doxorubicin,¹⁰ we focused our attention on diketals of 20-hydroxyecdysone and related derivatives of poststerone.

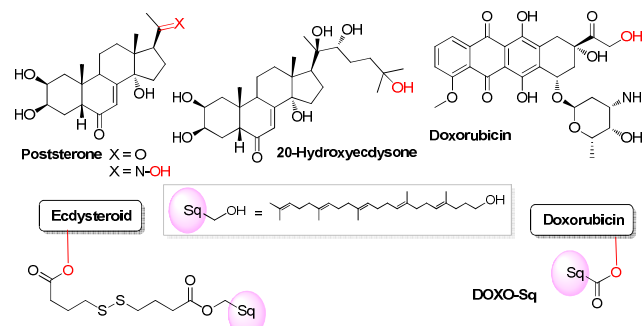
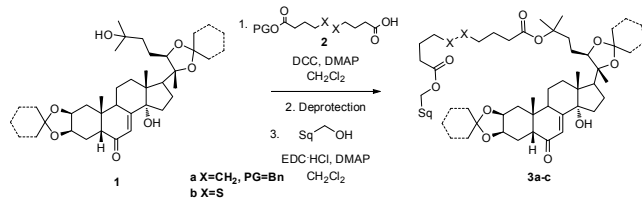
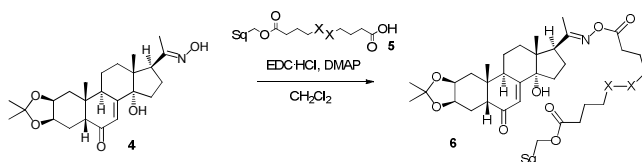


Chart 1. Schematic representation of the building blocks and obtained conjugates.

Five different conjugates were prepared according to Schemes 1 and 2 (see Supp. Info.). 20-Hydroxyecdysone derivatives **3a-c** were synthesized following a three-step procedure (Scheme 1): a) condensation reaction between the protected 20-hydroxyecdysone **1** and the proper acid **2**, b) deprotection of the carboxylic group and c) final condensation with 1',2-trisnorsqualene alcohol. Compounds **6a** and **6b** were prepared by a condensation reaction between compounds **5**⁴ and **4**.



Scheme 1. Synthesis of the conjugates **3a-c**.



Scheme 2. Synthesis of the conjugates **6a,b**.

Starting material	X	PG	Deprotection	Product
1 -diacetonide	CH ₂	Bn	H ₂ , Pd(OH) ₂	3a
1 -di(cyclohexylacetal)	CH ₂	Bn	H ₂ , Pd(OH) ₂	3b
1 -diacetonide	S	(CH ₂) ₂ SiMe ₃	TBAF	3c
4	CH ₂	-	-	6a
4	S	-	-	6b

Table 1. Ecdysteroid conjugates and corresponding starting material.

Next, the ability of the conjugates to self-assemble in nanoparticles was investigated. Briefly, compound solution in THF was added dropwise into ultrapure water. The spontaneous self-assembly into NPs is a consequence of local interactions between the hydrophobic molecules, mainly guided by squalene chains interactions. Subsequently, the organic solvent was removed under reduced pressure (see Supp. Info.). The nano-suspensions were characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS analysis confirmed the formation of nanoassemblies in aqueous *medium*. The assemblies were monodisperse (*PdI* < 0.2) with sizes around 200 nm, except for the **6a** NPs that exhibited a twofold larger hydrodynamic diameter (366.3 nm ± 20.17 nm). The highly negative ζ-potential values (< -20.0 mV) suggested that the electrostatic repulsion between NPs contributed to the stability of the nanoassemblies in aqueous *medium*.

NPs	h.d. ± S.D (nm)	P.I.	ζ-pot. ± S.D. (mV)
3a	198.1 ± 0.9	0.106 ± 0.041	-49.8 ± 0.9
3b	205.1 ± 0.6	0.050 ± 0.026	-43.0 ± 0.9
3c	214 ± 3.4	0.086 ± 0.046	-30.0 ± 4.6
6a	366.3 ± 20.17	0.161 ± 0.032	-21.5 ± 4.55
6b	221.8 ± 4.879	0.081 ± 0.088	-21.7 ± 1.50

Table 2. NPs characterization by dynamic light scattering (ZetaSizer, Malvern). *h.d.*: hydrodynamic diameter; *P.I.*: Polydispersity Index; *ζ-pot.*: ζ-potential.

Compounds **3a-c** and **6a,b** were further mixed with squalene-conjugated doxorubicine (**DOXO-Sq**) that is well known to

have a better therapeutic index than doxorubicin¹² and to form hetero-nanoparticles (H-NPs) according to our recent paper.⁷ H-NPs were prepared by mixing the compound solutions (125 μl, 250 μg, 2 mg ml⁻¹) with DOXO-Sq solution (2 mg ml⁻¹) in THF with a molar ratio compound/DOXO-Sq of 50 and by dropping the organic solution into 250 μl of ultrapure water as previously described. The mixture became opalescent suggesting the possible formation of particles. H-NPs filtered with a 0.45 μm filter were characterized by DLS and TEM. DLS analysis (Table 3) confirmed the formation of H-NPs in aqueous *medium*. The assemblies were monodisperse (*PdI* < 0.2), but larger compared to the homogeneous NPs, except for the **6a,b** NPs where **DOXO-Sq** resulted in a decrease of (or did not alter) the particle dimension, respectively. Also, the ζ-potential values decreased.

X:DOXO-Sq 50:1	h.d. ± S.D (nm)	P.I.	ζ-pot.±S.D. (mV)
3a	533.1 ± 26.60	0.160 ± 0.104	-9.73 ± 0.95
3b	538.2 ± 33.41	n.d.	-9.39 ± 2.91
3c	579.2 ± 104.4	0.226 ± 0.276	-20.3 ± 2.52
6a	187.7 ± 14.48	0.223 ± 0.069	-13.5 ± 9.65
6b	298.7 ± 11.43	0.264 ± 0.014	-11.1 ± 3.48

Table 3. H-NPs characterization by dynamic light scattering (ZetaSizer, Malvern). *h.d.*: hydrodynamic diameter, *P.I.*: Polydispersity Index; *ζ-pot.*: ζ-potential.

Figure 1 reports TEM analysis for NPs obtained by self-assembly of compound **6b** and the H-NPs **6b/DOXO-Sq** (50:1). The images show compact nanoparticles whose diameter is smaller if compared with the data obtained by DLS but similar in the case of H-NPs.

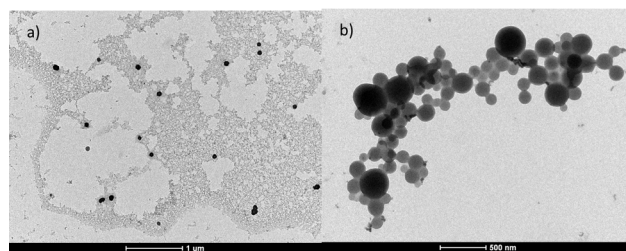


Figure 1. TEM analysis for NPs-**6b** (a, scale reported 1 μm) and H-NPs **6b/DOXO-Sq** (b, scale reported 500 nm)

The ability of both NPs and H-NPs to inhibit cell growth was assayed on A2780ADR cells, a human ovarian carcinoma doxorubicin-resistant cell line. The obtained results are shown in Table 4 and are expressed as GI₅₀ values, i.e. the concentration of the test agent inducing 50% reduction in cell number compared with control cultures.

A2780-ADR cells (GI ₅₀ , μM) ^a		
	NPs	X:DOXO-Sq 50:1
3a	> 50	0.50 ± 0.02
3b	> 50	0.78 ± 0.02
3c	> 50	0.69 ± 0.15
6a	19 ± 3	0.34 ± 0.08
6b	39 ± 4	0.26 ± 0.03
DOXO-Sq	1.17 ± 0.06	----

Table 4. Inhibition of A2780ADR cell growth.. ^aValues are the mean ± SD of at least four independent experiments.

Considering the NPs, it can be noted that **DOXO-Sq** exerts an interesting inhibitory effect on A2780ADR proliferation, showing a GI_{50} value of about 1 μ M. This capacity, notwithstanding about ten times lower with respect to that of doxorubicin ($GI_{50} = 0.16 \pm 0.01 \mu$ M) confirms, in accordance with previous studies,¹² the ability of the drug to induce an antiproliferative effect even following squalenylation. Otherwise, the NPs obtained by squalene-conjugated ecdysteroids provoke in the same experimental conditions a negligible effect on cells and indeed, only for the nanoassemblies **6a** and **6b** a detectable and quite low cytotoxicity on A2780ADR cells was demonstrated. Notably, the H-NPs obtained by mixing **3a-c** and **6a,b** with **DOXO-Sq** induce a significant antiproliferative effect, with GI_{50} values from 1.5 to more than 4 times lower with respect to that obtained for **DOXO-Sq**. These data suggest the ability of the H-NPs to overcome, at least partially, the resistance phenomenon. In this connection, it was of interest to investigate the effect of tamoxifen, a well-known inhibitor of the P-glycoprotein¹³ on the intracellular uptake of the H-NPs **3a/DOXO-Sq**, taken as an example, and of doxorubicin, used as reference. The cell internalization in A2780ADR was monitored by flow cytometry. As expected, the presence of tamoxifen significantly increased the drug accumulation in these cells. In detail, the intracellular mean fluorescence intensity of doxorubicin rose by 27% after exposure to the P-glycoprotein inhibitor. Otherwise, no difference in fluorescence was observed between the cells treated and those not-treated with tamoxifen. This result further supports the hypothesis that these new H-NPs can play a crucial role in drug resistance mechanism, suggesting the inability or at least the reduction of P-glycoprotein to mediate their efflux from resistant cells. The *in vitro* cell internalization was then further investigated by fluorescence microscopy to verify any change in the uptake pathway. For this purpose, A2780ADR cells were incubated for two hours in the presence of doxorubicin and the H-NPs **3a/DOXO-Sq**. The results are shown in Figure 2a and 2b, respectively.

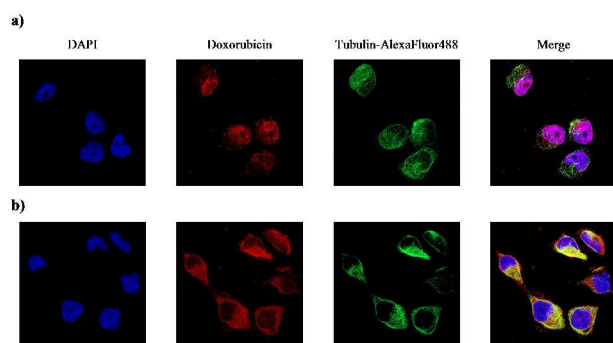


Figure 2. Fluorescence microscopy of A2780ADR cells incubated for 2h in the presence of doxorubicin (a) or H-NPs **3a/DOXO-Sq** (b) at 5 μ M. The nucleus and cytoplasm were stained with DAPI and antibody anti-tubulin conjugated with AlexaFluor488, respectively.

The cell penetration of both agents is rapid, nevertheless, the intracellular localization differs: for the drug a major localization in the nuclei is observed (a), while for the H-NPs a staining almost exclusive in the cytoplasm appears (b).

A more in depth microscopy analysis (Figure 3), highlights in the cytoplasm of A2780ADR cells treated with the H-NPs **3a/DOXO-Sq**, the occurrence of vesicles characterised by a

mean diameter of about 0.9 μ m, a value significantly higher with respect to the hydrodynamic diameter of the corresponding H-NP (538.2 ± 33.41 , Table 3). Such observation suggests that the H-NPs could enter into cell through an endosome-mediated pathway, a mechanism already demonstrated for **DOXO-Sq** in a human pancreatic cell line.¹²

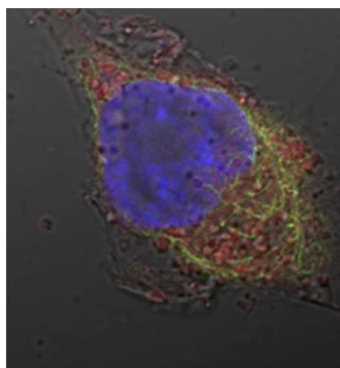


Figure 3. Fluorescence microscopy of A2780ADR cell treated for 2h with H-NPs **3a/DOXO-Sq**.

The obtained results highlight the effectiveness of self-assembled H-NPs to face cancer resistance and show a general strategy that could be applied in various pathologies where combined therapy could be beneficial.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. It contains the experimental details regarding: preparation of compounds **3a-c** and **6a,b**, nanoparticles preparation and characterization, biological evaluation. (cell cultures, inhibition growth assay, cytofluorimetric analysis, confocal microscopy analysis)

AUTHOR INFORMATION

Corresponding Author

*E-mail: danielle.passarella@unimi.it

*E-mail: lisa.dallavia@unipd.it

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. *These authors contributed equally.

ACKNOWLEDGMENT

D. Passarella, G. Lesma, A. Silvani express their gratitude to the family of Prof. Bruno Danieli (who passed away in 2014) for a research grant to financially support a fellowship for G. Giorgi. L.Dalla Via is grateful to the financial support provided by Dipartimento di Scienze del Farmaco-Università di Padova-Progetti di Ricerca di Dipartimento PRID 2017. A.H. acknowledges the National Research, Development and Innovation Office, Hungary (NKFIH; K119770). M.V. was supported by the New National Excellence Program of the Ministry of Human Capacities, Hungary (UNKP-17-3) and by an STSM grant from COST Action CM1407, Challenging organic syntheses inspired by nature - from natural products chemistry to drug discovery.

REFERENCES

- (1) Xing, P.; Zhao, Y. Multifunctional Nanoparticles Self-Assembled from Small Organic Building Blocks for Biomedicine. *Adv. Mater.* **2016**, *28*, 7304–7339.
- (2) Arias, J. L.; Reddy, L. H.; Othman, M.; Gillet, B.; Desmaele, D.; Zouhiri, F.; Dosio, F.; Gref, R.; Couvreur, P. Squalene Based Nanocomposites: A New Platform for the Design of Multifunctional Pharmaceutical Theragnostics. *ACS Nano* **2011**, *5*, 1513–1521.
- (3) Fumagalli, G.; Marucci, M.; Christodoulou, M. S.; Stella, B.; Dosio, F.; Passarella, D. Self Assembly Drug Conjugates for Anticancer Treatment. *Drug Discov. Today* **2016**, *21*, 1321–1329.
- (4) Borrelli, S.; Christodoulou, M. S.; Ficarra, I.; Silvani, A.; Cappelletti, G.; Cartelli, D.; Damia, G.; Ricci, F.; Zucchetti, M.; Dosio, F.; Passarella, D. New Class of Squalene-Based Releasable Nanoassemblies of Paclitaxel, Podophyllotoxin, Camptothecin and Epothilone A. *Eur. J. Med. Chem.* **2014**, *85*, 179–190.
- (5) Borrelli, S.; Cartelli, D.; Secundo, F.; Fumagalli, G.; Christodoulou, M. S.; Borroni, A.; Perdicchia, D.; Dosio, F.; Milla, P.; Cappelletti, G.; Passarella, D. Self-Assembled Squalene-based Fluorescent Heteronanoparticles. *ChemPlusChem* **2015**, *80*, 47–49.
- (6) Fumagalli, G.; Mazza, D.; Christodoulou, M. S.; Damia, G.; Ricci, F.; Perdicchia, D.; Stella, B.; Dosio, F.; Sotiropoulou, P. A.; Passarella, D. Cyclopamine–Paclitaxel-Containing Nanoparticles: Internalization in Cells Detected by Confocal and Super Resolution Microscopy. *ChemPlusChem* **2015**, *80*, 1380–1383.
- (7) Fumagalli, G.; Stella, B.; Pastushenko, I.; Ricci, F.; Christodoulou, M. S.; Damia, G.; Mazza, D.; Arpicco, S.; Giannini, C.; Morosi, L.; Dosio, F.; Sotiropoulou, P. A.; Passarella, D.

Heteronanoparticles by self-Assembly of Doxorubicin and Cyclopamine Conjugates *ACS Med. Chem. Lett.* **2017**, *8*, 953–957.

(8) Fumagalli, G.; Christodoulou, M. S.; Riva, B.; Revuelta, I.; Marucci, C.; Collico, V.; Prosperi, D.; Riva, S.; Perdicchia, D.; Bassanini, I.; García-Argáez, A.; Dalla Via, L.; Passarella, D. Self-assembled 4-(1,2-diphenylbut-1-en-1-yl)aniline Based Nanoparticles: Podophyllotoxin and Aloin as Building Blocks. *Org. Biomol. Chem.* **2017**, *15*, 1106–1109.

(9) a) Sotiropoulou, P. A.; Christodoulou, M. S.; Silvani, A.; Herold-Mende, C.; Passarella, D. Chemical Approaches to Targeting Drug Resistance in Cancer Stem Cells. *Drug Discov. Today*, **2014**, *19*, 1547–1562; b) Marucci, C.; Fumagalli, G.; Calogero, F.; Silvani, A.; Christodoulou, M. S.; Martinet, N.; Passarella, D. Natural Products and Cancer Stem Cells. *Curr Pharm Des.* **2015**, *21*, 5547–5557.

(10) Martins, A.; Tóth, N.; Ványolós, A.; Béni, Z.; Zupkó, I.; Molnár, J.; Báthori, M.; Hunyadi, A. Significant Activity of Ecdysteroids on the Resistance to Doxorubicin in Mammalian Cancer Cells Expressing the Human ABCB1 Transporter. *J. Med. Chem.* **2012**, *55*, 5034–5043.

(11) Savchenko, R. G.; Kostyleva, S. A.; Meshcheryakova, E. S.; Khalilov, L. M.; Parfenova, L. V.; Odnokov, V. N. Synthesis of Novel α -Aminoecdysteroids via Regio- and Stereoselective Oximation/Hydrogenation of 20-Hydroxyecdysone Derivatives. *Can. J. Chem.* **2017**, *95*, 130–133.

(12) Maksimenko, A.; Dosio, F.; Mougin, J.; Ferrero, A.; Wack, S.; Reddy, L. H.; Weyn, A.-A.; Lepeltier, E.; Bourgaux, C.; Stella, B.; Cattel, L.; Couvreur, P. A Unique Squalenoylated and Nonpegylated Doxorubicin Nanomedicine with Systemic Long-Circulating Properties and Anticancer Activity. *Proc. Natl. Acad. Sci. U S A* **2014**, *111*, E217–E226.

(13) Callaghan, R.; Higgins, C. F. Interaction of Tamoxifen with the Multidrug Resistance P-Glycoprotein. *Brit. J. Cancer* **1995**, *71*, 294–299.

Table of Contents (TOC)

