New Insights into Tolytoxin Effect in Human Cancer Cells: Apoptosis Induction and the Relevance of Hydroxyl Substitution of Its Macrolide Cycle on Compound Potency

Kateřina Delawská,[a, b] Petra Divoká,[a] David Sedlák,[c] Marek Kuzma,[d] Kumar Saurav,[a] Markéta Macho,[a, b] Gabor Steinbach,[e] and Pavel Hrouzek*[a]

Scytophycins, including tolytoxin, represent a class of actin disrupting macrolides with strong antiproliferative effects on human cells. Despite intense research, little attention has been paid to scytophycin-induced cell death or the structural features affecting its potency. We show that tolytoxin and its natural analogue, 7-*O*-methylscytophycin B, lacking the hydroxyl substitution in its macrolactone ring, differ substantially in their cytotoxic effect. Both compounds increase the level of caspases 3/7, which are the main executioner proteases during apoptosis, in HeLa wild-type (WT) cells. However, no caspase activity was detected in HeLa cells lacking Bax/Bak proteins crucial for caspase activation via the mitochondrial pathway. Obtained data strongly suggests that scytophycins are capable of inducing mitochondria-dependent apoptosis. These findings encourage further research in structure-activity relationships in scytophycins and highlight the potential of these compounds in targeted drug delivery.

The cytoskeletal proteins forming microfilaments, intermediate filaments, and microtubules are crucial for maintaining the shape and structure of a cell. They are also important in cell movement and vesicular transport as well as in tumor cell migration, invasion, and metastasis.[1]

Therefore, cytoskeleton targeting compounds are generally an attractive option for drug discovery. Microtubules as part of the mitotic spindle and microfilaments assembling the contractile ring are essential for cell division. Thus, malignant cells are more prone to cytoskeletal damage due to their enhanced cell proliferation.[2] Antimitotic compounds with microtubule-destabilizing effects are nowadays used as anticancer drugs in clinics,[3] however, not a single microfilament interfering agent has found a clinical application so far.

Scytophycins are cytotoxic macrolides isolated from cyanobacteria.[4,5] Tolytoxin, 6-hydroxy-7-*O*-methylscytophycin B (TLX), was the first described member of this family and its full chemical structure was revealed in 1990 (Figure 1A).[5] It was proved to disturb the microfilament organization of mammalian cells.[6] The mechanism of TLX action was inferred from the effect of other structurally similar macrolides such as reidispongiolide, jaspisamide A, and kabiramide C, all sharing with TLX a highly similar linear polyketide chain. These compounds bind to the barbed end of F-actin, in particular inside the hydrophobic cleft placed between actin subunits 1 and 3.[7–9] Consequently, their linear polyketide tail is buried in the cleft which normally serves for the DNase I-binding loop of the actin monomer during filament growth. This insertion results in filament capping or disruption and sequestration of G-actin. Thus the tail region apparently plays a more important role in the binding and disruption of actin than the macrolide ring.[7,10] This finding

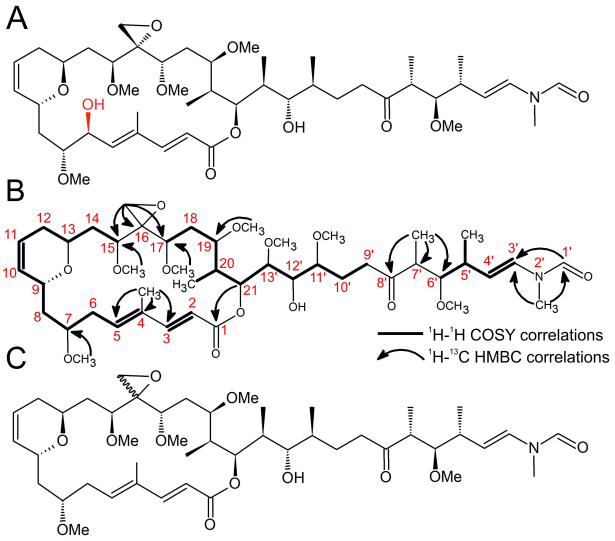

Figure 1. Chemical structure of TLX (A) and its structural variant 7-OMeSB lacking hydroxy- group on position 6 (B, C). The crucial NMR correlations in 7-OMeSB are depicted in (B) and the stereochemical assignment as obtained by comparative NMR measurement in (C).

[a] *K. Delawská, P. Divoká, Dr. K. Saurav, M. Macho, Dr. P. Hrouzek*
Institute of Microbiology of the Czech Academy of Sciences – Center Algatech
Novohradska 237, 37981, Trebon (Czech Republic)
E-mail: hrouzek@alga.cz
[b] *K. Delawská, M. Macho*
Faculty of Science, University of South Bohemia in České Budějovice
Branišovská 1760, 37005, České Budějovice (Czech Republic)
[c] *Dr. D. Sedlák*
CZ-OPENSCREEN: National Infrastructure for Chemical Biology
Institute of Molecular Genetics of the ASCR, v. v. i.
142 20 Prague 4 (Czech Republic)
[d] *Dr. M. Kuzma*
Laboratory of Molecular Structure Characterization
Institute of Microbiology, Academy of Sciences of the Czech Republic
Videnska 1083, 142 20 Prague (Czech Republic)
[e] *Dr. G. Steinbach*
Institute of Biophysics, Biological Research Center
6726 Temesvári krt. 62. Szeged (Hungary)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbic.202100489

was supported by the fact that synthetically prepared derivates of the linear polyketide tail exhibit actin disrupting activity.[8] However, it is worth noting that the presence of the macrolide ring is necessary for maintaining the high effectivity of the actin binding and disruption.[8]

TLX was demonstrated to exhibit a cytostatic effect against seven cancer cell lines at very low doses (0.5–8.4 nM) and was highly toxic to mice (LD_{50} – 1.5 mg/kg).[11] TLX-induced cytostatic activity was also observed in human epidermoid carcinoma (KB; retrospectively determined as HeLa contamination),[12] murine lymphocytic leukemia (L1210),[6] and interestingly also in the multi-drug resistant cell line SKVLB1 (ovarian carcinoma).[13]

Although the ability to induce apoptosis, a form of programmed cell death, is one of the essential features of chemotherapeutic drugs, no characterization of cell death modality and cell fate has been performed in TLX treated cells. Some authors even highlight that deeper investigation in this field is necessary for scytophycin family members.[2]

In the present contribution, we investigate the mechanism of cell death induced by TLX and its structural variant 7-*O*-methylscytophycin B (7-OMeSB, Figure 1A, B) and we compare their potency against a panel of cancer and primary cell lines. We show for the first time the induction of apoptosis by TLX and 7-OMeSB in cancer cells.

The TLX used was isolated from the soil cyanobacterium *Nostoc* sp. 5/96.[14] and 7-OMeSB from the benthic cyanobacterium *Anabaena* sp. ČIRK A. The identity of TLX (Figure 1A) was confirmed by the NMR measurement in acetone-d_6 and subsequent comparison with the literature data[5] which provided a good fit in δ_C values (Table S1) suggesting identical stereochemistry as described previously (Figure 1A). The chemical structure of 7-OMeSB was elucidated in 2015.[15] The mass spectrum of 7-OMeSB isolated in this study featured $[M+H]^+$ ion at 834.5359, which corresponds to the molecular formula $C_{46}H_{75}NO_{12}$ (Δ 1.07 ppm). The compound NMR was measured in CD_3OD, and therefore exchangeable protons were not detected. The spectra contain two components due to the presence of *cis/trans* isomers of the amidic bond. The assignment of ^1H and ^{13}C NMR spectra of 7-OMeSB is listed in Table S2 and shown in Figures S1 and S2. The 7-OMeSB ^{13}C NMR spectrum contains forty-six carbon signals for the main component. Multiplicity-edited ^1H-^{13}C HSQC (Figure S3) revealed their spin multiplicity as follows, twelve methyls, eight methylenes, twenty-two methines, and four quaternary carbons. The particular residues were identified by a combination of COSY, TOCSY, and ^1H-^{13}C HSQC-TOCSY (Figures S4–S6). The crucial correlations are depicted in Figure 1B. Finally, the ^1H-^{13}C HMBC spectrum (Figure S7) allowed us to assign quaternary carbons and put the particular structure together, as shown in Figure 1B. The stereochemistry of 7-OMeSB was assessed by comparison of the ^1H and ^{13}C NMR data acquired for TLX in CD_3OD (Supporting results, Tables S3 and S4). A comparison led to the conclusion that the linear polyketide chain, C21–C19, and the pyrano region possess identical stereochemistry to TLX. The C21–C1 region exhibited ROE correlations (Figures S8 and S9), which are consistent with the X-ray crystallography-based model of previously published scytophycin C di-*p*-bromobenzoate methanol solvate.[4] However, the ROE correlations of 16-CH_2 were not fully conclusive for the epoxy-functional group.

In the past, the effect of TLX against cancer and primary cell lines was investigated to a very limited extent.[11] Therefore, we tested TLX and its structural variant 7-OMeSB against 18 cancer and 3 normal human cell lines. The applied concentration range was from 0.1 nM to ~14 μM and the cytotoxic potency of compounds was determined using the Cell Titer Glo™ homogeneous assay (as a measure of the ATP level) after 72 h of exposure time. The potencies of these compounds in different cell lines varied considerably and IC_{50} could be found in the range starting at 16.3 nM and going beyond 14 μM. TLX, in comparison with 7-OMeSB, exhibited an exceptionally stronger cytotoxic effect (Table 1, Figure S10) with a 5.2 times higher potency in RPE-1 and BJ cells. A similar ratio (4- to 7-times) could be found in 4 colorectal and 3 leukemic lines and even higher sensitivity (11.6- and 9.3-times) was found in ovarian

Table 1. Obtained IC_{50} values of TLX and 7-OMeSB in cancer and primary (highlighted in bold) human cell lines (X – IC_{50} not reached in 14 μM).

Human cell line (origin, classification)	IC_{50} [nM][a] TLX	7-OMeSB
HL-60 (blood, leukemia)	16.31 ± 0.91	103.6 ± 13.08
D-283 Med (brain, blastoma)	29.75 ± 2.51	172.74 ± 3.3
U-937 (blood, lymphoma)	31.16 ± 9.57	232.66 ± 86.063
MDA-MB-231 (breast, carcinoma)	42.69 ± 14.05	348.83 ± 30.12
RKO (colon, carcinoma)	59.16 ± 0.77	347.69 ± 17.48
RPE-1 (retina, normal)	**82.55 ± 4.86**	**431.16 ± 28.5**
BJ (foreskin, normal)	**112.04 ± 11.41**	**582.52 ± 32.21**
Capan-2 (pancreas, carcinoma)	117.47 ± 20.12	1067.05 ± 148.29
Caov-3 (ovary, carcinoma)	139.35 ± 15.57	1622.55 ± 180.93
K-562 (blood, leukemia)	148.52 ± 38.48	798.86 ± 249.84
HT-29 (colon, carcinoma)	177.21 ± 33.79	1169.53 ± 167.29
BxPC-3 (pancreas, carcinoma)	217.07 ± 11.56	1830.05 ± 44.71
U-2OS (bone, sarcoma)	257.26 ± 10.77	1860.05 ± 185.88
DU-145 (prostate, carcinoma)	472.63 ± 14.02	3191.71 ± 129.02
SW-480 (colon, carcinoma)	658.39 ± 30.88	2567.8 ± 196.27
SJRH-30 (muscle, sarcoma)	682.54 ± 48.86	3349.89 ± 357.19
AsPC-1 (pancreas, carcinoma)	703.98 ± 53.02	2950.09 ± 506.12
HCT-116 (colon, carcinoma)	1864.34 ± 115.61	8101.24 ± 520.15
Hep G2 (liver, carcinoma)	X	X
HEK-293 (kidney, normal)	**X**	**X**
MCF-7 (breast, carcinoma)	X	X

[a] Values are expressed as mean ± SD, n = 3.

Caov-3 and pancreatic Capan-2 cell lines. Both compounds showed the highest potency against HL-60 (blood, leukemia), D-283 Med (brain, blastoma), U-937 (blood, lymphoma), and MDA-MB 231 (breast, carcinoma) cell lines in comparison with normal cells RPE1 (retina, epithelial cells) and BJ (foreskin, fibroblast) with a promising selectivity index (SI) in HL-60, D-283 Med and U-937 cells (Table 2). It is worth noting that MCF-7 and HEK-293 were resistant to the effect of both compounds in such a way that no toxic effects have been recorded up to the highest concentration. For IC_{50} of all 21 tested cell lines see Table 1.

TLX was previously demonstrated to lack hemolytic activity.[11] Accordingly, both TLX and 7-OMeSB exhibited only a negligible effect on red blood cells (Figure S11) leading to a maximum of 15% hemolysis up to the highest concentration tested (500 nM).

The difference in activity of TLX and 7-OMeSB is striking as the compounds differ mostly in the presence of a hydroxyl group at C6 (Figure 1A and 1 C) of the macrolactone ring which is thought to anchor the scytophycin molecule to actin via hydrophobic interaction with amino acid residues at the entrance of the hydrophobic cleft.[7] Thus, other than hydrophobic interactions could play a role in the anchoring of TLX to actin. Notably, lobophorolide, the compound sharing with TLX identical macrolactone ring, was demonstrated to interact with G-actin in a stoichiometric ratio 2:2 when two lobophorolide molecules tether together two actin monomers via interaction of both the ring and the tail region. Most interestingly, the suspected hydroxyl is predicted to stabilize the interaction by the formation of a hydrogen bond with Ala 144 of G-actin.[16] This fully supports our theory and shows that TLX can act in a similar way. As the stereochemical arrangement of the epoxy-group at the C16 of 7-OMeSB macrolide ring was not determined it might contribute to different compound bioactivity as well. Nevertheless, we show that the substituents present on the macrolide ring are important for scytophycin potency which was not previously considered. It is also important to note that while in kabiramide and jaspisamide the polar hydroxyl substituents are present in the same or close position as in TLX (C5 and C7, respectively), in reidiospongiolide the polar substituents are entirely missing on the macrolactone ring.[7,8] The fact that all these molecules exhibit low nanomolar IC_{50} values suggests that the macrolide ring is interacting with the entrance of the actin subdomain 1/3 hydrophobic patch in a compound-specific way. Such a finding encourages more detailed structure-activity relationship studies aimed at the substituent present at the macrolactone moiety.

Table 2. Selectivity index (SI) values of TLX and 7-OMeSB in the most sensitive cell lines (SI > 1) with BJ fibroblasts as a reference normal cell line. Values are expressed as mean ± SD, n = 3.

Cancer cell line (origin, classification)	Selectivity index TLX	7-OMeSB
HL-60 (blood, leukemia)	6.9 ± 0.91	5.73 ± 0.95
D-283 Med (brain, blastoma)	4.02 ± 1.55	3.37 ± 0.22
U-937 (blood, lymphoma)	3.8 ± 0.61	2.82 ± 0.88
MDA-MB-231 (breast, carcinoma)	2.83 ± 0.65	1.67 ± 0. 08
RKO (colon, carcinoma)	1.89 ± 0.21	1.67 ± 0.01

Although the induction of apoptosis has been demonstrated for various macrolides such as cytochalasin D[2] leptolyngbyolides[17] and sphinxolides[18] it has never been explored for scytophycins. Apoptosis can be induced by various stimuli which consequently trigger intrinsic or extrinsic signaling pathways. While the extrinsic pathway relies on receptor-ligand interactions, the intrinsic pathway is associated with mitochondria and pro-apoptotic Bcl-associated X protein (Bax) and Bcl-2 antagonist/killer1 (Bak). The Bax/ Bak proteins form a mitochondrial membrane permeabilization pore which leads to the release of cytochrome c and subsequent activation of effector caspases 3 and 7 directly executing the proteolytic cleavage of the cells.[19]

We have studied the elevation of caspase 3/7 in HeLa WT and Bax/Bak double knockout (Bax/Bak DKO) cells under compound treatment at 50 and 500 nM which were selected according to IC_{50} data obtained from HeLa WT (TLX, 7-OMeSB, respectively; Figure S12). Initially, we monitored differences in effectivity of TLX/7-OMeSB in time (24, 48, and 72 h) against HeLa WT (Figure 2A) using the Cell Titer Glo™ homogeneous assay.Interestingly, a decrease in viability was not observed in 7-OMeSB (50 nM) treated cells even after 72 h of exposure in comparison with a pronounced drop in viability after TLX treatment (50 nM). A significant decrease in viability was observed in TLX (500 nM) treated cells compared to the milder effect of 7-OMeSB. These data support the results obtained for other cell lines (Table 1) and shows a clear difference between compound potencies. The viability of the Bax/Bak DKO cells in time followed the same trend as observed for HeLa WT, although with slightly higher values (Figure 2B).

7-OMeSB, in contrast to TLX, manifested a lower actin depolymerization potential as demonstrated using immunofluorescence confocal microscopy in HeLa cells. While the residual condensed actin spots and focal actin can be observed in 7-OMeSB treated cells the actin signal is entirely lacking after TLX treatment (Figure 3).

The caspase 3/7 activity in treated HeLa WT cells was evaluated using Caspase-Glo® 3/7 luminescence assay in time (24, 48, 72 h). Data were completed by quantification of the total number of cells per well using Hoechst 33342 nuclear stain and an automated Fiji[20] macro developed in our laboratory. Subsequently, the obtained caspase 3/7 relative luminescence units (RLU) were adjusted to the cell count and normalized to the control (Figure 2C). The main onset of caspase 3/7 activity was recorded mainly at 48 h after treatment of both compounds which was followed by its decline at 72 h, suggesting that the cells underwent apoptosis mainly at 48 h (Figure 2C and Figure S13). Staurosporine (STS) 0.5 μM, inductor of caspase-dependent apoptotic cell death, was used as a positive control.

To determine the involvement of the intrinsic apoptotic pathway, HeLa Bax/Bak DKO cells were treated with compounds

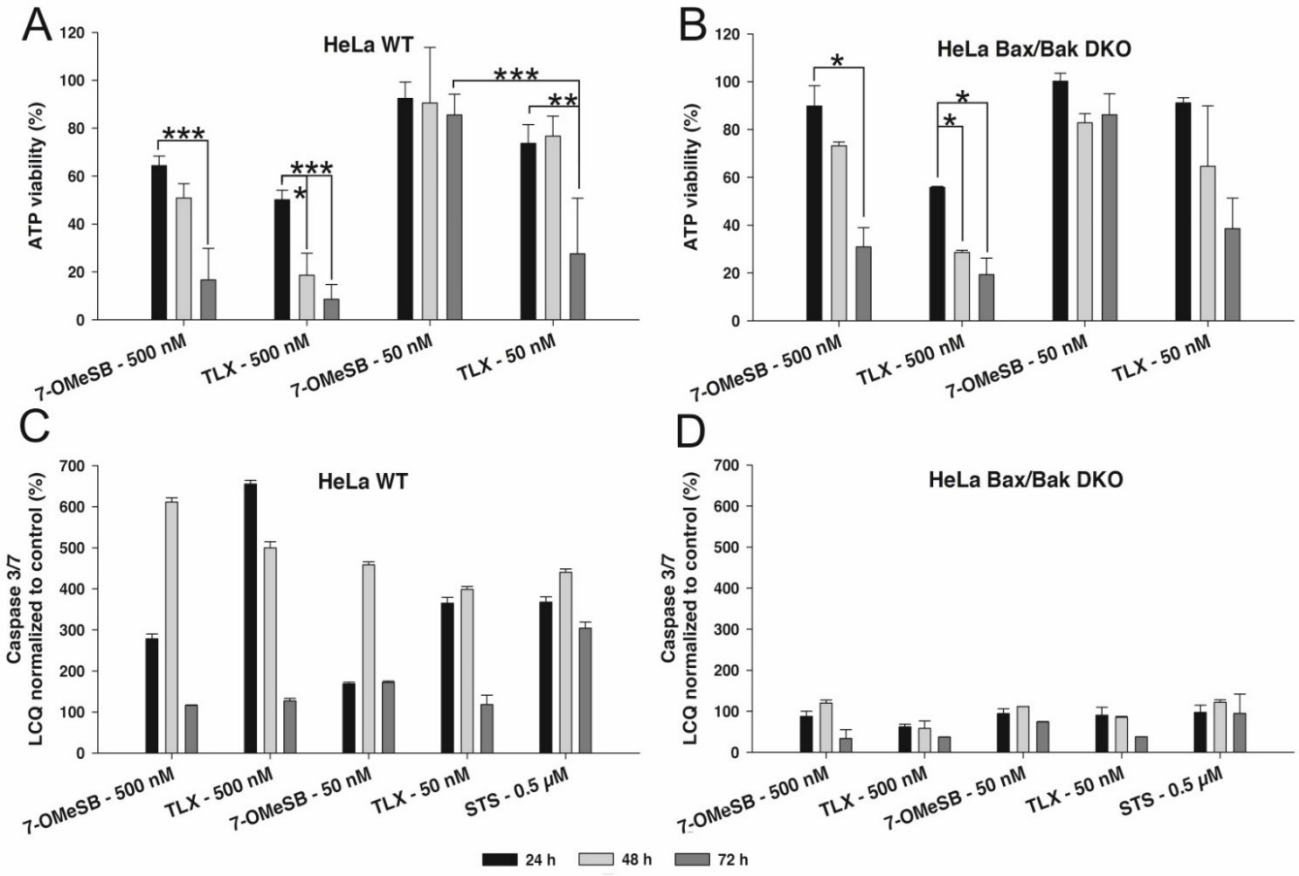

Figure 2. The viability of HeLa WT and Hela Bax/Bak DKO assessed by ATP assay after TLX and 7-OMeSB treatment (A, B). Cell viability was calculated as the percentage of viable cells in compounds-treated cells related to control cells at 24, 48, and 72 h. The caspase 3/7 activity of HeLa WT and HeLa Bax/Bak DKO cells treated by TLX, 7-OMeSB, and staurosporine (STS) was evaluated by Caspase-Glo® 3/7 assay (C, D). LCQ (luminescence cell quota) was obtained by normalizing the luminescence values to the number of cells and divided by control values at 24, 48, and 72 h. STS (0.5 μM) was used as a positive control. For panels (A, B, and D) the values are expressed as mean ± SD, n = 3. For caspase 3/7 activity in HeLa WT (C) data obtained as mean ± SD, n = 1 are depicted. For the remaining data see Figure S13.

and caspase-3/7 activity was evaluated by Caspase-Glo® 3/7 Assay at 48 h. Our data shows the reduction of caspase activity in HeLa Bax/Bak DKO suggesting the involvement of the mitochondria-dependent intrinsic apoptotic pathway (Figure 2D). In addition to these data, we also observed prominent nuclear condensation under TLX and 7-OMeSB treatment starting at 24 h, followed by compromised cytokinesis and progressing with nuclear fragmentation at 48 and 72 h similarly as in STS (Figure S14).Although we have proved that TLX and 7-OMeSB can induce apoptosis, the poor cytoprotection obtained in Bax/Bak DKO (Figure 2B) demonstrate that other cell death pathways can take part when apoptosis is compromised.

Taking the obtained data together, considering the cytotoxic activities in cancer and normal cells and the capability to induce apoptosis, we conclude that TLX is a promising candidate for further trials as an anticancer lead compound.Despite the initial *in vivo* trials which have shown substantial toxicity in mice models, the features of the compound discussed above indicate that TLX could be an appropriate candidate for selective drug delivery.

The antibody-drug conjugate employing a cathepsin-cleavage linker for selective delivery of the antitubulin compound monomethyl auristatin E (chemical analogue of cyanobacterial peptide dolastatin) has been developed and is currently in clinical use against Non-Hodgkin's lymphoma.[21] As we have shown above, TLX possesses enhanced potency against several therapeutically relevant cancers (e.g. of blood leukemia, brain blastoma) in which selective delivery may be considered.

Finally, macrolide ring substitution has not been considered of special importance in macrolide-actin interactions so far. However, our data has uncovered that the substituents present on the TLX macrolide ring significantly affect its potency and it opens the possibility for further research in this field.

Acknowledgements

This work was financially supported by the Czech Grant Agency (Discovery of promising chemotherapeutic candidates in cyanobacteria using high-throughput screening: mode of action and molecular targets. – project No. 21-05649K) and further by the

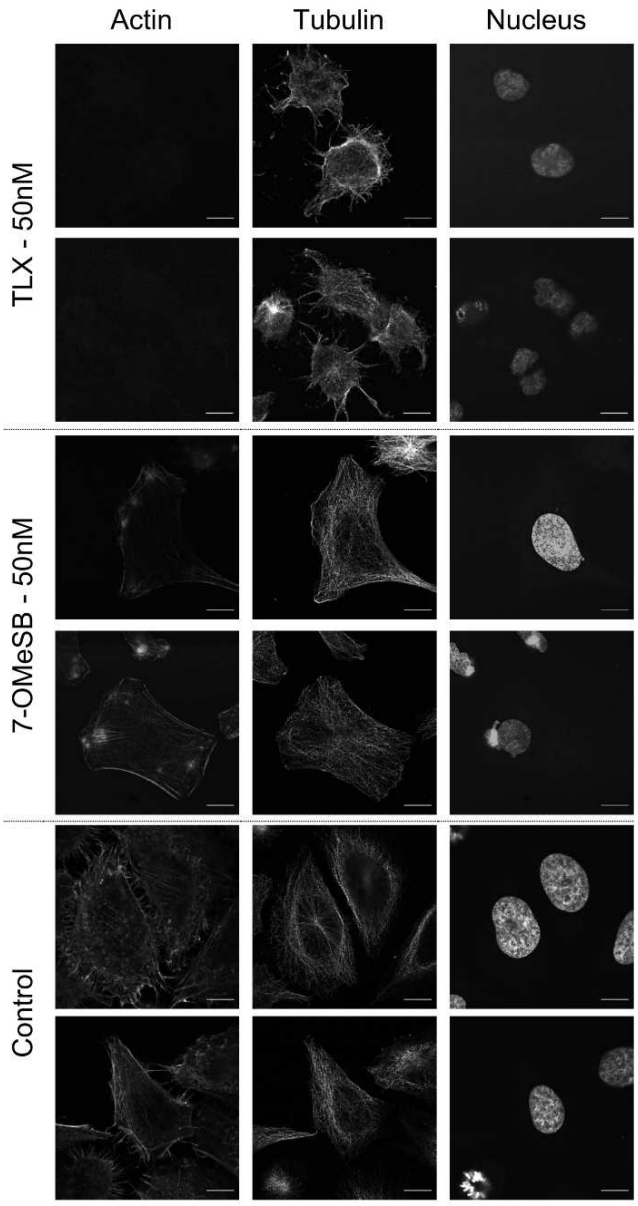

Figure 3. The immunolabeling of actin and tubulin in combination with nucleus staining visualized by confocal microscopy in HeLa.

Ministry of Education, Youth and Sports of the Czech Republic (project Algatech Plus LO1416 and RVO: 68378050-KAV-NPUI at CZ-OS), National infrastructure for chemical biology LM2018130, Cross-Border cooperation Czech-Bavaria project no. 41 and GINOP-2.3.2-15-2016-00001 grant. We want to thank Jindriska Paichlova, Veronika Brožová, Olga Martínková, Tomáš Langhammer and Martin Popr for technical assistance. Prof. Andreas Villunger, Ph.D. (Medical University Innsbruck) is acknowledged for providing cell lines HeLa WT and Bax/Bak DKO.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: actin disruption · apoptosis · cancer · scytophycines · tolytoxin

[1] C. M. Fife, J. A. McCarroll, M. Kavallaris, *Br. J. Pharmacol.* **2014**, *171*, 5507–5523.
[2] M. Trendowski, *Biochim. Biophys. Acta Rev. Cancer* **2014**, *1846*, 599–616.
[3] F. Pellegrini, D. R. Budman, *Cancer Invest.* **2005**, *23*, 264–273.
[4] M. Ishibashi, R. E. Moore, G. M. L. Patterson, C. F. Xu, J. Clardy, *J. Org. Chem.* **1986**, *51*, 5300–5306.
[5] S. Carmeli, R. E. Moore, G. M. L. Patterson, *J. Nat. Prod.* **1990**, *53*, 1533–1542.
[6] G. M. L. Patterson, C. D. Smith, L. H. Kimura, B. A. Britton, S. Carmeli, *Cell Motil. Cytoskeleton* **1993**, *24*, 39–48.
[7] V. A. Klenchin, J. S. Allingham, R. King, J. Tanaka, G. Marriott, I. Rayment, *Nat. Struct. Biol.* **2003**, *10*, 1058–1063.
[8] R. D. Perrins, G. Cecere, I. Paterson, G. Marriott, *Chem. Biol.* **2008**, *15*, 287–294.
[9] J. S. Allingham, V. A. Klenchin, I. Rayment, *Cell. Mol. Life Sci.* **2006**, *63*, 2119–2134.
[10] R. Ueoka, A. R. Uria, S. Reiter, T. Mori, P. Karbaum, E. E. Peters, E. J. N. Helfrich, B. I. Morinaka, M. Gugger, H. Takeyama, S. Matsunaga, J. Piel, *Nat. Chem. Biol.* **2015**, *11*, 705-++.
[11] G. M. L. Patterson, S. Carmeli, *Arch. Microbiol.* **1992**, *157*, 406–410.
[12] L. Vaughan, W. Glanzel, C. Korch, A. Capes-Davis, *Cancer Res.* **2017**, *77*, 2784–2788.
[13] C. D. Smith, S. Carmeli, R. E. Moore, G. M. L. Patterson, *Cancer Res.* **1993**, *53*, 1343–1347.
[14] J. Tomsickova, M. Ondrej, J. Cerny, P. Hrouzek, J. Kopecky, *Chem. Nat. Compd.* **2014**, *49*, 1170–1171.
[15] T. K. Shishido, A. Humisto, J. Jokela, L. W. Liu, M. Wahlsten, A. Tamrakar, D. P. Fewer, P. Permi, A. P. D. Andreote, M. F. Fiore, K. Sivonen, *Mar. Drugs* **2015**, *13*, 2124–2140.
[16] J. C. Blain, Y. F. Mok, J. Kubanek, J. S. Allingham, *Chem. Biol.* **2010**, *17*, 802–807.
[17] J. Cui, M. Morita, O. Ohno, T. Kimura, T. Teruya, T. Watanabe, K. Suenaga, M. Shibasaki, *Chem. Eur. J.* **2017**, *23*, 8500–8509.
[18] X. Q. Zhang, L. Minale, A. Zampella, C. D. Smith, *Cancer Res.* **1997**, *57*, 3751–3758.
[19] S. Elmore, *Toxicol. Pathol.* **2007**, *35*, 495–516.
[20] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D. J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, *Nat. Methods* **2012**, *9*, 676–682.
[21] D. S. Pereira, C. I. Guevara, L. Jin, N. Mbong, A. Verlinsky, S. J. Hsu, H. Avina, S. Karki, J. D. Abad, P. Yang, S.-J. Moon, F. Malik, M. Y. Choi, Z. An, K. Morrison, P. M. Challita-Eid, F. Donate, I. B. J. Joseph, T. J. Kipps, J. E. Dick, D. R. Stover, *Mol. Cancer Ther.* **2015**, *14*, 1650–1660.

Manuscript received: September 16, 2021
Revised manuscript received: November 16, 2021
Accepted manuscript online: November 25, 2021
Version of record online: December 9, 2021