Published in final edited form as:

J Nat Prod. 2017 March 24; 80(3): 676–683. doi:10.1021/acs.jnatprod.6b00963.

Cytotoxicity and Mechanism of Action of the Marine-Derived Fungal Metabolite Trichodermamide B and Synthetic Analogues

Petra E. Jans^{†,⊥}, Adelphe M. Mfuh^{§,⊥}, Hadi D. Arman^{§,⊥}, Corena V. Shaffer^{†,⊥}, Oleg V. Larionov^{§,*}, and Susan L. Mooberry^{†,‡,*}

[†]Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-3900, United States

[‡]Cancer Therapy & Research Center, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-3900, United States

[§]Department of Chemistry, The University of Texas at San Antonio, San Antonio, Texas 78249-0698, United States

Abstract

The trichodermamides are modified dipeptides isolated from a wide variety of fungi, including *Trichoderma virens*. Previous studies reported that trichodermamide B (**2**) initiated cytotoxicity in HCT-116 colorectal cancer cells, while trichodermamide A (**1**) was devoid of activity. We recently developed an efficient total synthesis for the trichodermamides A–C (**1–3**). Multiple intermediates and analogues were produced, and they were evaluated for biological effects to identify additional structure activity relationships and the possibility that a simplified analogue would retain the biological effects of **2**. The antiproliferative effects of 18 compounds were evaluated and the results show that **2** and four other compounds are active in HeLa cells, with IC₅₀ values in the range of 1.4–21 μM. Mechanism of action studies of **2** and the other active analogues revealed different spectra of activity. At the IC₈₅ concentration, **2** caused S-phase accumulation and cell death in HeLa cells, suggesting response to DNA double strand breaks. The analogues did not cause S-phase accumulation or induction of DNA damage repair pathways, consistent with an alternate mode of action. The mechanistic differences are hypothesized to be due to the chlorohydrin moiety in **2** which is lacking in the analogues, which could form a DNA-reactive epoxide.

Graphical abstract

*Corresponding Authors: Tel: +1 (210) 567-4788. Fax: +1 (210) 567-4300. Mooberry@uthscsa.edu. *Tel: +1 (210) 458-6050. Fax: +1 (210) 458-7428. oleg.larionov@utsa.edu.

[†]Author Contributions: P. E. Jans, A. M. Mfuh, H. D. Arman, C. V. Shaffer contributed equally.

Supporting Information: The Supporting Information is available free-of-charge on the ACS Publications website at DOI 10.1021/acs.jnatprod.6b00963.

Dedicated to Professor Phil Crews, of the University of California, Santa Cruz, for his pioneering work on bioactive natural products.

Notes: The authors declare no competing financial interest.

Natural products have long been an outstanding source for biologically active compounds, and some of the most effective anticancer drugs are natural products or are analogues of natural products. Newman and Cragg found that 55% of small molecules approved worldwide for the treatment of cancer are natural products or derived from natural products.¹ Marine environments continue to offer an attractive source for future natural product-derived pharmaceuticals, and systematic investigation of marine resources for drug candidates began relatively recently as compared to plants, for example.² Marine fungi are an excellent source of biologically active secondary metabolites, but as of yet they have not yielded numerous drug candidates³ despite the identification of multiple marine fungal metabolite leads, for example, plinabulin and sorbicillactone-A.⁴⁻⁶ The continued investigation of marine-derived fungi for chemotherapeutic drug leads is warranted.

The modified dipeptides trichodermamides A (**1**) and B (**2**) were first isolated from *Trichoderma virens* associated with the marine ascidian *Didemnum molle* and a green alga, *Halimeda* sp.⁷ *T. virens* is a well-known cosmopolitan fungus that had previously been isolated from terrestrial soil collections yielding a number of other bioactive compounds.⁸⁻¹⁰ Crews and colleagues additionally isolated the vertinoid polyketides from the marine-derived *Trichoderma longibranchiatum*.¹¹ Following the isolation of **1** and **2**, trichodermamide C (**3**) was identified in the terrestrial fungus *Eupenicillium* sp.¹² Biological evaluations of **1** and **2** found that only the latter displayed significant cytotoxicity against the HCT-116 human colorectal cancer cell line, with an IC₅₀ value of 0.32 µg/mL (710 nM).⁷ The cytotoxicity of **2** was hypothesized to be initiated by the chlorohydrin moiety, which could form an epoxide in situ to yield a reactive compound.⁷ Compound **1** and the similar compound aspergillazine A (**4**) were also isolated from the marine fungus *Spicaria elegans* KLA-03 by Liu et al.¹³ They reported IC₅₀ values of 89 and 84 µM respectively for **1** and **4** in HL-60 cells, and IC₅₀ values above 100 µM in P388 and A549 cells.¹³ Davis et al. reported that **3** is cytotoxic to both HCC-116 colorectal and A549 lung cancer cell lines, with IC₅₀ values of 0.68 µg/mL (1.5 µM) and 4.28 µg/mL (9.59 µM), respectively. Further studies showed that biosynthesis of **2** was promoted in *S. elegans* KLA-03 in high-salinity conditions,¹⁴ demonstrating the ability of the environment to alter these fungal biosynthetic pathways.

We recently developed a general approach for the total synthesis of compounds **1-3**,¹⁵ and several synthetic analogues and intermediates were generated (Table 1). Our synthetic strategy is based on the efficient construction of the *cis*-fused 1,2-oxazadecalin core of the trichodermamides. This goal was accomplished by base-mediated one-step synthesis of enone intermediate **5**. Subsequent elaboration of the carbocyclic portion of enone **5** and appendage of the 2-amidocoumarin moiety completed the synthesis of compounds **1-3** in the

racemic form. Our next goal was to investigate the influence and importance of the structural elements of trichodermamides on cytotoxicity. For example, it was of interest to determine if the 2-amidocoumarin moiety was important for biological activity. In addition, transposition of the electrophilic sites and the double bond in the carbocyclic ring of trichodermamide could lead to a change in the cytotoxic effects. Finally, we were interested in the development of simplified analogues of trichodermamides that could be readily accessed along the established synthetic route. Due to the notable instability of some of the trichodermamide derivatives, for this study we have focused on the analogues that are sufficiently stable and exhibit significant potential for further structural diversification.

Herein we detail evaluations of the cytotoxicity of **2**, its synthetic intermediates and analogues in HeLa cells, and the mechanisms of action of the active compounds. The results show that **2** and compounds **5–8** are cytotoxic with IC₅₀ values of 1.4–21 μ M. Mechanism of action studies were completed, and the results suggest that the analogues, which each lack the C-4/C-5 chlorohydrin moiety of **2**, have a different mechanism of action. Furthermore, evidence is presented to suggest that **2** causes DNA double-strand breaks leading to accumulation of cells in the S phase of the cell cycle and subsequent initiation of apoptosis.

Results and Discussion

Synthesis and Chemical Characterization of Trichodermamide Analogues **6** and **8**

Enone **5** and distal epoxide **7** were key synthetic intermediates in the synthesis of compounds **1–3**.¹⁵ Synthesis of amide **6** commenced with the saponification of the ester group in enone **5** (Scheme 1). Significant decomposition of the enone was observed under a variety of typical basic and acidic hydrolysis conditions (e.g., alkali metal hydroxides, acids, TMSONa). Hence, enzymatic hydrolysis was carried out under neutral conditions (pH 7.14) using porcine kidney acylase. The enzymatic hydrolysis afforded the hydrolysis product as a racemate. Amidation of the hydrolysis product with aminocoumarin **9** in the presence of 1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU) and *sym*-collidine afforded racemic amide **6** in 41% yield over two steps.

Racemic epoxy ketone **8** was prepared in 70% yield by a base-mediated epoxidation with hydrogen peroxide. Use of alkali metal hydroxides as bases led to significant hydrolysis of the ester group. On the other hand, 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) emerged as an optimal base that minimized the undesired saponification. The structure of epoxy ketone **8** was confirmed by means of X-ray crystallography. Compounds **9–18** as well as **1–3** were prepared and characterized as we described previously.¹⁵ All compounds were prepared in racemic form. With the library of trichodermamides and their structural analogues **5–18** we set out to investigate their cytotoxicity and mechanisms of action.

Biological Evaluations of Trichodermamide **B (2)** and Analogues

Compounds **1–3**, **5–18** were screened at 10 μ M for activity in HeLa cells using the sulforhodamine B assay.^{16, 17} Compounds **2** and **5–8** showed activity at this concentration and were pursued further, while the remaining compounds were not evaluated in detail. The

antiproliferative and cytotoxic potencies of **2** and **5-8** were determined in HeLa cells, and the IC₅₀ and the IC₈₅ concentrations were calculated from concentration-response curves. The results show that **2** has an IC₅₀ value of $3.1 \pm 0.5 \mu\text{M}$ in HeLa cells (Table 1). The most potent analogue was **8**, with an IC₅₀ value of $1.4 \pm 0.1 \mu\text{M}$, which was more potent than the natural product (**2**). Analogues **5-7** were less potent than **2**, and the least potent was **7**, with an IC₅₀ value of $21 \pm 2 \mu\text{M}$ (Table 1). The shapes of the concentration-response curves were evaluated, and while the curve for **2** is biphasic (Figure 1), the concentration-response curves for each of the analogues were monophasic. Representative curves with compounds **2** and **5** are shown for comparison (Figure 2). The shapes of these curves suggest potential differences in the mechanisms of action of **2** and **5-8**. The dotted line indicates the cell density at time zero and the time of drug addition, and the concentration response curves drop below this line, indicating that both **2** and **5** are cytotoxic in HeLa cells.

The cellular persistence of **2** and **5-8** was evaluated *in vitro* using a colony formation assay with a brief, 4 h, treatment followed by drug washout. This assay provides an initial indication of cellular uptake and retention and rapid target engagement, two desirable features for chemotherapeutic agents. Persistence of this type can be indicative of better efficacy *in vivo*, where bioavailability challenges increase.^{18, 19} HeLa cells were treated with vehicle (DMSO) or the IC₈₅ concentration of the active compounds for 4 h followed by drug washout and colony growth for 10 days. Compound **2** caused complete inhibition of colony formation (Figure 2), and **5** also caused statistically significant inhibition of colony formation. The analogues **6-8** caused only modest inhibition of colony formation, which was not statistically significant. These results show that **2** and **5** have excellent cellular persistence following a short drug exposure, suggesting that they might have properties that would contribute to *in vivo* efficacy.

Many anticancer drugs engage targets involved in normal cell cycle progression, leading to the accumulation of cells in various phases of the cell cycle. Flow cytometry was used to evaluate the effects of **2** on HeLa cell cycle distribution. HeLa cells were treated for 4, 8, 16, or 24 h with the IC₈₅ concentration, and cell cycle distribution was evaluated. Compound **2** caused a significant accumulation of cells in the S phase of the cell cycle after 16 and 24 h (Figure 3). Interestingly, compound **5** caused a small, yet significant accumulation of cells in the G₂/M phase of the cell cycle after 24 h, while compounds **6-8** had no significant effects on cell cycle distribution. These data suggest that cells treated with **2** are unable to complete the DNA replication required for continuation through the cell cycle, resulting in S-phase arrest. Cells with damaged DNA are unable to complete genomic replication;²⁰ therefore we hypothesized that **2** causes DNA damage, leading to S-phase arrest.

To determine whether S-phase accumulation after treatment with **2** is due to double-strand DNA breaks, we probed the phosphorylation of Ser139 of histone H2A.X ($\gamma\text{H2A.X}$). H2A.X is phosphorylated at S139 by the ataxia telangiectasia mutated (ATM) kinase in response to DNA double-strand breaks, initiated by endogenous damage or cytotoxic drugs.²¹ After treatment with the IC₅₀ of **2** or **5-8** for 24 h, HeLa cells were fixed and probed with a $\gamma\text{H2A.X}$ antibody and visualized by immunofluorescence microscopy. Compared to

the vehicle control, there was a large increase in visible γ H2A.X foci in the nuclei of cells treated with **2**, which was not present in cells treated with **5–8** (Figure 4).

To confirm the activation of cellular DNA damage repair pathways, Western blot analyses were used to probe for checkpoint kinase 1/2 (Chk1/2) phosphorylation. Chk1 is phosphorylated at Ser345 by ATM-Rd3-related (ATR) in response to disruption of DNA replication, while Chk2 is phosphorylated at Thr68 by ATM, primarily in response to double-strand DNA breaks.²² HeLa cells were treated for 24 h with vehicle or compounds **2** and **5–8** at the IC₅₀ concentration, and then lysed to retrieve the cellular proteins. Consistent with the previous experiments, **2** caused increased phosphorylation of Chk1/2 as compared to vehicle control or **5–8** (Figure 5A). Western blot analyses were then conducted to identify if this DNA damage culminated in apoptosis. The presence of cleaved poly ADP ribose polymerase (PARP) is one method to detect cells undergoing apoptosis. The IC₈₅ concentration of **2** caused the appearance of cleaved PARP as early as 4 h following treatment (Figure 5B). However, treatment with **5** did not cause the induction of cleaved PARP until approximately 16 h following treatment, and no cleaved PARP was visible within 24 h of treatment with compounds **6–8** (Figure 5B).

Structure–Activity Relationships—The biological evaluations show that **2** and **5–8** have antiproliferative and cytotoxic effects in HeLa cells and there are modest differences in potency but striking differences in their mechanisms of action. The data suggest that **2** is a DNA-damaging agent that causes double-strand breaks, leading to S-phase arrest and subsequent apoptotic cell death. It is reasonable to assume that the chlorohydrin group on **2** is likely responsible for the biological activity. This is consistent with data from the original study of **1** and **2**, which demonstrated a loss of cytotoxic activity when the chlorohydrin in **2** was substituted by a trans vicinal diol in **1**.⁷ Garo et al. predicted that the chlorohydrin moiety was a precursor to a reactive epoxide in the active form of the compound.⁷ Compounds **7** and **8** lack a chlorohydrin and, instead, contain epoxide moieties. Although these compounds retain the cytotoxic activity of **2**, our data suggest that they do not cause DNA damage. Thus, we hypothesize that an epoxide is not sufficient to produce the DNA double-strand breaks that are proposed to be generated by the chlorohydrin moiety of **2**.

Structural comparison of **1–3** indicates that the chlorohydrin moiety is necessary whether the central amide is secondary or tertiary. The replacement of the reactive chlorohydrin of **2** by another reactive moiety, for example, the epoxide of **12**, does not maintain cytotoxic activity in our model. Several simplified structures containing only one of the two ring systems were also tested, compounds **5**, **7–9**, and **15–17**, and it was determined that structural simplicity alone cannot predict potency. While the simplified structure of **5** is still effective at producing cytotoxicity, the addition of the second ring system as in **6** increases the activity. The most potent analogue, **8**, is a simplified ring system with both an epoxide and ketone replacing the chlorohydrin. This combination of an epoxide and a ketone is more potent than either alone as in compound **17** or **5**, respectively. The potency of **8** is abolished by the addition of the second ring system in **18**, indicating the ketone/epoxide combination is not beneficial to all mechanisms of action.

The 1,2-oxazadecalin core of the trichodermamide molecules is a rare moiety in nature, but has also been reported in another group of compounds, the epidithiodiketopiperazines, which includes several “pretrichodermamides” (**19–23**, Figure 6).^{23, 24} One of these compounds, N-methylpretrichodermamide B (**19**), was found to be cytotoxic in highly drug-resistant prostate cancer cell lines ($IC_{50} = 0.51\text{--}5.11\text{ }\mu\text{M}$)²³ as well as in a murine lymphoma cell line ($IC_{50} = 2\text{ }\mu\text{M}$),²⁴ while only cytotoxic to nonmalignant murine cells at much higher concentrations.²³ Interestingly, the cytotoxic pretrichodermamide **19** contains a chloride moiety at carbon 5, while the noncytotoxic compounds **20–23** lack this feature. This is analogous to the pattern of activity seen with **2** and its analogues. While mechanistic studies were not performed with N-methylpretrichodermamide B (**19**),²³ we hypothesize that given the similarities in structure and IC_{50} values to **2**, **19** may have the same mechanism of action and that the pretrichodermamide may be a precursor of **2**, ultimately leading to DNA damage. This hypothesis is supported by the facile conversion of pretrichodermamide A to trichodermamide A, as previously demonstrated by Seephonkai et al.²⁵ Further studies will be necessary to elucidate the exact mechanism of DNA damage induced by **2** and determine whether **2** or its related compounds are suitable for *in vivo* antitumor studies.

Conclusion

Taken together, our data show that **2** and **5–8** are active at low micromolar concentrations in HeLa cells, but that **2** and its synthetic precursors and analogues have a different mechanism of action. These studies were made possible by the initial discovery of **1–3** from marine and terrestrial fungi. The continued search for marine fungal-derived anticancer compounds is likely to uncover many previously uncharacterized compounds with promising activities. Marine fungi are poised to produce secondary metabolites that are biologically active due to the extreme conditions in which they survive; likely high salinity, low temperature, and low light. These extreme conditions promote the production of agents that act as a defense mechanism against other organisms as they compete for resources.²⁶

Experimental Section

Cell Culture

HeLa cells were purchased from the American Type Culture Collection and cultured in Basal Medium Eagle (Sigma-Aldrich), supplemented with 10% fetal bovine serum (Corning, Corning, NY, USA), 2 mM Glutamax (Gibco), and 50 $\mu\text{g/mL}$ gentamycin (Gibco). Cells were maintained at 37 °C and 5% CO_2 .

In Vitro Cytotoxicity Assays

The antiproliferative and cytotoxic actions of the compounds were measured using the sulforhodamine B (SRB) assay.^{16, 17} Concentration response curves were generated for **2** and **5–8** and fit using Graphpad Prism 6 with a four-parameter nonlinear regression. The concentrations that caused 50% or 85% inhibition of cell density as compared to vehicle-treated controls were calculated as the IC_{50} and IC_{85} concentrations, respectively. The cytotoxicity line corresponds to cell density at the time of compound addition.

Cell Cycle Analysis by Flow Cytometry

Flow cytometry was used to evaluate the effects of **2** and **5–8** on HeLa cell cycle distribution. The cells were treated with vehicle (DMSO) or the IC₈₅ of the compounds for 4, 8, 16, and 24 h. DNA was then stained with Krishan's reagent²⁷ and analyzed using a BDLSRII flow cytometer (BD Biosciences). FlowJo V.10.1 (FlowJo LLC) was used to calculate the percentage of cells in each phase of the cell cycle. Statistical significance was determined using a two-way ANOVA with GraphPad Prism 6.

Clonogenic Assays

HeLa cells were plated at low density (150 cells/60 mm³) and then treated with DMSO or the IC₈₅ concentration of compounds **2** and **5–8** for 4 h. Media was removed, plates were washed with phosphate-buffered saline (PBS), and fresh media was added. Cells were allowed to grow for 10 days, after which they were washed with PBS, fixed, and stained with 0.5% crystal violet in 10% methanol. Colonies were imaged on a Geliance 600 using the GeneSnap software (PerkinElmer), and the number of colonies per plate were counted using Gene Tools software (PerkinElmer). Statistical significance was determined by one-way ANOVA with GraphPad Prism 6.

Immunofluorescence Microscopy

Indirect immunofluorescence techniques were used to evaluate the initiation of the Ser139 phosphorylation of H2A.X (γ H2A.X), a measure of DNA-double strand breaks, in HeLa cells treated with compounds **2** or **5–8**. Cells were treated with the IC₅₀ concentrations of **2** and **5–8** or 0.6% DMSO vehicle control for 24 h. Cells were fixed with 100% ice cold MeOH for 5 min and blocked in 10% bovine calf serum (HyClone Laboratories) in PBS for 20 min at room temperature. The primary H2A.X (Ser139) antibody (Cell Signaling Technology) was prepared in a 1% (w/v) mixture of bovine serum albumin/PBS (Sigma-Aldrich), and fixed cells were incubated with antibody for 2 h at room temperature. An Alexa Fluor 594-conjugated secondary antibody (ThermoFisher Scientific) was used, and cells were incubated for 1 h at room temperature. The DNA was visualized using Hoechst 33342 (Molecular Probes NucBlue Live ReadyProbes Reagent, Fisher Scientific). Images were obtained using a Nikon Eclipse 80i microscope and NIS Elements Advanced Research imaging software (Nikon Instruments).

Whole Cell Lysates and Western Blot Analysis

Prior to lysis, HeLa cells were treated with the IC₅₀ (for analysis of Chk1/2) or IC₈₅ (for analysis of cleaved PARP) concentrations of compounds **2** and **5–8**, or DMSO vehicle control for 24 h or a 4, 8, 16, and 24 h time course. Cells were collected by scraping and lysed with cell extraction buffer (Invitrogen) supplemented with protease inhibitor cocktail (Sigma-Aldrich), 50 mM NaF, 200 μ M Na₃VO₄ (Sigma-Aldrich), and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich). Protein concentration was determined by a Coomassie Plus assay kit (Life Technologies) and equal amounts of protein were resolved by SDS-PAGE on NuPage Bolt 12% Bis-Tris gels (Life Technologies) and transferred to Immobilon-FL PVDF membranes (Millipore). Membranes were probed with primary antibodies diluted in Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE,

USA) for Chk1, p-Chk1 (Ser345), Chk2, cleaved PARP, and GAPDH at 1:1000 and p-Chk2 (Thr68) at 1:500 (Cell Signaling Technology). IRDye 680 or 800 secondary antibodies were used at 1:20000 (LI-COR Biosciences), and the fluorescence signals were imaged on an Odyssey FC (LI-COR Biosciences).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This study was funded by grants to S.L.M from the Greehey Distinguished Chair in Targeted Molecular Therapeutics and O.L.V. from the Welch Foundation (AX-1788), the NSF (CHE-1455061), NIGMS (SC3GM105579), and NIMHD (G12MD007591). Data were generated in the Flow Cytometry Shared Resource Facility, which is supported by UTHSCSA, NIH-NCI P30CA054174, and UL1 TR001120.

References

1. Newman DJ, Cragg GM. *J Nat Prod.* 2016; 79:629–661. [PubMed: 26852623]
2. Newman DJ, Cragg GM. *Planta Med.* 2016; 82:775–789. [PubMed: 26891002]
3. Jin L, Quan C, Hou X, Fan S. *Mar Drugs.* 2016; 14
4. Bhatnagar I, Kim SK. *Mar Drugs.* 2010; 8:2673–2701. [PubMed: 21116414]
5. Millward M, Mainwaring P, Mita A, Federico K, Lloyd GK, Reddinger N, Nawrocki S, Mita M, Spear MA. *Invest New Drugs.* 2012; 30:1065–1073. [PubMed: 21327495]
6. Bringmann G, Lang G, Muhlbacher J, Schaumann K, Steffens S, Rytik PG, Hentschel U, Morschhauser J, Muller WE. *Prog Mol Subcell Biol.* 2003; 37:231–253. [PubMed: 15825646]
7. Garo E, Starks CM, Jensen PR, Fenical W, Lobkovsky E, Clardy J. *J Nat Prod.* 2003; 66:423–426. [PubMed: 12662106]
8. Stipanovic RD, Howell CR. *J Antibiot (Tokyo).* 1982; 35:1326–1330. [PubMed: 6890954]
9. Hanson JR. *Nat Prod Rep.* 1995; 12:381–384. [PubMed: 7666979]
10. Itoh Y, Kodama K, Furuya K, Takahashi S, Haneishi T, Takiguchi Y, Arai M. *J Antibiot (Tokyo).* 1980; 33:468–473. [PubMed: 7191847]
11. Sperry S, Samuels GJ, Crews P. *J Org Chem.* 1998; 63:10011–10014.
12. Davis RA, Longden J, Avery VM, Healy PC. *Bioorg Med Chem Lett.* 2008; 18:2836–2839. [PubMed: 18424045]
13. Liu R, Gu QQ, Zhu WM, Cui CB, Fan GT. *Arch Pharm Res.* 2005; 28:1042–1046. [PubMed: 16212235]
14. Wang Y, Lu Z, Sun K, Zhu W. *Mar Drugs.* 2011; 9:535–542. [PubMed: 21731548]
15. Mfuh AM, Zhang Y, Stephens DE, Vo AX, Arman HD, Larionov OV. *J Am Chem Soc.* 2015; 137:8050–8053. [PubMed: 26084356]
16. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S, Boyd MR. *J Natl Cancer Inst.* 1990; 82:1107–1112. [PubMed: 2359136]
17. Boyd MR, Paull KD. *Drug Devel Res.* 1995; 34:91–109.
18. Risinger AL, Mooberry SL. *Cell Cycle.* 2011; 10:2162–2171. [PubMed: 21597323]
19. Towle MJ, Salvato KA, Wels BF, Aalfs KK, Zheng W, Seletsky BM, Zhu X, Lewis BM, Kishi Y, Yu MJ, Littlefield BA. *Cancer Res.* 2011; 71:496–505. [PubMed: 21127197]
20. Zhou BB, Elledge SJ. *Nature.* 2000; 408:433–439. [PubMed: 11100718]
21. Kuo LJ, Yang LX. *In Vivo.* 2008; 22:305–309. [PubMed: 18610740]
22. Smith J, Tho LM, Xu N, Gillespie DA. *Adv Cancer Res.* 2010; 108:73–112. [PubMed: 21034966]

23. Yurchenko AN, Smetanina OF, Ivanets EV, Kalinovsky AI, Khudyakova YV, Kirichuk NN, Popov RS, Bokemeyer C, von Amsberg G, Chingizova EA, Afiyatullof S, Dyshlovoy SA. *Mar Drugs*. 2016; 14
24. Orfali RS, Aly AH, Ebrahim W, AbdelAziz MS, Müller WEG, Lin W, Daletos G, Proksch P. *Phytochem Lett*. 2015; 11:168–172.
25. Seephonkai P, Kongsaree P, Prabpai S, Isaka M, Thebtaranonth Y. *Org Lett*. 2006; 8:3073–3075. [PubMed: 16805555]
26. Valliappan Karuppiah, FZaZL. *Handbook of anticancer Drugs from Marine Origin*. Kim, S-K., editor. Springer; 2015. p. 253-267.
27. Krishan A. *J Cell Bio*. 1975; 66:188–193. [PubMed: 49354]

Figure 1.

Concentration response curves show both antiproliferative and cytotoxic effects of trichodermamide B (**2**) and analogue **5**. HeLa cells were treated with a range of concentrations of compounds for 48 h, and an SRB assay was used to determine cell survival relative to a vehicle control. The results represent the mean $\pm$ SEM, $n=4$ independent experiments each conducted in triplicate. The dashed line represents the cell density at time zero. Points below this line represent concentrations that are cytotoxic.

Figure 2.

Effects of compounds on colony formation. HeLa cells were treated with the IC₈₅ of compounds **2** and **5–8** or vehicle (DMSO) control for 4 h followed by drug washout and were allowed to grow for 10 days before fixing, staining, and counting of colonies. (A) Bar graph of the number of colonies $\pm$ SEM representative of $n=3-5$ experiments, each performed in duplicate. (B) Representative images of plates after colony formation. * $p = 0.0008$ and ** $p = 0.0483$ compared to vehicle.

Figure 3.

Effects of compounds **2** and **5–8** on cell cycle distribution. HeLa cells were treated with the IC₈₅ of compounds or vehicle control for 4, 8, 16, or 24 h. Cells were stained with Krishan's reagent, and flow cytometry was used to determine the cell cycle distribution based on DNA copy number. Histograms are a representative experiment, n=4. Green, orange, blue, and red curves correspond to 4, 8, 16, and 24 h treatments, respectively. *p < 0.0001 compared to vehicle 16 h. ^p < 0.0001 compared to vehicle 24 h. #p = 0.0024 compared to vehicle 24 h.

Figure 4.

Effects of compounds on H2A.X phosphorylation at Ser139 (γ H2A.X), an indication of double-strand DNA breaks. HeLa cells were treated for 24 h with the IC₅₀ concentration of **2**, and **5–8** or vehicle (DMSO). Cells were stained for immunofluorescence with γ H2A.X (red) and Hoechst 33342 for DNA (blue). n=2 independent experiments done in duplicate.

Figure 5.

Activation of DNA damage repair pathways and apoptosis. (A) HeLa cells were treated for 24 h with the IC₅₀ concentration of **2** and **5–8** or 0.1% DMSO vehicle control. Whole cell lysates were prepared for Western blotting and probed for total and phosphorylated Chk1 at Ser345, total and phosphorylated Chk2 at Thr68, and a GAPDH loading control. (B) HeLa cells were treated with the IC₈₅ concentration of **2** and **5–8** or 0.1% DMSO vehicle control. Whole cell lysates were prepared for Western blotting and probed for cleaved PARP and GAPDH loading control. Results are representative of two independent experiments.

Figure 6.
 Structures of compounds **19–23**, compounds with a 1,2-oxazadecalin core.

Scheme 1
Synthesis of analogues **6** and **8**.

Table 1
Activities of Racemic Trichodermamides A–C (1–3) and Analogues

Compound	Structure	IC ₅₀ (μM)	IC ₈₅ (μM)
1 (Trichodermamide A)		>10	>10
2 (Trichodermamide B)		3.1 ± 0.5	18 ± 2
3 (Trichodermamide C)		>10	>10
5		12 ± 1	32 ± 5
6		5.7 ± 0.4	12 ± 2
7		21 ± 2	51 ± 6
8		1.4 ± 0.1	3.4 ± 0.3
9		>10	>10
10		>10	>10
11		>10	>10

Compound	Structure	IC ₅₀ (μM)	IC ₈₅ (μM)
12		>10	>10
13		>10	>10
14		>10	>10
15		>10	>10
16		>10	>10
17		>10	>10
18		>10	>10