Published in final edited form as:

Bioorg Med Chem Lett. 2016 July 1; 26(13): 3122–3126. doi:10.1016/j.bmcl.2016.04.090.

Synergistic potentiation of (–)-lomaiviticin A cytotoxicity by the ATR inhibitor VE-821

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Abstract

(–)-Lomaiviticin A (**1**) is a cytotoxic bacterial metabolite that induces double-strand breaks in DNA. Here we show that the cytotoxicity of (–)-lomaiviticin A (**1**) is synergistically potentiated in the presence of VE-821 (**7**), an inhibitor of ataxia telangiectasia and Rad3-related protein (ATR). While 0.5 nM **1** or 10 μ M **7** alone are non-lethal to K562 cells, co-incubation of the two leads to high levels of cell kill (81% and 94% after 24 and 48 h, respectively). Mechanistic data indicate that cells treated with **1** and **7** suffer extensive DNA double-strand breaks and apoptosis. These data suggest combinations of **1** and **7** may be a valuable chemotherapeutic strategy.

Graphical Abstract

Keywords

chemotherapy; DNA; natural product; cancer; lomaiviticin; synergism

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(–)-Lomaiviticin A (**1**) is an antiproliferative bacterial metabolite with half maximal inhibitory potencies in the low nanomolar–picomolar range against a panel of cultured human cancer cell lines (Figure 1).¹ The exquisite cytotoxicity of **1** derives from induction of DNA SSBs and DSBs in tissue culture.² The kinetics of DNA double-strand break induction and PI3KKs pathways-dependent phosphorylation of histone H2AX by **1** have been analyzed.^{2b} Reduction of histone H2AX phosphorylation induced by **1** was observed in K562 cells pretreated with inhibitors (KU55933,³ caffeine,⁴ and NU-7441⁵) of the major PI3KKs ATM, ATR, and DNA, respectively. In addition, **1** displayed selective toxicity toward BRCA2-, PTEN-, KU80-, DNA-PKcs, and ATM-deficient cell lines, all of which have been implicated in DSB repair.^{2a} BRCA2-deficient VC8 and PTEN-deficient U251 cell lines are particularly sensitive to **1** ($LC_{50} = 1.5 \pm 0.5$ and 2.0 ± 0.6 pM, respectively) with selectivities of >11.6 versus the isogenic cell lines transfected with and expressing functional BRCA2 and PTEN genes. Collectively, these results indicate activation of non-homologous end joining (NHEJ)⁶ and homologous recombination (HR)⁷ repair, the two canonical pathways by which DNA DSBs are ameliorated, in cells treated with **1** and suggest acceptable therapeutic indices may be attainable when **1** is applied toward NHEJ- or HR-deficient tumors.

These results led us to question if sensitization could be induced pharmacologically using small molecule inhibitors of DNA repair. The application of DNA repair inhibitors as chemo- and radiosensitizers is an active area of research, and several combinations of repair inhibitors and DNA damaging agents [small molecules, ionizing radiation (IR)] are undergoing clinical evaluation.⁸ Synergistic potentiation of **1** by DNA repair inhibitors could decrease required doses, resulting in fewer off-target effects and higher therapeutic indices across a range of tumor types, including DSB repair-proficient tumors. Herein, we report an evaluation of combinations of **1** and six inhibitors of DNA DSB repair. We report the discovery that the ATR inhibitor VE-821 (**7**) synergistically potentiates the cytotoxic activity of **1** in K562 cells. Combinations of **7** and **1** induce high levels of cell kill concentrations at which either agent alone is non-lethal.

We investigated six small molecules (Figure 1) that disrupt essential factors in DNA repair. AZD-2281 (Olaparib, **2**) is an inhibitor of PARP-1 ($IC_{50} = 5$ nM) and PARP-2 ($IC_{50} = 1$ nM).⁹ Following DNA damage, PARP-1 binds to DNA breaks leading to initiation of BER.¹⁰ BEZ-235 (Dactolisib, **3**) was originally designed as a combined PI3K-mTOR inhibitor¹¹ and was later shown to inhibit ATM and DNA-PKcs,¹² which have catalytic domains that are homologous to PI3Ks.¹³ Both DNA-PK¹⁴ and ATM¹⁵ are essential components of NHEJ repair. BEZ-235 (**3**) has demonstrated anticancer activity in clinical trials as well as radiosensitizing activity in preclinical models,¹⁶ and is synthetic lethal with cancer-associated mutations through inhibition of ATR.¹⁷ NU-7441 (**4**) is a highly selective inhibitor of DNA-PK ($IC_{50} = 14$ nM)¹⁸ that causes persistence of doxorubicin- and IR-induced DNA DSBs and induces chemo- and radio-potentiation in DNA-PK-proficient (MO59-Fus-1) human tumor cells.¹⁹ SAHA (Vorinostat, **5**) is an inhibitor of the HDAC proteins HDAC1 ($IC_{50} = 10$ nM) and HDAC3 ($IC_{50} = 20$ nM).²⁰ Combinations of HDAC inhibitors with radiation and chemical agents enhance cell kill as a result of chromatin relaxation mediated by HDAC inhibition, allowing increased DNA damage in

transcriptionally active DNA regions.²¹ **5** has also been suggested to inhibit HR repair directly.²² TCS-2312 (**6**) is a Chk1 inhibitor ($EC_{50} = 60$ nM).²³ Chk1 regulates G2/M and S-phase cell-cycle checkpoints and is activated by phosphorylation in response to various types of DNA-damaging agents, including DNA-strand-breaking agents such as IR and topoisomerase inhibitors, and those agents that cause replication stress such as UV, hydroxyurea and 5-fluorouracil.²⁴ **6** enhances the cell killing of gemcitabine in breast and prostate cancer cell lines and displays antiproliferative activity in vitro.²³ VE-821 (**7**) is an inhibitor of ATR ($IC_{50} = 26$ nM) that also shows inhibition of H2AX phosphorylation.²⁵ **7** is highly selective for ATR (>600-fold over related kinases such as ATM or DNA-PK).²⁶ Inhibition of ATR by **7** is synthetic lethal with DNA-damaging agents in cancer cells deficient in ATM or p53.²⁵

We employed the CellTiter-Glo luminescent cell viability assay (Promega) to identify DNA repair inhibitors that synergistically potentiate the cytotoxicity of (–)-lomaiviticin A (**1**) in K562 cells. The assay is based on the measurement of ATP using firefly luciferase and is commonly used to estimate the number of viable cells in high-throughput screening applications.²⁷ Cells were incubated under optimal growth conditions in 96-well plates with either a DNA repair inhibitor alone, **1** alone, or combinations of each. After 24 or 48 h of treatment, the reconstituted CellTiter-Glo enzyme–substrate mixture was added and the luminescence was measured. Synergistic potentiation of the cytotoxicity of **1** in the presence of DNA repair inhibitors was assessed using the combination index (CI) theorem of Chou–Talalay²⁸ where $CI < 1$ indicates synergism, $CI = 1$ indicates an additive effect, and $CI > 1$ indicates antagonism.

Cell viability assays conducted with BEZ-235 (**3**), SAHA (**5**), or TCS-2312 (**6**) alone indicated that cell viability decreased significantly with increasing concentrations of **3**, **5**, or **6** (0.01–2.8, 0.25–50, 0.1–20 μ M, respectively, Figures S1–S3). Accordingly, synergism studies with (–)-lomaiviticin A (**1**) were conducted at fixed ratios of **3**, **5**, or **6** to **1**. Cell viability assays conducted with AZD2281 (**2**), NU-7441 (**4**), VE-821 (**7**) revealed that cell viability did not decrease significantly with increasing concentrations of **2**, **4**, or **7** (up to 2.5–10 μ M, Figure S4–S6).

Comprehensive data depicting the results of these studies at fixed and varying ratios of (–)-lomaiviticin A (**1**) and **2–6**, and varying incubation periods (24 or 48 h), are presented in the Supporting Information (Figures S7–S15). Selected CI and fraction affected (Fa) values as a function of dose and incubation time are shown in Table 1. Combinations of AZD-2281 (**2**), BEZ-235 (**3**), NU-7441 (**4**), or SAHA (**5**) displayed modest synergistic effects or antagonist effects ($CI = 0.81–2.1$) at 0.036–0.72 fraction affected (entries 1–6). However, TCS-2312 (**6**) and VE-821 (**7**) displayed high levels of synergism with **1** (entries 7–9). Treatment of K562 cells with 0.5 μ M **6** and 0.40 nM **1** for 24 h resulted in a combination index of 0.20 at a high fraction affected (0.78, entry 7). Treatment of K562 cells with 10 μ M **7** and 0.25 nM **1** resulted in a combination index of 0.069 at 0.65 fraction affected after a 24 h incubation (entry 8). By extending the incubation time to 48 h, high levels of synergism were observed using 0.050 nM (50 pM) **1** ($CI = 0.13$, $Fa = 0.60$, entry 9). It is difficult to explain why the PARP inhibitor **2** and the HDAC inhibitor **5** display antagonistic effects with **1**. It has been

shown that inhibition of PARP-1, which is primarily involved in BER, leads to activation of the key NHEJ kinase ATM.²⁹ Thus, DSB repair may be upregulated in K562 cells treated with **2**, but further investigation is required to fully establish this. On the other hand, we speculate that inhibition of HDAC proteins by **5** may lead to changes in chromatin structure that modulate the DNA cleavage activity of **1**.

The strong synergism observed between VE-821 (**7**) and (–)-lomaiviticin A (**1**) led us to focus on this combination. Cell viability data for each component and their mixtures are shown in Figure 2. Treatment of K562 cells with up to 10 μ M **7** alone for 24 or 48 h led to only a marginal decrease in cell viability (13%, Figure 2A). In addition, treatment of K562 cells with 0.05–0.5 nM **1** alone for 24 or 48 h did not lead to a decrease in cell viability (<5%), although increasing the concentration of **1** to 1 nM decreased cell viability by 34% after 48 h (Figure 2B). Based on these data, we selected 10 μ M **7** and up to 0.5 nM **1** for evaluation as combination therapies. Exposure of K562 cells to as little as 50 pM (–)-lomaiviticin A (**1**) and 10 μ M **7** for 24–48 h led to statistically-significant decreases in cell viability (18% and 40% after 24 and 48 h, respectively, Figure 2C). Increasing the concentration of **1** to 250 or 500 pM further decreased cell viability, with up to 96% cell kill observed after 48 h (500 pM **1**).

Plots of the combination index versus fraction affected²⁸ illustrate that strong synergism between (–)-lomaiviticin A (**1**) and VE-821 (**7**) manifests across a range of concentrations of **1** and duration of exposures (Figure 3A). Treatment of K562 cells with 10 μ M **7** and up to 0.025–0.5 nM **1** for 24 or 48 h led to CI values <0.3, which is indicative of strong synergism.²⁸ Exposure of K562 cells to 2.5 or 5.0 nM **1** and 10 μ M **7** for 24 hr, or 2.5 nM **1** and 10 μ M **7** for 48 h were also synergistic (CI = 0.3–0.7). For doses of **1** 250 pM (48 h) or 500 pM (24 h) very large Fa values (>0.75) were observed, indicating high overall levels of cell kill (see also Figure 3B).

The experiments outlined below provide insight into the basis of the synergism between (–)-lomaiviticin A (**1**) and VE-821 (**7**). As the cytotoxicity of **1** has been shown to derive from induction of DSBs in DNA,² we conducted a neutral comet unwinding assay³⁰ to probe for an increase in DSBs in cells treated with **1** and VE-821 (**7**). Consistent with the cell viability studies above (Figure 2B), treatment of K562 cells with 50–500 pM **1** alone did not lead to measurable levels of DSB production, as compared to control (Figure 4). However, combinations of 50–500 pM **1** and 10 μ M **7** led to a dose-dependent increase in the levels of DSBs. At 500 pM **1** and 10 μ M **7**, high levels of DSB production were observed (average and median tail moment = 34, 33, respectively).

Immunofluorescence assays reveal that treatment of cells with (–)-lomaiviticin A (**1**) and VE-821 (**7**) lead to induction of apoptosis (Figure 5). We monitored the production and colocalization of phosphor-SER139 H2AX (γ H2AX)³¹ and 53BP1³² foci as an indicator of DSB formation, as previously described.^{2a,2b,8d} In the presence of **1** alone (up to 250 pM) γ H2AX and 53BP1 foci were observed, as expected. However, in the presence of **7** (10 μ M), pan-nuclear staining of nuclear γ H2AX and the formation of apoptotic bodies (also displaying pan-staining) was observed, which is indicative of apoptosis.³³ Thus, it appears that the synergism observed between **1** and **7** derives from an increase in the levels of DSBs

and induction of apoptosis. Treatment with 10 μ M **7** alone did not induce apoptosis (Figure S16).

In summary, we have discovered that the ATR inhibitor VE-821 (**7**) synergistically potentiates the activity of the DNA-damaging natural product (–)-lomaivitin A (**1**). At levels of **1** that are non-lethal to K562 cells (0.5 nM), high levels of cell kill are observed in the presence of **7**. These data argue for preclinical evaluation of **1** and **7** and highlight the utility of small molecules to counteract cellular responses to molecular cytotoxic agents.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Financial support from the National Institute of General Medical Sciences (R01GM090000) is gratefully acknowledged.

Abbreviations

ATR	ataxia telangiectasia and Rad3-related protein
SSB	single-strand break
DSB	double-strand break
PI3KK	phosphatidylinositol 3-kinase-related kinase
ATM	ataxia telangiectasia mutated
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
BRCA2	breast cancer type 2
PTEN	phosphatase and tensin homolog
KU80	Ku autoantigen protein p80
NHEJ	non-homologous end joining
HR	homologous recombination
PARP-1	poly(ADP-ribose) polymerase-1
PARP-2	poly(ADP-ribose) polymerase-2
BER	base excision repair
PI3K	phosphoinositide 3-kinase
mTOR	mammalian target of rapamycin
IR	ionizing radiation

HDAC	histone deacetylase
Chk1	checkpoint kinase 1
γH2AX	phosphor-SER139 H2AX
53BP1	p53-binding protein 1

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Figure 1.

Chemical structures of (-)-lomaivictin A (**1**) and the DNA repair inhibitors AZD-2281 (Olaparib, **2**), NVP-BEZ235 (Dactolisib, **3**), NU-7441 (**4**), SAHA (Vorinostat, **5**), TCS-2312 (**6**), VE-821 (**7**).

Figure 2.

(–)-Lomaiviticin A (**1**) and VE-821 (**7**) are synthetic lethal in K562 cells. K562 cells were incubated under optimal growth conditions in the presence of varying concentrations of **7** (0.1, 1.0, 10.0 μM ; A), varying concentrations of **1** (0.05, 0.1, 0.5, 1.0 nM; B), or 10 μM **7** + varying concentrations of **1** (0.025, 0.05, 0.25, 0.5 nM; C) for 24 or 48 h. Cell viability was assessed using the Cell Titer-Glo Luminescent Cell Viability assay.

A. CI vs. Fa in cells treated with **1** (25 pM–5.0 nM) + **7** (10 μ M) for 24 or 48 h.

B. Fa vs. [1] in cells treated with **7** (10 μ M).

Figure 3.

A. Plot of combination index (CI) versus fraction affected (Fa) in K562 cells treated with VE-821 (**7**, 10 μ M) and (–)-lomaiviticin A (**1**, 0.025–5.0 nM) for 24 or 48 h. CI < 0.3 is indicative of strong synergism, CI = 0.3–0.7 indicates synergism. B. Plot of fraction affected (Fa) versus [1] in K562 cells treated with **7** (10 μ M) and **1** (0.025–5.0 nM) for 24 or 48 h.

Figure 4.

Tail moment in a neutral comet unwinding assay employing K562 cells treated with 0–500 pM (–)-lomaivitin A (**1**) in the presence or absence of VE-821 (**7**, 10 μ M). Drug exposure was 24 h. Cross denotes mean tail moment (89–153 cells), box denotes 2nd and 3rd quartile, lines in the middle of the box denote the median. Error bars represent 10th and 90th percentile, values individually plotted are outliers. Tail moment represents the extent of DNA cleavage and is defined as the product of the tail length and the fraction of DNA in the tail.

Figure 5.

Immunofluorescence imaging of γ H2AX and 53BP1 foci in K562 cells treated with (–)–lomaivitin A (**1**, 25–250 pM) and VE-821 (**7**, 0 or 10 μ M). Rows (top to bottom): γ H2AX (green), 53BP1 (red), DNA (blue), and merge. Columns (left to right): 25 pM **1**, 50 pM **1**, 250 pM **1**, 25 pM **1** + 10 μ M **7**, 50 pM **1** + 10 μ M **7**, 250 pM **1** + 10 μ M **7**. Drug exposure was 48 h. K562 cells were in exponential growth phase immediately before treatment. Immunological detection was performed using a primary antibody (rabbit polyclonal anti-53BP1 antibody (Novus Biologicals) and mouse monoclonal antiphosphohistone H2AX (SER139) antibody (Upstate)) and visualized with Alexa 488 (goat-antimouse IgG) and Alexa 594 (goat-antirabbit IgG). Mounting medium contained DAPI to visualize nuclear DNA.

Table 1

Selected values of the fraction affected (Fa) and combination indices (CI) for binary mixtures of (–)-lomaiviticin A (**1**) and the DNA repair inhibitors **2–7**.

entry	DDR inhibitor, dose	dose 1 nM	t	Fa	CI
1	AZD-2281 (2), 5.0 μ M	5.0	24 h	0.036	1.2
2	AZD-2281 (2), 5.0 μ M	5.0	48 h	0.56	2.1
3	BEZ-235 (3), 0.05 μ M	0.25	48 h	0.61	0.81
4	NU-7441 (4), 5.0 μ M	2.5	24 h	0.72	0.86
5	NU-7441 (4), 5.0 μ M	0.50	48 h	0.68	1.1
6	SAHA (5), 0.50 μ M	0.10	48 h	0.33	1.5
7	TCS-2312 (6), 0.50 μ M	0.40	24 h	0.78	0.20
8	VE-821 (7), 10 μ M	0.25	24 h	0.65	0.069
9	VE-821 (7), 10 μ M	0.050	48 h	0.60	0.13