ACS Medicinal Chemistry Letters

LETTER

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The Use of 3,5,4′-Tri-*O*-acetylresveratrol as a Potential Prodrug for Resveratrol Protects Mice from γ-Irradiation-Induced Death

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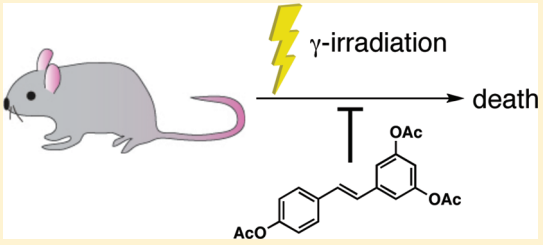
ABSTRACT: Currently, no drugs are available to protect humans from γ-irradiation-induced death. Because reactive oxygen species are produced upon exposure to γ-irradiation and directly responsible for the resulting death, we hypothesized that antioxidants found in foodstuffs may provide a safe and potent means of antioxidant-dependent radioprotection. Here, we describe our studies investigating the radioprotective properties of resveratrol and 3,5,4′-tri-*O*-acetylresveratrol. Each of these natural antioxidants was found to protect live cells after γ-irradiation. In mice, the use of 3,5,4′-tri-*O*-acetylresveratrol with Cremophor EL was particularly effective, indicating that this natural antioxidant may be a leading candidate for radioprotective drug development.



KEYWORDS: Antioxidants, resveratrol, 3,5,4′-tri-*O*-acetylresveratrol, radioprotection, Cremophor EL

Exposure to ionizing radiation (γ-irradiation), either from radiotherapy or by accidental contact, is deleterious and can cause severe cellular and organismal damage and even death. With respect to its medical uses, both normal and diseased cells are often injured, and many adverse side reactions accompany such treatments. Furthermore, in the event of a nuclear catastrophe, because of the long half-life of radioactive reagents, people would be predictably and continuously exposed to γ-irradiation from environmental samples. For instance, after the Chernobyl power plant accident on April 26, 1986, high levels of radioactivity were detected in rain even in remote countries such as Japan.[1] As a result, a continuous effort has been made to discover radioprotective agents that could be administered prior to irradiation to limit injuries caused by ionizing radiation.[2]

Early studies toward the development of radioprotective agents relied on the exploration of cysteine[3] and synthetic thiol derivatives. Because γ-irradiation generates superoxide ($O_2^{-\bullet}$), which is converted to hydroxy ($HO^{\bullet}$) and perhydroxy ($HOO^{\bullet}$) radicals,[4] researchers speculated that the sulfhydryl moiety of these compounds scavenged free radicals, thus serving as an antioxidant. One member of this class of radioprotective agents, amifostine (WR-2721), has been widely studied and was approved by the FDA to mitigate side effects in patients undergoing radiotherapy.[5] However, as observed with other thiol-containing compounds, amifostine exhibits many toxic side effects including nausea, vomiting, hypotension, and allergic reactions.[5]

As an alternative antioxidant-dependent approach, natural antioxidants and synthetic antioxidants have also been examined for their radioprotective properties.[6−9] Similar to amifostine, these compounds would presumably scavenge reactive free radicals to prevent damage to critical biomacromolecules. For example, vitamin E[10,11] and glutathione[11,12] have each demonstrated moderate protection against ionizing radiation in mice; however, the efficacy of these compounds requires further examination.

Inspired by this natural antioxidant-based approach, we hypothesized that natural polyphenol products, already found in a variety of foodstuffs, could provide a safe and potent means of antioxidant-dependent radioprotection, as it is well-documented that this class of natural products quench reactive oxygen species (ROS) by a suicidal mechanism.[13] To examine this concept, we tested the commercially available polyphenol natural products resveratrol, (−)-catechin gallate, and propyl gallate (Figure 1) in vitro. Although none of these compounds mitigated γ-irradiation-induced cell death (data not shown), resveratrol showed promising protective activities (Table 1 and Figure 2). Specifically, when 32D cl 3 cells were pretreated with 10 μM resveratrol, increased cell survival was observed 7 days after irradiation from 0 to 7 Gy.

Resveratrol is found naturally in grapes, nuts, fruits, and red wine and is one of the most intensively studied natural products today due to its cancer preventive activity[14] and potential lifespan-extending properties,[15,16] among others.[17−21] In spite of the great therapeutic interest in resveratrol, its pharmacokinetic and bioavailability properties are known to be poor, and it has a short half-life (∼8−14 min) and is metabolized extensively.[22,23] As a result, accurate dosage of this compound is difficult, diminishing its

Received: July 2, 2010
Accepted: January 14, 2011
Published: January 25, 2011

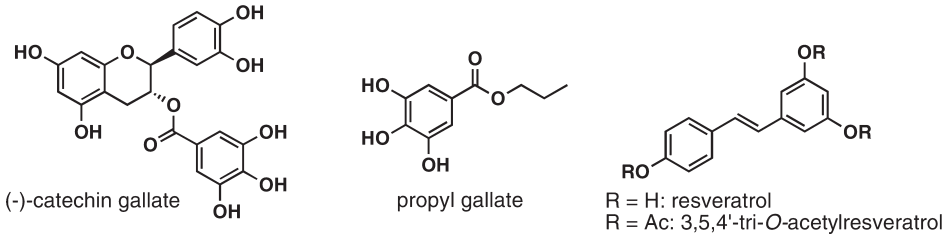


Figure 1. Structures of antioxidant natural products used in this study.

Table 1. Cells Incubated with Resveratrol or Acetylated Resveratrol Have Increased Radioresistance as Seen by an Increased Shoulder on the Survival Curve ($\tilde{n}$)[a]

compound	concentration (μM)	$\tilde{n}$
control (DMSO)		3.6 ± 1.4
resveratrol	1	1.2 ± 0.1
	5	1.1 ± 0.1
	10	33.2 ± 5.7 ($p = 0.0122$)
3,5,4′-tri-*O*-acetylresveratrol	1	1.3 ± 0.3
	5	1.1 ± 0.1
	10	57.5 ± 9.9 ($p = 0.0072$)

[a] Analysis of dose and $\tilde{n}$ demonstrates that incubation of 32D cl 3 cells with resveratrol or acetylated resveratrol (10 μM) before irradiation results in increased radioresistance. $\tilde{n}$ is a measurement of the shoulder on the irradiation survival curves. Those with a larger $\tilde{n}$ have a larger shoulder (Figure 2) and thus are more resistant to irradiation.

likelihood of being considered as a radioprotective drug. Excited by our findings, however, we were intrigued by the possibility of using the resveratrol analogue and natural product, 3,5,4′-tri-*O*-acetylresveratrol,[24] which may act as a more stable resveratrol prodrug. Additionally, because of the widespread interest in resveratrol, this analogue could provide insight beyond that of its radioprotective properties alone. 3,5,4′-Tri-*O*-acetylresveratrol was prepared according to literature procedure,[25] and its activity was examined in the in vitro irradiation assay. As Table 1 and Figure 2 show, 3,5,4′-tri-*O*-acetylresveratrol was slightly more potent than resveratrol.

To further test our hypothesis, we next examined the potential applicability of resveratrol and its triacetate analogue in an animal model involving γ-irradiated mice. Briefly, each natural antioxidant in 1:1:8 (v/v/v) EtOH/Cremophor EL[27]/H_2O was intraperitoneally injected into mice (n = 13 or 14 for each dose), which were subsequently exposed to γ-irradiation (9.75 Gy) for 10 min before or after the injection (Figure 3). When 3,5,4′-tri-*O*-acetylresveratrol was given at 10 mg/kg prior to γ-irradiation, a significant protective activity was noted with an 80% survival rate (p = 0.0089 as compared to 9.75 Gy only, Figure 3a). Use of a lower dose of 3,5,4′-tri-*O*-acetylresveratrol (1 mg/kg) or either dose (1 or 10 mg/kg) of resveratrol showed no change in survival as compared to the control group that received no antioxidant treatment. These findings can be corroborated with reports from other studies exploring the possibility of using resveratrol as a radioprotective agent for the chemoprevention of skin cancer and damage and a protector against radiotherapy injuries.[28] Similar to our in vitro study results, neither compound, when used after irradiation, mitigated irradiation-induced death (Figure 3b). It can be speculated that the diminished activity of resveratrol may be due to the poor bioavailability of resveratrol or metabolism of its hydroxy groups.[29,30] Thus, our findings give credence to our hypothesis of potentially using 3,5,4′-tri-*O*-acetylresveratrol as a potent resveratrol

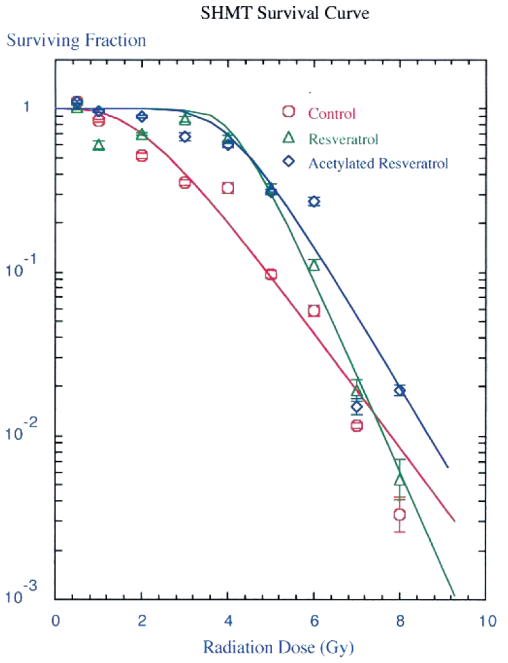


Figure 2. Irradiation survival curve for 32D cl 3 cells incubated with 10 μM resveratrol or 3,5,4′-tri-*O*-acetylresveratrol prior to irradiation. The data were analyzed using linear quadratic or single-hit, multitarget models.[26] Incubation in either resveratrol or 3,5,4′-tri-*O*-acetylresveratrol resulted in an increase in radioresistance as seen by an increased shoulder on the survival curve.

prodrug in vivo. In addition to this study, 3,5,4′-tri-*O*-acetylresveratrol has been shown to be slightly more potent than resveratrol in vitro by others.[31] However, our data presented here show the clear superiority of 3,5,4′-tri-*O*-acetylresveratrol over resveratrol in vivo. These data are encouraging not only for antiradiation therapy but also for other areas of research because despite promising in vitro data with resveratrol, this compound has not shown equally compelling data in vivo.[32]

Why is 3,5,4′-tri-*O*-acetylresveratrol superior to resveratrol in vivo? To gain insight into this question, we examined the stability of 3,5,4′-tri-*O*-acetylresveratrol and resveratrol in mouse serum. Resveratrol was stable in mouse serum for more than 27 h (data not shown). Next, a solution of 3,5,4′-tri-*O*-acetylresveratrol in DMSO was added to mouse serum. In this case, the compound was completely hydrolyzed to resveratrol within 20 s (data not shown). In contrast, when a solution of 3,5,4′-tri-*O*-acetylresveratrol in the vehicle used in the animal study (1:1:8 mixture of EtOH/Cremophor EL/H_2O) was added to mouse serum, the half-life of the compound was 48 min (Figure 4), a dramatic improvement as compared to the solution in DMSO. From these results, it is plausible, although further pharmacokinetic studies are

dx.doi.org/10.1021/ml100159p |*ACS Med. Chem. Lett.* 2011, 2, 270–274

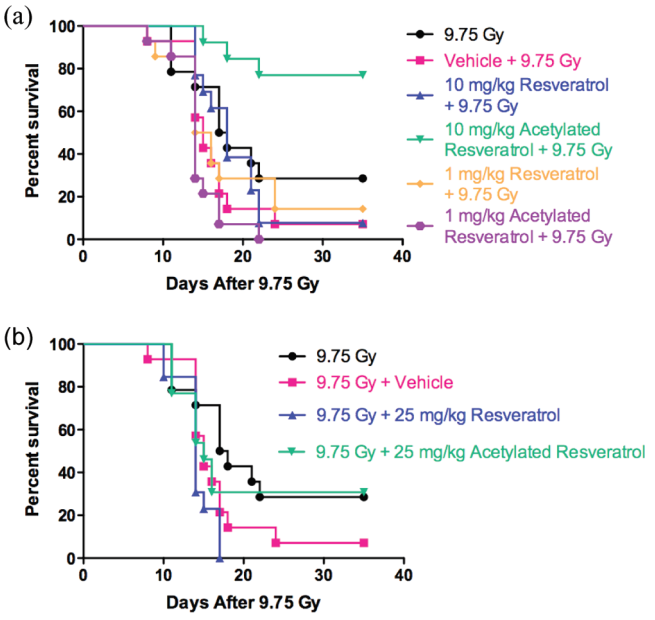


Figure 3. Acetylated resveratrol protects against total body irradiation in vivo. Resveratrol and 3,5,4′-tri-*O*-acetylresveratrol were dissolved in a 1:1:8 v/v solution of EtOH/Cremophor EL/H_2O. (a) C57BL/6NHsd mice (n = 13 or 14 for each dose) were injected intraperitoneally 10 min before 9.75 Gy total body irradiation with 1 or 10 mg/kg of resveratrol or 3,5,4′-tri-*O*-acetylresveratrol. Mice injected with 10 mg of 3,5,4′-tri-*O*-acetylresveratrol had a significant increase in survival as compared to the control 9.75 Gy mice. (b) In a separate experiment, C57BL/6NHsd mice were irradiated to 9.75 Gy and injected intraperitoneally 10 min after irradiation and daily for 4 days with resveratrol or 3,5,4′-tri-*O*-acetylresveratrol. There was no significant difference in the survival of the mice.

needed in vivo, that the vehicle protects 3,5,4′-tri-*O*-acetylresveratrol from the ester hydrolysis that would result in metabolically unstable resveratrol. The longer half-life of 3,5,4′-tri-*O*-acetylresveratrol may also assist more rapid distribution to tissues.

Regarding the mechanism of action, if resveratrol or its triacetate were acting merely as free radical scavengers, the other polyphenols tested would have also protected irradiated cells. As this did not prove to be the case, it is possible that only the resveratrol type polyphenols were able to localize to free radical-rich intracellular domains, which would be necessary to kinetically compete with free radical-mediated cellular damages.[32] With the available data, however, we cannot exclude other possible mechanisms such as the inactivation of COX-1[21] and the perturbation of gene expression[33] as part of resveratrol's antiradiation mode of action.

Recently, studies with the peptide drug CBLB502, a Toll-like receptor 5 agonist derived from *Salmonella* flagellin, provided perhaps the most promising in vivo radioprotetction data to date.[34] A single dose of CBLB502 (0.2 mg/kg) prior to irradiation (13 Gy) was enough to rescue 87% of mice from radiation-induced death. Radioprotective activity was also noted in lethally irradiated rhesus monkeys. Because of the potential efficacy of this peptide, the FDA is currently examining the use of CBLB502 in the treatment of acute radiation syndrome. Despite these positive results, as the mechanism of action for this drug involves apoptotic suppression, use of a CBLB502-based therapy in cancer patients receiving radiotherapy is risky. In addition, large-scale production of biological therapeutic agents such as CBLB502 is difficult and not suited for emergencies. Because of the potential

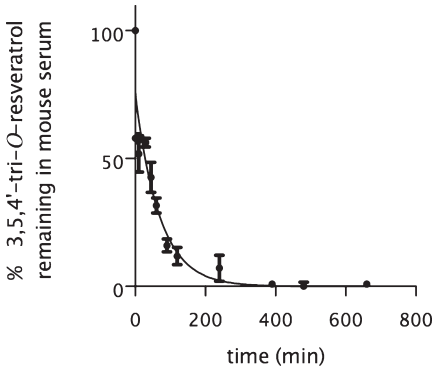


Figure 4. Hydrolysis of 3,5,4′-tri-*O*-acetylresveratrol in mouse serum at 37 °C. Rhodamine B was used as an external standard. The concentrations of these compounds were measured by HPLC analysis. Each data point represents the mean value ± standard deviation of two independent experiments.

mass administration problems and risky side effects of this peptide drug, the use of 3,5,4′-tri-*O*-acetylresveratrol may provide a valuable alternative, as it is a small-molecule drug that can be easily prepared in one step from resveratrol, it has the potential for mass oral delivery, and to date, it has shown very few, if any, detrimental side effects. Furthermore, this study implies that 3,5,4′-tri-*O*-acetylresveratrol may be used as a radioprotector in radiation therapy for cancer patients,[35] although this optimism might not be supported unless this compound is shown to selectively protect normal tissues.

In summary, we have discovered the potential of 3,5,4′-tri-*O*-acetylresveratrol as a promising lead to act as a potent radioprotective agent in mice. Although the activities of this compound and resveratrol were similar in vitro, they were quite distinct in vivo. Further studies suggested that the better protective activity of 3,5,4′-tri-*O*-acetylresveratrol in vivo might be related to the significantly improved stability of the ester groups in blood against hydrolysis when it was bound to Cremophor EL. Considering intensive studies associated with resveratrol, the use of Cremophor EL and 3,5,4′-tri-*O*-acetylresveratrol might have broader implications in other areas besides radiation biology. Further studies should reveal important properties of this compound (pharmacokinetics, ADME, plasma stability, etc.) necessary for establishing its applicability in human trials for both radiotherapy patients and as a protector against acute radiation syndrome.

EXPERIMENTAL PROCEDURES

(−)-Catechin gallate was purchased from Sigma-Aldrich (catalog number C0692) and used without further purification. Propyl gallate was purchased from Fisher (catalog number AC13158-1000) and used without purification. Resveratrol was purchased from Fisher (catalog number NC9382296) and used without further purification. 3,5,4′-Tri-*O*-acetylresveratrol was synthesized according to the literature[25] and used after recrystallization (purity >97% as determined by ^{1}H NMR analysis).

Cells (the number of cells per plate: 500 for 0−3 Gy, 1000 for 4−6 Gy, and 5000 for 7−8 Gy) from the murine hematopoietic progenitor cell line 32D cl 3 were incubated in the presence of 1, 5, or 10 μM resveratrol or 3,5,4′-tri-*O*-acetylresveratrol in Dulbecco's modified Eagle's medium (DMEM) for 1 h at 37 °C. The cells were irradiated using a JL Shepherd Mark I-68 cesium irradiator at a dose rate of 75 cGy/min to doses ranging from 0 to 8 Gy. The irradiated cells were plated in

2.2% methylcellulose in DMEM containing 15% WEHI-conditioned media as a source of interleukin 3, 30% fetal bovine serum, 1% deionized bovine serum albumin, 0.1% mercaptoethanol, and 0.07% $NaHCO_3$ in 35 mm plates as previously described.[36,37] The cells were incubated in a humidified chamber with 5% CO_2 at 37 °C for 7 days, at which time colonies of greater than 50 cells were counted by using a microscope. This experiment was performed in triplicate, and the mean values and standard deviation are shown in Figure 2. The data were analyzed using linear quadratic and single-hit, multitarget models using the Fit version 2.5 computer program.[26]

Resveratrol or 3,5,4′-tri-*O*-acetylresveratrol was dissolved in 100% ethanol and diluted 1:1 (v/v) with Cremophor EL and then diluted in water to give a 1:1:8 (v/v/v) mixture of EtOH/Cremophor EL/H_2O. Thirteen or fourteen C57BL/6NHsd female mice of 6−8 weeks of age were injected intraperitoneally with resveratrol or 3,5,4′-tri-*O*-acetylresveratrol at 1 or 10 mg/kg 10 min before irradiation (Figure 3a). In a separate experiment, 13 or 14 C57BL/6NHsd mice were irradiated to 9.75 Gy and injected intraperitoneally 10 min after irradiation and daily for 4 days with resveratrol or 3,5,4′-tri-*O*-acetylresveratrol at 25 mg/kg. The mice were irradiated in a JL Shepherd Mark I-68 cesium irradiator at a dose rate of 75 cGy per min to a dose of 9.75 Gy and monitored for the development of the hematopoietic syndrome at which time the mice were sacrificed. The data were analyzed using a log rank test (GraphPad Prism 5 Statistical Package).

Stock solutions of resveratrol in DMSO (5 mM), 3,5,4′-tri-*O*-acetylresveratrol in DMSO (5 mM) and 3,5,4′-tri-*O*-acetylresveratrol in a 1:1:8 (v/v/v) mixture of EtOH/Cremophor EL/H_2O (5 mM) were stored in a −20 °C freezer until further usage. The solution of the test compound (40 μL) was added to a glass vial containing mouse serum (1.96 mL), resulting in a final concentration of 100 μM of the test compound. This mixture was vortexed for 30 s and then immersed in a 37 °C water bath. For each time point, an aliquot (100 μL) of each serum sample was mixed with cold MeCN (100 μL) and centrifuged at 13000 rpm for 10 min at 23 °C. The supernatant was collected, and to it was added an additional cold MeCN (100 μL). The resulting mixture was centrifuged at 13000 rpm for 10 min at 23 °C. Subsequently, the supernatant (100 μL) was transferred to a glass vial, and a DMSO solution of rhodamine B was added to it (the final concentration of rhodamine B was 300 μM).[38] The final mixture was passed through a 0.22 μm filter, and the filtered solution was used for HPLC analysis. For time point 0 min, water was used instead of mouse serum.

Aliquots (10 μL each) of serum extracts were injected directly into the HPLC system equipped with a photodiode array detector. HPLC monitoring was performed on a Zorbax XDB-C_{18} column, 4.6 mm × 7.5 cm, 1.0 mL/min, 10% MeCN (containing 1% HCO_2H) in H_2O (containing 1% HCO_2H) to 100% MeCN (containing 1% HCO_2H) linear gradient elution from 0.5 to 15 min, followed by 100% MeCN (containing 1% HCO_2H) from 15 to 18 min, monitored at 309 nm. For the quantitative analysis of resveratrol and 3,5,4′-tri-*O*-acetylresveratrol, the resulting HPLC data were normalized by dividing the ratios of resveratrol or 3,5,4′-tri-*O*-acetylresveratrol/rhodamine B by the ratio of the first data point. The retention times of resveratrol, rhodamine, and 3,5,4′-tri-*O*-acetylresveratrol were 5.2, 8, and 9.5 min, respectively. The data were analyzed by using a one-phase exponential decay (GraphPad Prism 5.0a Statistical Package). The experiment was performed in duplicate. The mean values and the standard deviation are shown in Figure 4.

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Funding Sources

This work was supported by the National Institutes of Health Grant (U19 AI068021).

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