Antioxidative stress effects of vitamins C, E, and B₁₂, and their combination can protect the liver against acetaminophen-induced hepatotoxicity in rats

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Background: Several vitamins, including C, E, and B₁₂, have been recognized as antioxidants and have shown hepatoprotective effects against the hepatotoxicity caused by acetaminophen (APAP) overdose. The current investigation aims to study the effect of these vitamins and their combination in protecting the liver from APAP hepatotoxicity in rats.

Materials and methods: An in vitro model of freshly isolated rat hepatocytes was utilized for assessing hepatocyte mitochondrial activity conducted by cell proliferation assay (MTT). The isolated hepatocytes were treated with vitamin C, vitamin E, vitamin B₁₂, and their combination, with and without further addition of toxic concentrations of APAP. In addition, an in vivo experiment was carried out on Sprague Dawley rats treated intraperitoneally for 8 days with emulsions of the vitamins or their combination prior to injecting them with APAP.

Results: In vitro results showed that vitamins C and B and the combination preparation significantly increased the percentage of hepatocyte mitochondrial activity, both with and without the addition of APAP (P<0.01). The mitochondrial activity in the isolated cultured hepatocytes was further enhanced with APAP addition. In vivo, the vitamins and their combination effectively reduced APAP-induced serum liver enzymes levels, namely ALT, AST, and ALP, and also attenuated oxidative stress and lipids peroxidation confirmed by the results of glutathione, superoxide dismutase, and malondialdehyde.

Conclusion: Pretreatment with vitamins C, E, B₁₂, or their combination was found to be beneficial in preventing in vivo hepatic oxidative stress induced by APAP overdose. Vitamin C on its own showed superior protection against APAP-induced liver injury in rats compared to the other vitamins. The proliferation of APAP-intoxicated liver cells in vitro was highest when protected with the vitamins’ combination.

Keywords: glutathione, hepatocytes, proliferation, hepatoprotective, superoxide dismutase, primary cell culture

Introduction
Drug-induced hepatotoxicity is encountered in clinical practice and rises within easily accessible compounds like over-the-counter drugs. Acetaminophen, N-acetyl-para-aminophenol (APAP) or paracetamol, was originally introduced to the market as an analgesic drug by Von Mering in 1893, mostly used as an analgesic and antipyretic for relieving mild and moderate pain and fever. APAP-induced liver injury is translated to acute liver failure, with admission of more than 30,000 patients every year. The spectrum of injury caused by APAP triggers the liver all the way down to its...
frequent cause of drug-induced liver failure, dietary supplement. One of cobalamin forms that is available commercially as a reported. antioxidative properties and the ability of cobalamins in myelination of the white matter and nerves. and the brain, as it plays a considerably critical role in the ing the normal functions of the peripheral nervous system cobalamin) is a water-soluble vitamin essential for maintain inflammation, and fibrosis. genes responsible for its own metabolism, cell adhesion, NADPH-oxidase activation, as well as the expression of also regulates cell proliferation, platelet aggregation, and antioxidants, vitamins C, E, and B function mainly by two principle mechanisms of action; initially by donating electrons to free radicals: the chain-breaking mechanism. The second mechanism involves removal of initiators of reactive oxygen or nitrogen species, acting as secondary antioxidants by quenching a chain-initiating catalyst. Antioxidants may exert their effects on biological systems by several mechanisms including electron donation, chelation of metal ions, regulation of gene expression, or by acting as co-antioxidants. Among biologically important antioxidants, vitamins C, E, and B function mainly by two principle mechanisms of action; initially by donating electrons to free radicals: the chain-breaking mechanism. The second mechanism involves removal of initiators of reactive oxygen or nitrogen species, acting as secondary antioxidants by quenching a chain-initiating catalyst. Vitamin C (VC), also known as ascorbic acid, is a potent naturally occurring water-soluble antioxidant. Vitamin E (VE), a potent lipid-soluble antioxidant, is found in the plasma and tissues as an isomer, namely, α-tocopherol, the most important antioxidant in cell membranes. It also regulates cell proliferation, platelet aggregation, and NADPH-oxidase activation, as well as the expression of genes responsible for its own metabolism, cell adhesion, inflammation, and fibrosis. Vitamin B (VB) (cyano cobalamin) is a water-soluble vitamin essential for maintaining the normal functions of the peripheral nervous system and the brain, as it plays a considerably critical role in the myelination of the white matter and nerves. The potential antioxidative properties and the ability of cobalamins in regulating inflammatory cytokines and have been previously reported. Furthermore, the synthetic cyanocobalamin is one of cobalamin forms that is available commercially as a dietary supplement.

Since APAP-induced liver injury represents the most frequent cause of drug-induced liver failure, and such injury is mainly attributed to acute induction of oxidative stress in hepatocytes, the aim of the present study was to evaluate the hepatoprotective effects of VC, VE, and VB on APAP-induced hepatotoxicity. Moreover, we also wanted to investigate whether a preparation comprising a combination of all three vitamins could augment the expected hepatic protection.

Materials and methods

Materials and chemicals

All the materials used for the experiment were of analytical grade. VC, VE, and VB, EDTA, MgCl₂, FBS, streptomycin, penicillin, RPMI-1640 medium, olive oil, and GSH ELISA assay kit were all purchased from Sigma Aldrich (St Louis, MO, USA). Hank’s balanced salt solution (HBSS) was obtained from Invitrogen (Carlsbad, CA, USA). Collagenase II and L-glutamine were purchased from Gibco BRL (Gaithersburg, MD, USA). Tween 80 and DMSO were obtained from Scharlan Chemie S.A. (Barcelona, Spain), whereas Tris base and TACS® MTT kit were purchased from Promega (Madison, WI, USA) and Trevigen (Gaithersburg, MD, USA), respectively. Acetaminophen and isoflurane were kind gifts from the Jordanian Pharmaceutical Manufacturing Company JPM (Amman, Jordan) and Hikma Pharmaceuticals (Amman, Jordan), respectively. ELISA kits for superoxide dismutase (SOD) and malondialdehyde (MDA) were purchased from MyBioSource, (San Diego, CA, USA).

Experimental animals

Adult male Sprague Dawley rats with average weights of 240±20 g were housed at the Animal House Unit of the University of Petra (Amman, Jordan). Rats were kept in air-conditioned environment under controlled temperatures, humidity, and photoperiod cycles and acclimatized for 10 days before experimenting. Rats were fasted overnight (for 18–22 hours) with free access to water, unless otherwise stated. All experiments were approved by the Research Council at the Faculty of Pharmacy and Medical Sciences (approval number: 15/2/2016) and conducted in accordance with the University of Petra Institutional Guidelines on Animal Use, which adopts the guidelines of the Federation of European Laboratory Animal Science Association.

Preparation of buffers

Liver perfusion buffers were freshly prepared under sterile techniques (Al Shaker et al26). Perfusion buffer I was prepared by adding 0.9 mM MgCl₂ (95.211 g/mol), 0.5 mM EDTA...
(292.24 g/mol), and 0.5 mM Tris base (121.14 g/mol) to 0.5 L HBSS (without Ca\textsuperscript{2+} and Mg\textsuperscript{2+}). Perfusion buffer II was prepared by adding 0.5 mM Tris base to 0.5 L HBSS (with Ca\textsuperscript{2+} and Mg\textsuperscript{2+}). Perfusion buffer II with collagenase II was prepared by dissolving appropriate amount of collagenase II equivalent to 1,000 IU in 300 mL of perfusion buffer II. RPMI medium was prepared by the addition of 2 mM of L-glutamine, 5% FBS, 100 IU/mL penicillin, and 100 mg/mL of streptomycin to RPMI-1640 medium.

Preparation of emulsions and suspensions

Owing to the nature and solubility of the tested vitamins, an oil-in-water (o/w) emulsion was found to be a suitable carrier for the vitamins and their combination. All vitamins were prepared as o/w emulsions, using 10% olive oil as the oily phase and 2.5% Tween 80 as the emulsifying agent. Primary emulsions were prepared using dry gum method under the same conditions. VC and VB\textsubscript{12}, being water-soluble vitamins, were dissolved in distilled water (the aqueous phase). On the other hand, VE, being a lipid-soluble vitamin, was dissolved in olive oil followed by the addition of Tween 80. The resultant oily phase was then mixed with the aqueous phase using a vortex mixer. The final volume of each prepared emulsion was completed with sufficient quantity of distilled water. All preparations were freshly made before use.

The in vivo dose selections of 500 mg/kg VC, 200 mg/kg VE, 10 mg/kg VB\textsubscript{12}, and their combination were based on previous studies.\textsuperscript{19,22,23}

An oral dose of 2,800 mg/kg/d of APAP was used to induce hepatotoxicity in rats.\textsuperscript{9} APAP stock was freshly prepared before use by weighing appropriate amount of the drug and suspending it in distilled water for in vivo testing or in RPMI media for in vitro intoxications of isolated hepatocytes with a concentration of 10 mM APAP.\textsuperscript{24}

Surgical and liver perfusion protocols

In order to perfuse the liver with collagenase enzyme, a small animal general anesthesia system (SomnoSuite, Kent Scientific Corporation, Torrington, CT, USA) linked to an oxygen concentrator (Dual Flow Oxygen Concentrator, Longfian Scitech Co., Ltd. Hebei, People’s Republic of China), a water bath (Elmasonic S, Elma, Singen, Germany), a peristaltic perfusion pump (Langer Instruments Corporation, Boonton, NJ, USA), and a portable phlegm suction unit (Ningbo Sofine Electric Co., Ltd., Zhejiang, People’s Republic of China) were used.

The adult rat was placed on a surgical board (Plas Labs, Lansing, MI, USA), above a heating pad maintained at 37°C, and anesthetized by isoflurane (5% for induction and 2.5% for maintenance).\textsuperscript{25} A midline longitudinal incision was made to expose the rat’s viscera. Hepatic portal vein was sighted and cannulated with an 18-gauge angiocath (Becton Dickinson, Mountain View, CA, USA) and perfused with a prewarmed (37°C) perfusion buffer I at a flow rate of 10 mL/min. The perfused buffer was allowed to efflux from the body by cutting the inferior vena cava from the right kidney side. Thereafter, perfusion was switched to perfusion buffer II plus collagenase II, and the flow rate was increased to 25 mL/min and proceeded until the liver became mushy and pale in color. The liver was then dissected and placed into a sterile stoppered container with buffer II plus collagenase II, to be transferred to a sterile cell culture hood.

Hepatocyte isolation

Liver cells were dispersed gently under laminar flow, using sterile cell scrapers (Fisher Scientific Ltd., Loughborough, UK) in a sterile petri dish containing buffer II plus collagenase II. Connective tissues and undigested tissue fragments were removed from the cell suspension and centrifuged at 100 RCF for 3 minutes at 4°C. Cells were then resuspended in RPMI medium, and centrifuged at 200 RCF for 10 minutes at 4°C. After centrifugation, the supernatant was aspirated again, and cells were resuspended in 10 mL warm RPMI medium and centrifuged at 200 RCF for 10 minutes at 15°C. Finally, the resultant cell suspension was counted under light microscope using a hemocytometer (Marienfeld-Superior Co., Lauda-Königshofen, Germany).

Hepatocyte culture

Hepatocytes were diluted with appropriate amount of warm RPMI medium to the desired concentration in order to reach 70%–80% confluence. Hepatocytes were seeded into sterile 96 well plates and allowed to settle for 30 minutes before incubation in a humidified CO\textsubscript{2} incubator overnight at 37°C to allow cells to recover and grow in monolayer prior to testing.

In vitro testing protocol and cell proliferation assay

After 24 hours of incubation, the seeded plates were treated with the freshly prepared emulsions, at concentrations containing 500 μM VC,\textsuperscript{26} 180 μM VE,\textsuperscript{27} 100 μM VB\textsubscript{12},\textsuperscript{19} or their combination, with or without further addition of APAP, followed by 3 hours incubation. Later, RPMI media with and without 10 mM APAP\textsuperscript{24} was added to its corresponding hepatocyte-containing wells and further incubated for 48 hours. For assessing the mitochondrial activity for cultured hepatocytes, which also resembles cellular proliferation,
TACS MTT cell proliferation assay was performed according to the manufacturer’s instructions (Trevigen TACS MTT assay and Promega Glomax® Microplate Reader).

In vivo protocols of hepatoprotection
An in vivo study was performed on 56 adult male Sprague Dawley rats divided randomly into seven groups (n=8) and treated for 8 days. Control groups were injected intraperitoneally with placebo o/w emulsion (Groups I and II), whereas the other groups were treated with 500,20 20030, and 1000 mg/kg/d of VC, VE, and VB12, respectively (Groups III, IV, and V). Groups VI and VII were treated with the combination of the three vitamins either intraperitoneally or orally, respectively, by suspending equivalent amount of vitamins to obtain the same doses per vitamin as mentioned for the preceding treatment groups. On the other hand, systemic liver toxicity was induced by administering APAP (2,800 mg/kg) twice,9 at days 7 and 8, for all groups except Group I (negative control). At day 9, all rats were sacrificed and blood samples were withdrawn by cardiac puncture and left at room temperature for 15 minutes to clot completely. Blood samples were centrifuged at 6,000 rpm for 10 minutes at room temperature to obtain serum and stored at −20°C until requested for analysis. In addition, serum samples were referred for analysis of liver function biomarkers (ALT, AST, ALP, albumin, and cholesterol) at Al-Mahabbah Hospital, Madaba, Jordan. Stress biomarkers (SOD, MDA, and total GSH) were assessed in-house using ELISA kits, according to the manufacturers’ procedures and instructions.

Data analysis
Each data point represents the mean±standard error of the mean (SEM). Statistical comparisons were made using SPSS statistical software (version 20, IBM Company, Armonk, NY, USA), using one-way ANOVA followed by Tukey’s post Hoc Multiple Comparison test to compare between groups. P-values <0.05 were considered statistically significant (P<0.05).

Results
Cotreatment of hepatocytes with VC, VE, and VB12 enhances mitochondrial activity
The selected concentration of 10 mM of APAP significantly decreased the cellular proliferation of the isolated rat hepatocytes approximately by half (Figure 1). Figure 1 illustrates the mitochondrial activity of isolated rat hepatocytes treated with the vitamins when examined on nonintoxicated isolated hepatocytes compared to APAP-intoxicated cells. The tested vitamins did not show any notable proliferation changes in healthy isolated hepatocytes except for VE that significantly reduced hepatocyte mitochondrial activity (P<0.01). On the other hand, when APAP-intoxicated hepatocytes were pretreated with the vitamins or their combination, the mitochondrial activity was significantly increased (P<0.01), and highest proliferation activity was observed in the cells treated with the combination.

VC, VE, and VB12 ameliorate APAP-induced hepatotoxicity
As illustrated in Table 1, treating rats with a high hepatotoxic dose of APAP caused significant increase in serum levels of ALT, AST, and ALP (P<0.01), although it did not cause any pronounced change in serum cholesterol and albumin levels. The effect of VC on protecting the liver from APAP toxicity was quite noticeable, where it significantly reduced serum levels of all tested liver enzymes (P<0.01). On the other hand, VE and VB12 caused a statistically significant decrease (P<0.01) in the levels of ALT and ALP. A decrease in AST levels was also noticed, although not statistically significant. Orally administered combination prior APAP intoxication in rats decreased the overproduction of ALT, AST, and ALP in their serum while intraperitoneal administered combination reduced only ALT and AST levels (Table 1).

Vitamins C, E, and B12 ameliorate APAP-induced oxidative stress
Serum levels of MDA, SOD, and total GSH are illustrated in Figures 2–4, respectively. When rats were intoxicated with an
overdose of APAP, a marked increase in serum MDA, SOD, and total GSH levels was observed ($P<0.01$). Pretreatment of rats with VC, VE, VB$_{12}$, or their combinations prior to APAP intoxication has significantly prevented such increase ($P<0.01$). Although the decrease in APAP-induced lipid peroxidation, observed by MDA formation, is lowered by pretreatment with mentioned vitamins, they did not show complete reversal down to control basal levels. The significant reversal of APAP-induced increase in GSH to norm was observed in both VC and VB$_{12}$. However, all vitamin reversed SOD production in APAP-injected rats.

**Discussion**

Vitamins have been reported to have pharmacologically protective effects owing to their antioxidant and various cell regulatory roles. APAP, in animal models, can induce hepatic injury, usually pointed out as hepatocyte apoptosis and necrosis. High doses of APAP have also been shown to decrease hepatocyte viability and survival.\(^{30}\) Having known that mice are more susceptible to APAP-induced injury and rats show more resistance unless high doses are employed,\(^{31}\) the current study investigates the potential hepatoprotective effects of specific vitamins in inhibiting such injury. It was demonstrated that the tested vitamins, namely, VC, VE, and VB$_{12}$, significantly reduced APAP-induced liver injury in Sprague Dawley rats. Their combination also significantly enhanced cell viability and mitochondrial activity in vitro for both APAP-treated and untreated hepatocytes. All vitamins, alone or in combination, exerted statistically significant increase in cellular activity and proliferation in APAP-treated cell

**Table 1** Serum levels (mean±SEM) of liver function biomarkers

<table>
<thead>
<tr>
<th>Group (n=8)</th>
<th>Treatment</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
<th>Albumin (g/dL)</th>
<th>Cholesterol (mg/dL)</th>
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<tbody>
<tr>
<td>I</td>
<td>Placebo emulsion</td>
<td>45±2</td>
<td>115±9</td>
<td>171±39</td>
<td>2.7±0.1</td>
<td>79±4</td>
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<tr>
<td>II</td>
<td>Placebo emulsion+APAP</td>
<td>4,350±1,171</td>
<td>4,065±1,194</td>
<td>503±39</td>
<td>2.3±0.1</td>
<td>81±5</td>
</tr>
<tr>
<td>III</td>
<td>500 mg/kg VC+APAP</td>
<td>300±112</td>
<td>1,000±332</td>
<td>256±48</td>
<td>3.4±0.5</td>
<td>78±5</td>
</tr>
<tr>
<td>IV</td>
<td>200 mg/kg VE+APAP</td>
<td>970±217</td>
<td>2,130±453</td>
<td>251±17</td>
<td>2.6±0.1</td>
<td>96±8</td>
</tr>
<tr>
<td>V</td>
<td>10 mg/kg VB$_{12}$+APAP</td>
<td>692±217</td>
<td>1,342±274</td>
<td>209±17</td>
<td>2.6±0.1</td>
<td>86±5</td>
</tr>
<tr>
<td>VI</td>
<td>Intraperitoneal combination+APAP</td>
<td>548±51</td>
<td>1,318±360</td>
<td>373±14</td>
<td>2.5±0.1</td>
<td>83±4</td>
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<tr>
<td>VII</td>
<td>Oral combination+APAP</td>
<td>610±115</td>
<td>1,148±221</td>
<td>231±29</td>
<td>3.0±0.1</td>
<td>93±5</td>
</tr>
</tbody>
</table>

**Notes:** \(P<0.01, \*P<0.05\) compared to APAP alone treated group (II).

**Abbreviations:** APAP, acetaminophen; SEM, standard error of the mean; VB$_{12}$, vitamin B$_{12}$; VC, vitamin C; VE, vitamin E.

**Figure 2** Serum levels of MDA in rats pretreated with vitamins prior to systemic intoxication with APAP.

**Notes:** Data points are presented as mean±SEM (n=4). The effect of vitamins or their combinations on reducing serum MDA levels of APAP-intoxicated rats were found significant ($^{**}$P<0.01).

**Abbreviations:** APAP, acetaminophen; IP, intraperitoneal; MDA, malondialdehyde; SEM, standard error of the mean; VB$_{12}$, vitamin B$_{12}$; VC, vitamin C; VE, vitamin E.

**Figure 3** Serum levels of SOD in rats pretreated with vitamins prior to systemic intoxication with APAP.

**Notes:** Data points are presented as mean±SEM (n=4). The effect of vitamins or their combinations on reducing serum SOD levels of APAP-intoxicated rats were found significant ($^{**}$P<0.01).

**Abbreviations:** APAP, acetaminophen; IP, intraperitoneal; SEM, standard error of the mean; SOD, superoxide dismutase; VB$_{12}$, vitamin B$_{12}$; VC, vitamin C; VE, vitamin E.
sections of ~2-fold in comparison to untreated control cells (P<0.01). Nevertheless, the results indicated that VC and VB_{12} had a moderate influence on mitochondrial activity when tested on normal hepatocytes (Figure 1). Such an enhancement might be sufficient to protect the cells from oxidative stress and excess free radical and ROS formation, as reported in previous studies.

Liver transaminases, namely, ALT and AST, remain the gold standards for the assessment of liver injury and have been used for a long time as the biomarkers of choice. These enzymes are released into blood after hepatic cell injury, and their elevated serum levels are indicative of cellular leakage and loss of functional integrity of hepatocyte cell membranes. The increase in ALT levels is more specific for liver damage than AST, since it is mainly found in the liver, whereas AST is also found in other tissues such as heart, skeletal muscles, kidney, red blood cells, and brain.

Intoxication of rats with APAP highly increased the levels of serum ALT and AST, indicating liver injury. However, pretreatment of rats with 500 mg/kg VC, 200 mg/kg VE, 10 mg/kg VB_{12}, or their combination, daily for 8 days, significantly inhibited the increase of ALT serum levels; however, VC and the combination group significantly decreased AST serum levels unlike VE and VB_{12}, which showed insignificant effect. These results are in line with many previously reported studies where such reduction in liver enzymes indicates the possession of membrane-stabilizing activity in the tested vitamins and might indicate their ability to induce regeneration of liver cells, therefore reducing the leakage of the liver enzymes into the blood.

In contrast to ALT and AST, serum level of ALP is related to the function of hepatic cell rather than its integrity. In this study, ALP levels were significantly elevated in APAP group (Group II), while pretreatment with the vitamins or their combinations inhibited such increase, although not statistically significant in the intraperitoneal combination group (Group VI).

Usually, injury of the liver inhibits synthesis of major proteins including albumin. A decrease in albumin levels was noted in rats treated with high doses of APAP alone. This can be endorsed to APAP-induced disruption in hepatocyte integrity, which either in turn leads to decreased hepatic capacity for protein synthesis, or increased proteolysis and degradation activities. VC showed the highest protection against APAP-induced albumin deprivation unlike the other groups, which exhibited an insignificant effect. This might be linked to its considerable ability to protect hepatocytes from injury, thereby reserving its role in albumin levels modulation.

Besides albumin, cholesterol is one of many molecules synthesized by the liver and thereafter released to the bloodstream. To date, there is no clear and constant published report confirming the effect of APAP overdose on modulating in vivo cholesterol levels. Some previous studies reported an increase in cholesterol serum levels after APAP overdose, whereas few others reported decreased levels, whereas few other reports claimed no remarkable change. This variability in results might be related to the dose and frequency of APAP administration which affects the extent of liver damage on many enzymes including cholesterol 7-α-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid biosynthesis that converts cholesterol to bile acid. However, in this study, no noticeable change in cholesterol levels was observed after administration of the selected toxic dose of APAP.

It is well documented that liver tissue contains relatively high content of polyunsaturated fatty acids, which are very sensitive to peroxidative damage. Peroxidation of polyunsaturated fatty acids results in MDA formation, a biochemical indicator of necrosis. MDA is a biomarker for oxidative stress that indicates a state of damage when detected.
mechanism against stress induced by APAP overdose. Such results are in line with the previous work of Aycan et al.

on Wistar rats where serum ALT, AST, GSSG, and SOD activity, as well as the serum and tissue MDA levels were found to be higher in the APAP group (500 mg/kg) than in the control group.

Conclusion
Pretreatment with VC, VE, and VB₁₂ or their combination was shown to protect the livers of rats, both in vitro and in vivo, and prevented APAP-induced liver damage. The survival of APAP-intoxicated liver cells in vitro was highest when protected with the vitamin combination followed by VC, VB₁₂, and VE, respectively. Yet, it seems that VC on its own may have a superior protection against APAP-induced liver injury compared with the other vitamins. VC has also shown significant protection actions on the isolated liver cells. The outcome of this research suggests that the use of one or more of the investigated vitamins can protect from hepatotoxicity or liver-induced injury which may occur from APAP overdose; however, such claims must be tested clinically. Therefore, future investigations are needed to approve the potential protective effects of the tested vitamins on APAP-induced injury clinically in humans compared to other available APAP antidotes and further elucidate the molecular mechanisms behind such capability.

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Disclosure
The authors report no conflicts of interest in this work.

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