Annonaceous Acetogenins as a new anticancer agent

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ABSTRACT

In spite of the many advances in modern scientific medicine, plant-based traditional medicine still plays an important role in the health care. The acetogenins of Annonaceae are known by their potent cytotoxic activity. In fact, they are promising candidates as a new future generation of antitumoral drugs to fight against the current chemiotherapic resistant tumors. The present review will describe the source and chemistry of Annonaceous acetogenins, their diverse biological activities and their anticancer mechanism of action with highlighting on their toxicity and previously reported total synthesis.

Keywords: Annonaceous acetogenins, Annonacin, antitumor agents, cytotoxic agent, HIF-1 inhibitors, Natural products.

1. Natural product as a source of new drugs:

Natural products (NPs) and their synthetic derivatives have been widely used in drug discovery because of their structural diversity and their highly selective and specific biological activities [1-3]. Numerous NPs, semi-synthetic NPs and modified NPs compounds are undergoing clinical testing [4]. Worldwide, between 2005 and 2010, nineteen new NP-based drugs were approved for marketing [5]. Cragg and Newman continuously present updates on NP drugs based on traditional medicine both as pharmaceutical agents and/or as leads for bioactive molecules against serious diseases [1, 2, 6]. It is anticipated that nature will continue to be a major source of new structural leads, and effective drug development will depend on multidisciplinary collaborations. A wide number of studies, including in vitro, in vivo and enzymatic experiments, have reported effective activity in NP extracts and have isolated compounds from them[7-9].

There are a significant number of NPs drugs in development. It is reported that 100 NPs and NPs-derived compounds and Antibody Drug Conjugates (ADCs) with a NPs-derived cytotoxic component being evaluated in clinical trials or in registration at the end of 2013.Thirty eight of these NPs compounds and thirty three ADCs are being investigated as potential oncology treatments, twenty six as anti-infectives, nineteen for the treatment of cardiovascular and metabolic diseases, eleven for inflammatory and related diseases and six for neurology, Figure 1[1, 4, 10]
It is estimated that well over 50% of cancer patients use at least one or more complementary and alternative medicine (CAM) therapies as part of their treatment[11]. Among the alternative medicines, products (e.g., PawPaw Cell-Reg, Graviola Max, Royal Graviola, Graviola Liquid Extract) that contain twig extracts of paw paw (Asiminatriloba) or Brazilian paw paw (Annonamuricata, graviola or soursop) are reported to have exhibited antitumor efficacy both in animal models and in a limited number of clinical studies. However, the lack of rigorously controlled clinical trials has cast a shadow on the observation that the administration of paw paw capsules rich in acetogenins decreased tumor size, reduced tumor blood flow, suppressed metastasis, and improved survival in cancer patients[12, 13].

2. Acetogenins
2.1. Annonaceous Plants
The Annonaceae, or custard-apple, family consists of many different genera including Annona and Asimina, each containing several species. Asiminatriloba (paw paw), **Figure 2**, is the only temperate species; the rest of the family is tropical or subtropical. The United States Department of agriculture provides a complete list of the 205 Annonaceae genera on the Germplasm Resources Information Network (GRIN, http://www.ars-grin.gov/cgi-bin/npgs/html/exgnlist.pl) including Annona and Asimina individual species taxonomy.

The fruits of pawpaw have nourished wild animals and mankind in Eastern North America for thousands of years, and Paw paw festivals are to be found in September throughout the Midwestern United States. Many of the tropical species bear edible fruits and have been naturalized from central and South America to other warm climates in Asia and Africa. The latest articles[14, 15] has summarized the economic potential of the annonaceous fruits and noted that certain parts of several species are poisonous and/or pesticidal. Soursop (graviola, guanabana) and Cherimolia, **Figure 3(a & b)** are two of the best known annonaceous fruits and are sold either fresh or in processed forms; a much amount of the seeds of these commercial species, with their rich concentrations of acetogenins, are discarded during the processing.[16, 17]

Annonacin (1a) has been reported to be present in the bark and seeds of Asiminatriloba (paw paw, prairie banana, poor man’s banana, Ozark banana, Banango, also commonly referred to as its native states ‘‘banana’’, e.g. Indiana/Hoosier banana, Kentucky banana, etc.) which grows throughout the Eastern United States and is used as a potential alternative cash crop to tobacco. Furthermore, there is (1a) containing commercial supplement made from paw paw twig extracts, Paw-Paw Cell-Reg TM, that is marketed as beneficial for overall health (www.naturessunshine.com) and as a safe complement to cancer therapy.[18]
2.2. Structures and nomenclature of acetogenins:

Natural compounds, known as Annonaceous acetogenins (ACGs), are considered the active ingredients in paw paw products. Found only in Annonaceae species, the ACGs are derivatives of fatty acids which are combined with an alkanolmoiety at the 2-position of 2,4-disubstituted γ-lactone[19]. Usually, one, two or three THF rings occur in the middle of the long hydrocarbon chain, which often contains a number of oxygenated moieties (hydroxyl, acetoxy, carbonyl, epoxide) and/or a double bond. Figure 4. All of the acetogenins have multiple stereocenters, and their relative and absolute configuration can be determined.[20]

The ACGs was classified according to the characteristic number of their oxygen-containing groups appeared on a long chain fatty acids moiety. Linear ACGs, epoxy ACGs, mono-tetrahydrofuran ACGs and bis-tetrahydrofuran ACGs can be found.[21]

The ACGs can be also classified according to the number and arrangement of the tetrahydrofuran rings within the molecule into subgroups as mono-THF, adjacent bis-THF, nonadjacent bis-THF, non-THF or tetrahydropyran (THP), nonadjacent THF and THP, and adjacent tris-THF ring compounds, according to the number and arrangement of the THF rings. The chemical structures of the ACGs are shown in Figure 5. Mono-THF compounds are the most popular components of ACGs. Some of them have two flanking hydroxyls, Figure 5, A, while others have only one. Adjacent bis-THF ring compounds are the second largest group. These ACGs are characterized by carrying variable number of hydroxyl groups or carbons, placement of the bis-THF rings and hydroxyl groups on the chain, the stereochemistry around the bis-THF rings, the type of terminal γ-lactone and/or the existence of some specific functional groups, Figure 5, B. Nonadjacent bis-THF acetogenins contain two nonadjacent THF rings which are usually separated by a four-carbon chain; one THF ring is flanked by two hydroxyl groups while the other THF ring has only one adjacent hydroxyl group which is positioned between the two THF rings, Figure 5, B. Mucocin(2) is a typical nonadjacent THF and THP acetogenin, and gonioin(3) is an example of adjacent tris-THF acetogenin. They contain a methylated α-β-unsaturated γ-lactone at one end, but do not have THF or THP rings. Diepomuricanin(4) is an example of a non-THF/non-THP acetogenin, Figure 5, C.[22]
2.3. Acetogenins biological activity

Quantitative evaluation of the published data on cytotoxic activity of these compounds has led to information on the structure-activity relationships. Generally, the data indicate that the most potent cytotoxic compounds possess an adjacent bis-THF ring subunit; the nonadjacent bis-THF ring acetogenins show less cytotoxicity. Mono-THF acetogenins are, in turn, more potent than the non-ring compounds. Acetogenins with different configurations usually show different selectivities or potencies in bioassays. Thus, the importance of determining their relative and absolute configuration is obvious. It should also be pointed out that chlorination decreases bioactivity but indicates some cytotoxic selectivity (Table 1 shows a brief list of bioactive ACGs)[23]
In particular, the inhibitory effect of acetogenins on mitochondrial NADH-ubiquinone oxidoreductase (complex I) is worthy of note for the following reasons: (a) the diverse biological activities are thought to be attributable to this effect; (b) some of the compounds, such as Bullatacin(6) are the most potent inhibitors of the enzyme identified to date [34, 35] IC50(1.2 nM (± 0.1) ] and (c) it is quite difficult to visualize structural similarities between the acetogenins and ordinary complex I inhibitors such as ptericidin A and rotenone, although the acetogenins act at the terminal electron transfer step of complex I similarly to the ordinary complex I inhibitors.[36]
As a result, the powerful cytotoxicity of these compounds indicated a myriad of potentially useful applications. Commercial development of these compounds uses natural mixtures of active components, incorporated into pesticide, topical and dietary supplement products. Successful applications and commercial products include a shampoo, highly effective in treating infestations of head lice, fleas, and ticks; a series of pesticidal sprays, which protects host plants against a diversity of pests; and an ointment for treatment of oral herpes (HSV-1) and other skin afflictions[27]. The extract (in capsule form) enhances a mixture of natural anthelmintics. In addition, an encapsulated extract has been effectively used by certain cancer patients as a botanical supplement product.[2]

2.3.1. Anticancer effects
Most of the acetogenins evaluated in vitro exhibited similar IC50 values (10^-2 to 10^-7 µg/ml). However, it was observed that some acetogenins appear to be selectively cytotoxic for certain cancer types. For example, cytotoxicity results for Asimicin(5) and Bullatacin(6) showed potent selectivity against A2780 human ovarian tumor cells. Other results demonstrating selectivity for certain cancer cell lines are summarized in Table 2[37]. The ACGs have also been shown to inhibit the growth of multidrug-resistant (MDR) tumor cells. The resistance of MCF-7/Adr human breast cancer cells and KBv200 human nasopharyngeal carcinoma cells to doxorubicin and vincristine, respectively, was about 100-fold compared to their parental sensitive cell lines MCF-7 and KB. Whereas the acetogenins exhibited potent cytotoxicity to both cell lines [38]. Bullatacin(6) was also found to be potent against MDR human mammary adenocarcinoma cells[39]. MDR tumor cells were resistant to apoptosis induced by natural anticancer drugs, however, the acetogenins induced apoptosis of both sensitive and MDR cells. It is concluded that adjuvant acetogenins therapy with anticancer drugs may be effective in treating MDR tumors.[40, 41]
ACGs have also been evaluated for in vivo antitumor activity. Increased life spans were observed in their in vivo studies using acetylgenins against 3PS and L1210 leukemia in mice. In addition, when xenografts of A2780 human ovarian tumor cells were established in athymic nude mice, acetylgenin treatment significantly inhibited tumor growth, Table 3. The in vivo antitumor efficacy of acetylgenins suggests that rather evaluation is warranted to define optimal routes and schedules of administration and to verify in vitro selectivities.[43]

Table 3. Antitumor efficacy of acetylgenins in mice.[43, 44]

<table>
<thead>
<tr>
<th>Comp. No.</th>
<th>Compound</th>
<th>Tumor cell line</th>
<th>(daily dose, mg/kg)</th>
<th>Antitumor activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a)</td>
<td>Annonacin</td>
<td>3PS</td>
<td>0.95</td>
<td>124 *</td>
</tr>
<tr>
<td>(5)</td>
<td>Asimicin</td>
<td>3PS</td>
<td>0.025</td>
<td>124 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L1210</td>
<td>0.2</td>
<td>131 *</td>
</tr>
<tr>
<td>(6)</td>
<td>Bullaticin</td>
<td>L1210</td>
<td>0.05</td>
<td>138 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2780</td>
<td>0.1</td>
<td>68 **</td>
</tr>
<tr>
<td>(21)</td>
<td>Bullaticine</td>
<td>L1210</td>
<td>0.4</td>
<td>144 *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A2780</td>
<td>0.13</td>
<td>52 **</td>
</tr>
<tr>
<td>(8)</td>
<td>Bullatulcin</td>
<td>A2780</td>
<td>1.0</td>
<td>75 **</td>
</tr>
<tr>
<td>(23)</td>
<td>Bullatulcin</td>
<td>L1210</td>
<td>0.63</td>
<td>113 *</td>
</tr>
<tr>
<td>(24)</td>
<td>Rolliastatin</td>
<td>3PS</td>
<td>0.25</td>
<td>128 *</td>
</tr>
<tr>
<td>(25)</td>
<td>Squanocin</td>
<td>L1210</td>
<td>0.63</td>
<td>113 *</td>
</tr>
<tr>
<td>(26a)</td>
<td>Uvanacin</td>
<td>3PS</td>
<td>1.4</td>
<td>157 *</td>
</tr>
</tbody>
</table>

*Increase of life span (%). **Tumor growth inhibitor (%) in athymic nude mice. 3PS and L1210: murine leukemia cell lines. A2780: human ovarian carcinoma cell line.
Although acetogenins are extremely potent against tumor cells in vitro, the effect on growth of noncancerous cells such as rat Gl epithelial cells is minimal. Table 4 shows that acetogenins display a much higher activity against KB human nasopharyngeal carcinoma cells as compared to Vero normal monkey epitheliodrenal cells. In vivo studies have also reported little weight loss with acetogenin treatment, indicating favorable drug tolerance among the test animals. In addition, a standardized acetogenin mixture from Asiminastriloba, was found to be non-mutagenic in the Ames test, suggesting that acetogenins would not be carcinogenic and therefore may have an added advantage over many current antitumor drugs. Furthermore, it has been shown that the NADH oxidoreductase activity of rat liver plasma membrane is largely unaffected by Bullatacin(6) whereas that of HeLa (human cervical carcinoma origin) and HL-60 (human promyloctic leukemia origin) plasma membranes is strongly inhibited, Table 5. These different responses of NADH oxidoreductase activity to Bullatacin(6) explain, in part, the ability of certain members of the Annonaceae family to selectively kill cancer cells while leaving normal cells unharmed.[37]

Table 4: Effect of acetogenins on the growth of tumor and normal cells in vitro[37, 45]

<table>
<thead>
<tr>
<th>Comp. No</th>
<th>Compound</th>
<th>OH-positions</th>
<th>THF- relative configuration</th>
<th>Molecular Formula</th>
<th>Ed50 (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(27a)</td>
<td>Corosolin</td>
<td>(th/th/th)</td>
<td>C20H2O8</td>
<td>0.3×10⁻²</td>
<td>10⁻³</td>
</tr>
<tr>
<td>(28)</td>
<td>Jetein</td>
<td>(th/ter)</td>
<td>C20H2O8</td>
<td>10⁻³</td>
<td>10⁻³</td>
</tr>
<tr>
<td>(29)</td>
<td>Molvizarin</td>
<td>(th/th/ter)</td>
<td>C20H2O8</td>
<td>10⁻⁷</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>(30)</td>
<td>Squamoxin</td>
<td>(th/th/ter)</td>
<td>C20H2O8</td>
<td>10⁻⁷</td>
<td>10⁻⁴</td>
</tr>
<tr>
<td>(31)</td>
<td>Spinecin</td>
<td>(th/th/ter-th)</td>
<td>C20H2O8</td>
<td>10⁻⁷</td>
<td>6×10⁻⁸</td>
</tr>
<tr>
<td>(32)</td>
<td>Carolin-A</td>
<td>(th/th/ter)</td>
<td>C20H2O8</td>
<td>10⁻⁷</td>
<td>2×10⁻³</td>
</tr>
<tr>
<td>(33)</td>
<td>Carolin-B</td>
<td>(th/th/ter)</td>
<td>C20H2O8</td>
<td>10⁻⁷</td>
<td>4×10⁻⁷</td>
</tr>
<tr>
<td>(34)</td>
<td>Carolin-C</td>
<td>(th/th/ter)</td>
<td>C20H2O8</td>
<td>2×10⁻⁷</td>
<td>5×10⁻⁷</td>
</tr>
</tbody>
</table>

KB: human nasopharyngeal carcinoma cell line. VERO: normal monkey epitheliodrenal cell line.

Table 5: Summary of inhibition of NADH oxidoreductase activity by acetogenins[35]

<table>
<thead>
<tr>
<th>Comp. No</th>
<th>Compound</th>
<th>Ed50(nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1a)</td>
<td>Annonacin</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>(5)</td>
<td>Asimicin</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>(6)</td>
<td>Bullatacin</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>(21)</td>
<td>Bullatacinone</td>
<td>&gt;10,000</td>
</tr>
</tbody>
</table>

Tamoxifen resistance is common in estrogen receptor-(ER)-positivebreast cancers. α-ER-positive MCF-7 cells, annonacin(1a) (half-effective dose Ed50 = 0.31 μM) and 4-hydroxytamoxifen (Ed50 = 1.13μM) decreased cell survival whereas annonacin(1a) (0.5-1 μM) increased cell death at 48 h. Annonacin(1a) induced growth arrest and apoptosis in ER-related pathways in MCF-7 cells. Annonacin(1a) and 4-hydroxytamoxifen were additives in inhibiting cell survival and ER transcriptional activity. Moreover, annonacin(1a) attenuated MCF-7 xenograft tumor growth while inhibiting ER, cyclin D1 and Bcl-2 protein expressions in nude mice.[46]

2.3.2. Potential mechanism of the anticancer action of ACGs
It is reported recently that the inhibitory effects exerted by paw paw crude extract and purified acetogenins on the important anticancer molecular target hypoxia-inducible factor-1 (HIF-1). The transcription factor HIF-1 activates the expression of more than 100 target genes that promote cellular adaptation and survival under hypoxic conditions. It is a heterodimer of the oxygen-regulated HIF-1α and the constitutively expressed HIF-1/ARNT subunits. The classical oxygen-dependent post-translational modification of HIF-1α protein includes the prolyl hydroxylation that tags HIF-1α for pVHL-mediated ubiquitination and subsequent proteasome degradation, and the asparaginyl hydroxylation that inactivates HIF-1 as shown in Figure 6.[11]
Solid tumors are often subjected to hypoxia and need to adapt to the hypoxic environment to sustain their rapid growth, **Figure 6**. As a transcription factor, HIF is the master regulator of the cellular hypoxia response. HIF is a heterodimer composed of a hypoxia-regulated subunit (HIF-α) and a constitutively expressed subunit HIF-1β. Among three HIF-α isoforms, HIF-1α is the most critical regulator of hypoxia responses in solid tumors, and its activity is indispensable for tumors to adapt to hypoxic conditions and recover from damages caused by hypoxic insult. While HIF-1β is constitutively expressed in the nucleus, HIF-1α remains at a low level through proteasome-dependent mechanisms under normoxia. Upon hypoxia, HIF-1α is quickly stabilized and translocated to the nucleus, where it forms a heterodimer with HIF-1β and subsequently binds to the hypoxia responsive element (HRE) (59-RCG TG-39), resulting in transactivation of more than 200 genes required for the cell to adapt to hypoxic conditions. Since many of the HIF-1 target genes can promote cell survival under hypoxic conditions, it is not surprising that HIF-1α is often overexpressed in various cancers, including breast, lung, pancreatic and renal cancer. Therefore, inhibition of HIF-1α activity represents an attractive strategy for cancer treatment. Indeed, small molecules targeting HIF-1α transcription, translation, and stabilization have been developed, and some of them (e.g., PX-478, Topotecan and BAY87-2243) have entered clinical trials for treating cancer patients.[48]

Both the HIF-1 inhibitory and cytotoxic/cytostatic activities of acetogenins and crude extract appear to be cell line-dependent. Tumor cell populations that rely solely on mitochondria to generate ATP are most sensitive to inhibitors of the electron transport chain. In contrast, aggressive and malignant tumor cells often utilize aerobic glycolysis to fuel cellular metabolism and function. Thus, they are less sensitive to antitumor agents that affect mitochondria. Although neither purified acetogenins nor paw paw crude extracts have been approved by the FDA for cancer treatment, dietary supplements that contain these substances are consumed by cancer patients as alternative medicines.[47, 49]

The toxicity of AGEs in both cases probably arises from the strong inhibitory ability of mitochondrial electron transport with specific action at complex I. DegliEsposti et al. [50] first used mammalian mitochondria to study the action of AGEs toward the NADH-ubiquinone oxoisoreductase (complex I) and reported that Bullatacin(6) inhibited the proton pumping function of complex I with similar efficiency under steady-state and nonsteady-state conditions, as compared to the action of piericidin and rotenone. Because of the ability to inhibit the mitochondrial complex I, the main gate of the energy production in the cells, AGEs have been regarded as candidates for future generations of antitumour drugs with different mechanisms. [51]
Besides blocking the NADH ubiquinone oxidoreductase (complex I) in the electron transport system, AGEs are also powerful inhibitors of the NADH oxidases peculiar to the plasma membranes of cancer cells. Both mechanisms of action result in the inhibition of ATP production and may account for the observation that AGEs are more effective at killing multiple-drug resistant (MDR) tumors than their nonresistant counterparts since the MDR pumps on the cell membranes require ATP to function.

In addition, Oberlies et al.[52] observed that AGEs could selectively inhibit the cell growth of cancerous cells by in vitro cell inhibition assays against three murine (P388, PO3, and M17/Adr) and two human (H8 and H125) cancer cell lines. Interestingly, the work of Oberlies et al. proposed that this class of natural products showed a certain biological activity against some drug-resistant cancers. Currently, multidrug-resistant cancers are hard to cure (43a).

1.7 nM for annonacin cell lines. Interestingly, the work of Oberlies et al. proposed that this class of natural products showed a certain advantage in the chemotherapeutic treatment of MDR tumors. Shimada et al. also proposed a model for explaining the action of AGEs. They suspected that the lactone ring alone could directly interact with the binding to complex I, mitochondrial complex I was lowest for squamocin, the action of AGEs. They suspected that the lactone ring alone could directly interact with the binding to complex I, and the THF rings with flanking OH groups function just as hydrophilic anchors at the membrane surface that allow lateral diffusion (or random distribution) of the lactone ring in the membrane interior.[11, 18]

Toxicity of ACGs
Graviola (A. muricata), fruta-de-conde (A. squamosa), and the paw paw (A. triloba) may cause atypical parkinsonism, due to the presence of annonacin(1a) and other neurotoxins.[54][17] A. muricata that contains high content of annonacin(1a) is enough to cause neurodegeneration in rats can also be attained in humans by regular consumption of fruits and tea made from the leaves. At first it was blamed on the presence of neurotoxic benzyltetrahydroisoquinolines. Subsequent authors identified annonacin(1a) and other ACGs as a more likely cause.

Like benzyltetrahydroisoquinolines, annonacin(1a) may cause tauopathies, including atypical parkinsonism due to their inhibition of mitochondrial complex I. The concentration that caused 50% inhibition (IC50) of the mitochondrial complex I was lowest for squamocin(25) (1.4 nM), compared to 54.8, 63.8, 100.3, 11.8, 2.1, 1.6, and 1.7 nM for annonacin(1a), annonacinone(4b), isoannonacin(4c), solamin(41), sootepensin B (42), tonkinesin A (43a), and tonkinin C (43b), respectively. The concentrations needed to induce cell death in 50% of the cultured neurons (EC50 death) were 134, 246, 121.0, 23.8, 1.7, 3.2, and 3.9 nm for annonacin(1a), annonacinone(1b), isoannonacin(1c), solamin(41), sootepensin B (42), tonkinesin A(43a) and tonkinin C (43b), respectively.

These ACGs also decreased ATP levels, with an EC50 of 134 nM for annonacin(1a) and 2.9 nM for squamocin(25), and values ranging from 4.0 to 246 nM for the others. These ACGs also induced neuronal cell death with EC50 values of 60.8 nM for annonacin(1a), rolliniastatin(24) and 1.1 nM for squamocin(25), and values ranging from 1.7 to 189.7 nM for the others. These ACGs also redistributed thetau protein in cultured neurons. The concentration needed to redistribute tauin 5% of the neurons (EC5) was (0.6 nM) for squamocin(25) and rolliniastatin(24), 44.1 nM for annonacin(1a), and values ranging from 1.0 to 134.9 nM for the others.[59, 60]
Recently, other workers found that the mechanism underlying the cytotoxicity of ACGs is modulated by the chelation of THF moieties of ACGs with Ca\(^{2+}\) to form hydrophobic complexes. As shown in Figure 7a, a representative example of annonacin (1a) - Ca\(^{2+}\) chelate. The ability of ACGs to bind Ca\(^{2+}\) correlated with their levels of cytotoxicity. Also, incubation of cells with bis-THFACGs also increased the mitochondrial Ca\(^{2+}\) concentration and decreased the mitochondrial membrane potential, which may be the main cause of cytotoxicity.[17]

![Figure 7. Annonacin - Ca\(^{2+}\) complex(61)](image)

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