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Corn rootworm-active RNA DvSnf7: Repeat dose oral toxicology assessment in support of human and mammalian safety

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ABSTRACT

Genetically modified (GM) crops have been developed and commercialized that utilize double stranded RNAs (dsRNA) to suppress a target gene(s), producing virus resistance, nutritional and quality traits. MON 87411 is a GM maize variety that leverages dsRNAs to selectively control corn rootworm through production of a 240 base pair (bp) dsRNA fragment targeting for suppression the western corn rootworm (*Diabrotica virgifera virgifera*) *Snf7* gene (DvSnf7). A bioinformatics assessment found that endogenous corn small RNAs matched ~450 to 2300 unique RNA transcripts that likely code for proteins in rat, mouse, and human, demonstrating safe dsRNA consumption by mammals. Mice were administered DvSnf7 RNA (968 nucleotides, including the 240 bp DvSnf7 dsRNA) at 1, 10, or 100 mg/kg by oral gavage in a 28-day repeat dose toxicity study. No treatment-related effects were observed in body weights, food consumption, clinical observations, clinical chemistry, hematology, gross pathology, or histopathology endpoints. Therefore, the No Observed Adverse Effect Level (NOAEL) for DvSnf7 RNA was 100 mg/kg, the highest dose tested. These results demonstrate that dsRNA for insect control does not produce adverse health effects in mammals at oral doses millions to billions of times higher than anticipated human exposures and therefore poses negligible risk to mammals.

1. Introduction

Ribonucleic Acids (RNA) are ubiquitous bio-molecules fundamental to life and, therefore, are abundant in food and feed that has an extensive history of safe consumption. This safe consumption includes RNA molecules of varying types ranging from single stranded RNA, such as messenger RNA (18 to >10,000 nucleotides for the SAMDC and titin transcripts, respectively), to more complex RNA structures such as transfer RNAs (typically 76–90 nucleotides) and ribosomal RNAs (approximately 1500 and 1800 nucleotides for 16S and 18S rRNAs, respectively). Long double stranded RNA (dsRNA) precursors (e.g. 200–400 base pairs) and small RNAs (e.g. 21–25 base pairs (Carthew and Sontheimer, 2009; Llave et al., 2002)) are involved in RNA interference (RNAi), a natural mechanism that modulates endogenous gene expression and is found widely across plants, animals, and fungi. As a naturally occurring, ubiquitous process in eukaryotes, RNAi and dsRNA molecules that modulate endogenous gene expression are present in plants and animals that have a substantial history of safe consumption. This safe consumption is remarkable since commonly consumed foods such as maize, soybean, rice, lettuce, and tomatoes contain both short (e.g. small interfering RNAs (siRNAs) and micro RNAs) and long dsRNAs encoding short RNAs with perfect sequence identity to human and mammalian genes (Ivashuta et al., 2009; Jensen et al., 2013).

Throughout the crop domestication and plant breeding processes, desirable crop phenotypes that are now known to be mediated through RNAi have been selected (Della Vedova et al., 2005; Tuteja et al., 2004). The RNAi mechanism has also been leveraged in the development of GM crops with quality traits and with virus resistance (Frizzi and Huang, 2010; Kamthan et al., 2015; Parrott et al., 2010) and has been recently developed as a highly selective tool for insect control (Bachman et al., 2013; Baum et al., 2007; Mao et al., 2007). In light of a long history of safe RNA consumption discussed above, the fact that RNAi does not represent a novel mechanism for developing crop traits, and the broader

Abbreviations: bp, base pair; dsRNA, double stranded RNA; GM, genetically modified; miRNA, micro RNA; siRNA, small interfering RNA; RNA, ribonucleic acid; RNAi, RNA interference.

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weight of the scientific evidence presented herein, it is reasonable to conclude that applications of dsRNA in agricultural biotechnology are safe (Petrick et al., 2013, 2015).

The safety of ingested nucleic acids, including RNA, is well understood from several perspectives, including the simple fact that humans consume significant amounts of RNA with every meal of plant and animal derived ingredients (Jonas et al., 2001). This safety is driven not by the sequence of the ingested RNA (recall that humans safely consume RNA with sequence matches to their genome and transcriptome as noted above) but rather, by a highly effective set of biological barriers that greatly limit the potential for biologically meaningful exposures to ingested RNAs (Petrick et al., 2013, 2015). Formidable barriers against exogenous RNAs are evident from investigative research into RNA therapeutics (reviewed by (Juliano et al., 2009; Juliano, 2016; O'Neill et al., 2011; Petrick et al., 2013)) and these barriers have limited the potential for systemic RNA therapeutics. More notably, these barriers have been the source of insurmountable challenges to the development of oral RNA-based therapeutics to date. These barriers necessitate the use of direct injections, chemical modifications/stabilization, and specialized delivery formulations for systemic drugs (Behlke, 2006) and ensure very low oral bioavailability of oligonucleotide drugs (i.e. <1%) after oral administration (Nicklin et al., 1998; Petrick et al., 2013).

The efficacy of biological barriers against ingested dietary RNAs *in vivo* is demonstrated by feeding studies with plant-derived materials/foods and/or dsRNAs (Dickinson et al., 2013; Petrick et al., 2015; Snow et al., 2013; Witwer and Hirschi, 2014; Witwer et al., 2013). Further evidence of the impact of these barriers is also provided by the noted difficulties in achieving oral delivery of nucleic acid drugs for diseases of the intestinal tract (e.g. local rather than systemic delivery) (Knipe et al., 2016). Barriers to ingested dsRNAs include pH extremes and nucleases in the gastrointestinal tract and in blood (Juliano et al., 2009; O'Neill et al., 2011; Petrick et al., 2013). However, even when RNA targeting a rat gene is injected intravenously (i.v.) into rats, bypassing several of the aforementioned gastrointestinal and membrane barriers, at doses up to 200 mg/kg body weight it does not produce adverse effects (Thompson et al., 2012). This provides strong evidence for the impact of exogenous dsRNA barriers beyond the gastrointestinal tract. For example, it has been shown that exogenous systemic dsRNAs are extensively degraded in the blood within minutes following i.v. dosing (even when chemically stabilized (Christensen et al., 2013);) and that these dsRNAs undergo rapid renal elimination (Molitoris et al., 2009; Vaishnaw et al., 2010).

Cellular transit of highly polar macromolecules such as dsRNAs is limited by a series of membrane barriers that separate the intestinal lumen from the vasculature, and the vasculature from any putative systemic target tissues (e.g. epithelial and endothelial cellular membranes) (Reviewed by (Juliano et al., 2009; O'Neill et al., 2011; Petrick et al., 2013). The possibility that orally ingested dsRNA could have a systemic effect is highly unlikely when one considers the ingested dsRNA has to: 1) be absorbed in a functionally intact form from the GI tract and in quantities suitable for mediating RNAi despite being subjected to a series of cellular membrane barriers that limit the transit of polar molecules and a hydrolytic environment that typically degrades nucleic acids, 2) avoid degradation by blood nucleases, 3) avoid renal elimination, 4) pass through the cellular membrane of the target cell, and 5) avoid sequestration in endosomes (since polar RNA molecules do not have direct cytoplasmic access, but are subject to endocytic vesicle uptake). Sequestration in endosomes is important to RNA fate because RNAs may be shunted to lysosomes (for degradation) and/or may undergo subsequent degradation by intracellular nucleases (Forbes and Peppas, 2012; Gilmore et al., 2004; Juliano, 2016). The sequestration of RNAs in endosomes, where the contents remain outside of the cytoplasmic space, represents a significant barrier to putative activity by any systemically absorbed exogenous dsRNAs, as only 1–2% of dsRNAs entering the cell are able to escape from this compartment (Gilleron et al., 2013). The weight of the scientific evidence, including *in vivo* testing, therefore supports the conclusion of rapid metabolism and clearance and low intracellular exposures resulting from exogenous dsRNA exposures. This weight of the evidence is especially strong for dsRNAs ingested from the diet which have a limited toxicity potential, due to additional barriers afforded by the gastrointestinal tract (Knipe et al., 2016) and is therefore particularly applicable to dsRNAs composed of naturally occurring nucleotides that would be expressed in GM crops (i.e., unformulated and unstabilized dsRNAs).

MON 87411 expresses the *Bacillus thuringiensis* (*Bt*)-derived toxin Cry3Bb1 and an RNA molecule, DvSnf7 RNA, to protect maize from corn rootworm damage. The DvSnf7 RNA expressed in MON 87411 is composed of a 968 nucleotide sequence containing a corn rootworm-active 240 base pair double stranded RNA (dsRNA) component plus the addition of a poly A tail (Urquhart et al., 2015). DvSnf7 RNA confers selective control (i.e., activity is limited to a subset of the Galerucinae subfamily) against corn rootworm through suppression of the insect Snf7 gene (Bachman et al., 2013). Therefore, although DvSnf7 is orally active against *Diabrotica virgifera virgifera*, Bachman and colleagues demonstrate that it is toxic to only a narrow spectrum of insects that are both closely related and susceptible to ingested RNA. Therefore, since a number of species that are closely related to Diabrotica but fall outside the *Galerucinae* subfamily of beetles are not susceptible to DvSnf7 RNA, based on both sequence divergence and biological barriers, activity or toxicity in more distantly related mammalian species would not be anticipated.

As an additional assurance of the absence of a hazard to mammals, the potential for toxicity of the 968 nucleotide rootworm-active RNA, DvSnf7, was evaluated in a 28-day repeat dose oral gavage toxicology study in mice at doses of 1, 10, or 100 mg/kg body weight. Following 28 days of consecutive treatment, there was no toxicity observed in this study and the No Observed Adverse Effect Level (NOAEL) of DvSnf7 RNA was therefore considered to be 100 mg/kg, the highest dose tested. The results of this study and exceedingly large margins of exposure relative to anticipated human exposures described in this paper demonstrate the safety of DvSnf7 RNA, a rootworm-active RNA expressed in MON 87411 and illustrate the lack of potential hazard or risk to humans or animals from dietary exposures to this RNA.

2. Materials and methods

2.1. Bioinformatics assessment

Eight total RNA isolations were conducted from conventional, non-transgenic maize, LH244: three from maize grain 25 days after pollination (DAP), two from maize grain 32 DAP, and three from maize grain 39 DAP. Small RNAs from these individual maize grain total RNA samples were isolated after separation on a polyacrylamide gel, followed by sequential ligation of cloning adaptors to these extracts (as described by Llave et al., 2002). This material was reverse transcribed and the cDNA libraries generated in this process were sent to 454 Life Sciences (Branford, CT) for deep sequencing via pyrosequencing. Computer algorithms written in the Perl programming language (Perl scripts) were used to identify small RNA inserts within the raw sequence data through identification and removal of sequences representing the cloning adaptors. The sequences from the eight maize grain sequencing libraries were combined into a single library. After removal of duplicate