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RISK ASSESSMENT FOR DSRNA-BASED PESTICIDES

The agricultural sector is currently facing challenges such as decreasing availability of conventional pesticides, rapid pesticide resistance evolution, and climate change impacts, leading to a growing demand for sustainable pest management solutions.

INTRODUCTION

Double-stranded RNA (dsRNA)-based pesticides have emerged as a promising alternative, leveraging RNA interference (RNAi) to precisely target and silence specific genes in pest organisms. dsRNA acts more selectively than conventional pesticides, which often harm non-target species. When dsRNA is taken up by eukaryotic cells, it is processed by Dicer endonucleases into 20-25 nt short interfering RNAs (siRNAs) (Figure 1).These siRNAs guide Argonaute (AGO) proteins in the RNA-Induced Silencing Complex (RISC) to identify and degrade complementary mRNAs, leading to post-transcriptional gene silencing (PTGS).
If the targeted gene is essential for organismal development, this can result in lethality. This specificity reduces the possibility of undesirable effects on non-target organisms, positioning dsRNA-based pesticides as a significant advancement in sustainable agriculture (1-3). However, RNAi-based pest control has limitations in efficacy against certain pest orders, such as lepidopterans (4), and rapid resistance development has been observed in coleopteran pests (5-8), indicating a need for resistance management strategies to protect the technology.
Given the promising potential of dsRNA-based pesticides, there is a pressing need for regulatory frameworks to adapt to this new technology.

POLICY BRIEF

Current regulations may not adequately address the unique properties and mechanisms of dsRNA products, while it is essential to ensure their safety, efficacy, and responsible use in agriculture. Establishing clear guidelines can help facilitate the integration of dsRNA solutions into modern pest management strategies, contributing to sustainable agricultural practices.

The regulatory landscape for dsRNA-based pesticides is complex, particularly in the European Union, where they are classified as chemical pesticides under Regulation (EC) No. 1107/2009.

Consequently, dsRNA products undergo the same stringent safety and efficacy assessments as conventional pesticides, delaying their approval and commercial availability (2, 3). This pesticide-based regulatory approach does not consider the biological specificity of dsRNA and its rapid degradation in the environment, which may make it safer than traditional chemicals.

In contrast, the U.S. Environmental Protection Agency (EPA) has begun approving dsRNA products like Calantha/Ledprona (CAS Number: 2433753-68-3, Docket ID: EPA-HQ-OPP-2021-0271), setting a precedent for more flexible regulatory frameworks that recognize the biological nature of dsRNA (9).

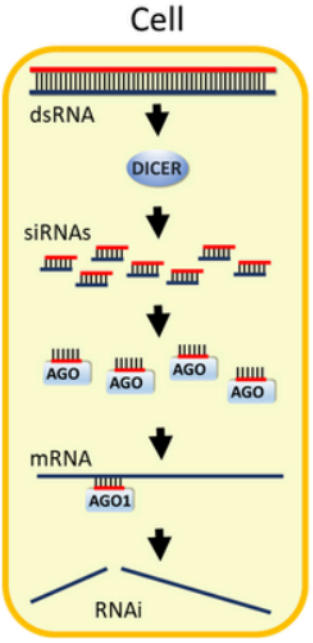

Figure 1. Schematic representation of the RNAi mechanism induced by dsRNAs

REGULATORY AND SCIENTIFIC CHALLENGES

The deployment of dsRNA-based pesticides faces a range of regulatory and scientific challenges that must be addressed to ensure their safe and effective use. The Organisation for Economic Co-operation and Development (OECD) has made significant contributions by publishing comprehensive documents that tackle both environmental and human health considerations related to dsRNA products (1,10). These reports underscore the critical importance of conducting thorough evaluations of potential toxicological hazards associated with dsRNA application. Specifically, assessments must focus on various exposure routes, the

POLICY BRIEF

potential risks presented to applicators, bystanders, and consumers who may come into contact with or consume treated product. Potential risk assessment challenges have to do with knowledge gaps related mainly to hazard identification and characterization. Pro-actively identifying off-targets and non-targets is of paramount importance and for this sake bioinformatics may prove extremely helpful, especially when considering the ever-growing availability of genome and transcriptome data. The golden standard for assessing the likelihood of off-target hits is the presence of a 20-nt sequence identity between a given dsRNA pesticide and the target's genome and/or transcriptome. Yet, more nuanced criteria need to be developed, because RNAi efficiency in various organisms is dependent more factors, including e.g. the overall size of sequence identity, the number of allowed mismatches between a small RNA and its mRNA target, the number of small RNA hits on the genome/transcriptome, the topology of hitting site. Clearly, the bioinformatics criteria need to be nuanced and are currently far from being established. A surprisingly unexplored facet of RNAi is a possible induction of unintended epigenetic events, especially at the crops of application. DsRNA may trigger DNA methylation at genome loci sharing an overall 70-80% sequence identity with the dsRNA (11). Then, de novo DNA methylation may occur at CG, CHG and CHH context; of these, methylation at CG context may be trans-generationally maintained (even at the absence of homologous dsRNA) and may affect endogenous transcription (12), This is not of minor importance and highlights that bioinformatics tools need to be optimized also for these worst-case scenarios. In that case, of course, these tools would need to employ not only on transcriptome but also genome data. Finally, besides RNAi-specific and sequence-dependent events, dsRNA may also trigger RNAi-unspecific and sequence-independent events, such as immune system stimulation and even saturation of the endogenous RNAi machinery, both of which may negatively affect the organism (2,13). Importantly, the scenario where a dsRNA affects non-target species in the environment needs to be addressed.

Besides hazard identification and characterization, risk assessment relies upon exposure assessment. DsRNA environmental persistence is influenced by various ecological factors, including microbial activity, levels of ultraviolet (UV) exposure, and the composition of the soil in which it is applied (2,3). While dsRNA typically undergoes rapid degradation in agricultural soils (e.g. DT90<35 h) (14) and aquatic microcosms (e.g. DT90 <96h) (15) its stability may be greatly increased upon the use of formulations such as LDH nanoclays, carbon dots, chitosan etc. (16). Such formulations are meant to increase dsRNA's bioactivity (stability and uptake) but may also increase

bioavailability to non-target organism or exhibit toxicity themselves. Evidently, the impact of formulation substances needs to be addressed in future risk assessment schemes, where (eco)toxicological tests and environmental persistence assays should (also) be performed with a given dsRNA in its formulated form.

Indeed, the OECD document highlights the necessity of understanding the stability and bioavailability of dsRNA in environmental contexts. These factors play a pivotal role in determining non-target organisms exposure levels and overall risk profiles associated with the use of dsRNA products (1,10). To this end, novel methods for accurate and specific dsRNA quantification need to be developed. So far, detection and quantification of dsRNA pesticides is achieved by QuantiGene assay, a hybridization probe-based method that allows the direct measurement of dsRNA molecules, using signal amplification rather than target amplification. Yet, although specific and sensitive, this method exhibits low versatility, since, for each new sequence/dsRNA, newly customized probes need to be designed, synthesized and provided to the user; moreover, the availability of expensive equipment such as Luminex instruments for the detection of the signal is required. But analytical methods, intended to support regulatory decision making, require a high level of standardization and versatility. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is a widely used method to detect and quantify different types of transcripts, such as messenger RNA, ribosomal RNA, non-coding RNA etc. As a method, RT-qPCR is characterized by established standardization protocols, reproducibility, versatility of detecting a wide range of dsRNA concentrations in divergent environmental matrices and low reaction costs. Development of personalized PCR-based protocols adjusted to the technical challenges of isolation and quantification of dsRNA from environmental samples will most likely prove extremely helpful. Coupling RT-qPCR assays with small RNA sequencing where detection of dsRNA degradation products such as small RNAs is achieved would provide the most stringent data on dsRNA (and its active degradation intermediates) persistence.

The potential effects of dsRNA targeting essential and highly conserved genes on non-target organisms, particularly beneficial species such as pollinators and essential soil organisms, remain a significant area for ongoing research. These considerations highlight the pressing need for thorough and comprehensive risk assessments that consult a wide range of parameters, including environmental persistence, degradation rates, uptake by non-target species, their susceptibility to RNAi, and the likelihood of off-target effects. This multifaceted approach is essential to ensure that dsRNA-based pesticides can be used safely and sustainably in modern agricultural practices (2,3).

POLICY BRIEF

ADVANCES IN RESEARCH AND APPLICATIONS

Recent developments in dsRNA-based pesticides are showing remarkable potential, but also its limitations in the pest management landscape. Field trials have provided compelling evidence of the effectiveness of dsRNA against notorious pests such as the Colorado potato beetle, whereas other pest groups such as lepidopterans and hemipterans are largely not amenable to RNAi under applied conditions (yet). Notably, these innovations cause minimal collateral damage to non-target species, emphasizing their ecological safety. Furthermore, dsRNA has shown the potential for not only controlling pest populations but also for tackling the pathogens they carry, which may significantly broaden its role in integrated pest management strategies.

A key driver of this progress is the innovation in formulation technologies, particularly the development of nanoparticle-based delivery systems and bioclay formulations. These novel approaches have dramatically improved the stability and bioavailability of dsRNA in agricultural settings (17).

One of the primary challenges faced by dsRNA-based pesticides is ensuring their effectiveness across varying environmental conditions, including fluctuations in UV exposure and microbial activity. Research has indicated that nanoparticles can shield dsRNA from accelerated degradation while simultaneously enhancing its uptake by insect pests, thus boosting its overall efficacy. Additionally, Bioclay formulations have been successfully utilized to stabilize dsRNA, further improving its performance in outdoor environments (17).

Despite the encouraging advancements in dsRNA formulation and field applications, the pathway to regulatory approval remains a vital hurdle. The approval of Calantha/Ledprona by the EPA marks a significant milestone, underscoring the ability of dsRNA-based products to conform to stringent safety and efficacy standards. This case serves as a crucial reference point for other regulatory bodies, illustrating the necessity for robust yet adaptable regulatory frameworks capable of accommodating these innovative pesticide solutions.

POLICY BRIEF

RECOMMENDATIONS FOR COMPREHENSIVE RISK ASSESSMENT

In light of the growing interest in deploying dsRNA as a pesticide, it is crucial to establish a robust framework for risk assessment that ensures both safety and efficacy in the long term. Several key recommendations must be meticulously considered:

Hazard identification and characterization:
Develop appropriate bioinformatic tools that will provide detailed and in-depth analysis of the potential for unintended effects by comparing dsRNA sequences with genomic and transcriptomic databases of non-target organisms. This analysis could be considered a basis for planning testing strategies of given dsRNA molecules destined for use in plant protection.

Determination of Envrionmental fate:
Assess the environmental persistence and degradation of dsRNA under various conditions (e.g., UV exposure microbial activity, soil type) to understand its stability, degradation kinetics, and potential for accumulation. Additionally, study its environmental fate, including mobility through runoff, leaching, or atmospheric deposition, and identify any degradation products that may pose ecological or human health risks.

The potential of mathematical models, currently available for conventional chemical pesticides, to determine environmental exposure levels for dsRNA molecules should be evaluated and new tools might be required in case low prediction accuracy is shown.

Non-target and Ecological Impact Assessment:
To address the unique characteristics inherent to dsRNA molecules, the adaptation of standardized testing protocols may be necessary. While the existing OECD test guidelines offer a variety of standard test species, it is important to verify whether potentially susceptible species identified through bioinformatics analysis are included, especially those related to ecologically significant organism groups and phylogenetically similar organisms. Investigate potential trophic transfer and potential long-term effects due to epigenetic effects.

Resistance Management and Human Health:
Monitor target pest populations for resistance development and assess gene silencing efficacy to ensure continued effectiveness. Evaluate potential human exposure routes (inhalation, dermal contact, ingestion)

and conduct toxicological studies to assess immunogenicity, allergenicity, and other adverse effects, particularly for agricultural workers and consumers.

Investment in Training and Capacity Building:
Regulatory agencies must prioritize the enhancement of their scientific and technical understanding of RNA interference mechanisms and the potential ecological ramifications of dsRNA applications. This investment entails providing targeted training programs and resources for regulatory personnel and stakeholders in the agricultural sector. An emphasis on interdisciplinary collaboration is vital, fostering partnerships between regulatory bodies, scientific researchers, and industry representatives. Such cooperation will ensure comprehensive evaluations of dsRNA products that consider both the direct impacts on target pests and the possible indirect effects on ecosystem dynamics, including predator-prey interactions and shifts in community structure.

Promoting Public Engagement and Transparency in dsRNA technology development and application:
For dsRNA-based pesticides to gain widespread acceptance, it is essential to foster public engagement and transparency in their development, assessment and use. Openly communicating the benefits, potential risks, and underlying science of dsRNA products is fundamental to building public trust. Engagement strategies should include informative outreach efforts directed at various stakeholders, including farmers, environmental groups, and the general public. Additionally, the establishment of open-access databases that share data on dsRNA sequences, risk assessments, and research findings will facilitate informed dialogue and collaborative efforts. By ensuring that the development of dsRNA products is transparent and participatory, the agricultural community can promote responsible usage and mitigate concerns regarding safety and environmental impact.

By implementing these detailed recommendations, the journey toward safe and effective utilization of dsRNA-based pesticides can be navigated with greater confidence and responsibility.

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POLICY BRIEF

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POLICY BRIEF

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