EUROPEAN MEDICINES AGENCY
SCIENCE MEDICINES HEALTH

23 July 2013
EMA/CVMP/430890/2013
Committee for Medicinal Products for Veterinary Use

European public MRL assessment report (EPMAR)

Double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus (Bees)

On 27 May 2013 the European Commission adopted a Regulation[1] establishing maximum residue limits for double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus in honey, valid throughout the European Union. These maximum residue limits were based on the favourable opinion and the assessment report adopted by the Committee for Medicinal Products for Veterinary Use.

Double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus is intended for the prevention of acute disease caused by Israeli acute paralysis virus in honeybees.

Beelogics Holdings Ltd submitted the application for the establishment of maximum residue limits to the European Medicines Agency, on 29 October 2010.

Based on the original and complementary data in the dossier, the Committee for Medicinal Products for Veterinary Use recommended on 13 April 2012, the establishment of maximum residue limits for double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus in honey.

On the 9 July 2012 the European Commission requested the Committee to reconsider its opinion in order to reflect on whether, due to the new type of veterinary active substance being examined, existing guidance relating to human medicines might be applied in the evaluation of this substance. The Commission also asked the Committee to provide further clarification in relation to the risk management considerations.

On 13 September 2012 the Committee for Medicinal Products for Veterinary Use, having considered the Commission's request, adopted a revised opinion recommending the establishment of maximum residue limits for double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus in honey.

[1] Commission Implementing Regulation (EU) No 489/2013, O.J. L 141, of 28 May 2013

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Subsequently the Commission recommended on 27 March 2013 that maximum residue limits in honey are established. This recommendation was confirmed on 17 April 2013 by the Standing Committee on Veterinary Medicinal Products and adopted by the European Commission on 27 May 2013.

Summary of the scientific discussion for the establishment of MRLs

Substance name:	Double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus
Therapeutic class:	Antiviral
Procedure number:	EU/10/182/BEE
Applicant:	Beeologics Holdings Ltd
Target species:	Bees
Intended therapeutic indication:	Prevention of acute disease caused by Israeli Acute Paralysis Virus in honeybees
Route(s) of administration:	Oral

1. Introduction

Double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus is an anti-viral agent for use in honeybees for the preventative treatment of infections caused by the Israeli Acute Paralysis Virus (IAPV), a single stranded ribonucleic acid (RNA) virus placed taxonomically within the family Dicistroviridae. The pharmacologically active agent consists of double stranded (unmodified and uncoated) ribonucleic acid (dsRNA) homologous to parts of the coat protein (CP) and intergenic region (IR) of the IAPV virus. The dsRNA molecule corresponds to 857 nucleotides from the viral genome.

Using immobile feeders inside the hive bees are treated with an intended commercial formulation containing the dsRNA, administered in sucrose solution (66 % w/v) at a dose of 10 mg/0.5 l/hive per month (approximately once every 4 weeks) at any time that bees are not storing surplus honey. An amount of 0.5 l of sugar solution is consumed by the bees of an active and healthy hive in 24 to 48 hours. Product application is limited to the autumn, winter and early spring months only, outside the honey flow period when bees are producing honey. A dose of 10 mg/hive corresponds to a calculated dose of approximately 1 µg of active/bee/treatment.

Beeologics Holdings Ltd submitted the application for the establishment of maximum residue limits to the European Medicines Agency, on 29 October 2010.

2. Scientific risk assessment

This risk assessment evaluates the consumer risk of potential residues in honey of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV and its derivatives.

2.1. Safety assessment

2.1.1. Overview of pharmacological properties

Pharmacodynamic properties including mode of action

The mode of action of the pharmacologically active agent is based on the principle of RNA interference (RNAi). RNAi is initiated by the cellular uptake of the double stranded RNA which is homologous to sequences of the viral genome (CP and IR regions). The dsRNA is subsequently cleaved by the RNAse III enzyme “DICER” into 21- to 24-nucleotide duplexes of short interfering RNAs (siRNA). These siRNAs are unwound and the more thermodynamically stable strand or (“passenger”) single strand is incorporated with the aid of “DICER” into a RNA-induced silencing complex (RISC) which targets complementary viral messenger RNA (mRNA) in the cytoplasm, leading to its degradation and thus prevention of protein translation. The RNAi technique mimics a naturally occurring defense mechanism of gene silencing (inactivation) which has been found in almost all eukaryotes. Double stranded RNAs are also naturally created inside bees as a result of IAPV infection.

The components of the dsRNA do not appear to share homology with any human gene product. To identify homologous regions between the nucleotide sequences of the proposed dsRNA molecules and human gene transcripts, a bioinformatic analysis (BLAST) was performed. No sequences of more than 20 base pairs (b.p.) homologous to human gene transcripts were found (for the IAPV dsRNA to be able to theoretically "silence" endogenous genes, derived siRNAs generated by endogenous “DICER” RNAses would have to generate 100 % of 21 to 26 nucleotide homologies with coding host sequences). Only two genes were found to have partial homology to the proposed dsRNA molecules.

Pharmacokinetic properties (mainly in laboratory animals)

Upon ingestion, the dsRNA enters the epithelial cells of honeybee mid-gut through a putative trans-membrane protein where it triggers a pathway that results in its degradation by bee “DICER” enzymes into small siRNAs of various sizes. The mid-gut cells are assumed to be target cells where most of the viral replication occurs. In the presence of a virus encoding enzyme called RNA dependent RNA polymerase (RdRP) and/or an IAPV template, the natural gene silencing mechanism amplifies the siRNAs and the RNAi reaction can persist for some time through this production of secondary siRNAs. Short interfering RNAs have been detected in bees for several weeks after the last feeding with the proposed commercial formulation. In the absence of RdRP or IAPV template, the two strands of the dsRNA are disjoined and rapidly degraded by endogenous RNAse enzymes to composite nucleotides which become integrated into the cells’ endogenous nucleotide pool.

No pharmacokinetic data in laboratory animals were available. Based on general pharmacokinetic considerations it may be assumed that uptake of dsRNA in mammalian cells will be very limited due to its relatively large molecular weight and its negative charge. In addition, in general, dsRNA and its derivatives are quickly degraded under physiological (acidic) conditions of the human gastrointestinal tract and/or rapidly degraded by RNases of the gastrointestinal tract or serum. The stability of the proposed dsRNA in the gastrointestinal tract was investigated in an *in vitro* digestion gut model with both a gastric and a duodenal phase. The proposed dsRNA was not detected in any of the samples subjected to digestion, in contrast to the findings in samples that were not subjected to gastric and duodenal conditions. These data suggest that oral bioavailability of intact dsRNA from honey in the human gastrointestinal tract is negligible.

2.1.2. Calculation of pharmacological ADI, if relevant

Given that:

- orally ingested dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV will not absorbed and will be rapidly broken down to its constituent nucleotides;
- the nucleotide sequence of the dsRNA is not sufficiently homologous with that of human genes to result in silencing of human genes or to otherwise interact with the human genome;

it can be concluded that orally ingested dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV would not induce pharmacological effects in the consumer. The derivation of a pharmacological ADI was therefore not considered necessary.

2.1.3. Overview of toxicology

Single-dose toxicity, where appropriate

No data have been provided from single-dose toxicity studies in mammalian laboratory animals and no information regarding the acute toxic potential of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV in mammals or humans have been provided.

Repeated dose toxicity

No repeated-dose toxicity studies in laboratory animals have been provided.

Reproductive toxicity, including developmental toxicity

No studies on reproductive toxicity including developmental toxicity were performed in laboratory animals. Some results from clinical trials in the target species evaluating adverse effects of the intended commercial formulation on queen reproductive capacity have been provided. It was concluded that treatment with the intended commercial formulation has no adverse effect on queen reproductive capacity and no negative outcome on foraging.

Genotoxicity

No studies on genotoxicity of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV were provided.

Carcinogenicity

No studies on carcinogenicity of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV were provided.

Studies of other effects including immunotoxicity and neurotoxicity

The potential for allergic reactions to dsRNA was considered. Consumers are exposed to exogenous RNA on a daily basis, yet there is no indication that this results in immunotoxicity. All known food allergens are proteins and not nucleic acids. It is therefore accepted that there is no indication of potential allergenicity to dsRNA.

No studies on neurotoxicity were provided.

2.1.4. Calculation of the toxicological ADI or alternative limit

Given that orally ingested dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV will not be absorbed and will be rapidly broken down to its constituent nucleotides it can be concluded that standard toxicology studies (including single dose, repeated dose, reproductive toxicity, genotoxicity and carcinogenicity studies) would not be appropriate as such studies would not be expected to reveal toxicological effects. A toxicological ADI was therefore not considered necessary.

2.1.5. Overview of microbiological properties of residues

Disruption of the colonisation barrier

No data were provided but as microbiological effects are not expected for this type of substance this is acceptable.

Increase of the population of resistant bacteria

No data were provided but as microbiological effects are not expected for this type of substance this is acceptable.

2.1.6. Calculation of microbiological ADI

No microbiological ADI was calculated as microbiological effects are not expected for this type of substance.

2.1.7. Observations in humans

No data on observations in human were available.

2.1.8. Findings of EU or international scientific bodies

No evaluations by other international committees were available.

2.1.9. Overall conclusions on the ADI

A standard safety package of pharmacology and toxicology studies performed with the proposed pharmacologically active material was not provided. The safety package consists of published literature reports relevant to the safety and stability of nucleic acid and double stranded RNA in general, along with a limited number of studies investigating the stability of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV and their sequence homology with human genes. This is acceptable because:

- the nature of the material, nucleic acid (dsRNA), is such that a standard pharmacological/toxicological approach including the setting of an ADI is not appropriate as:
  - consumers orally ingest nucleic acids on a daily basis and there is no inherent risk associated with this exposure;
  - due to the size and polar nature of RNA molecules, cell penetration is likely to be very limited;

- studies with the specific substance (ie dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV) reinforce the conclusion that it is not absorbed, and indicate that homology with human gene transcripts is not sufficient to result in silencing of human genes:
  - *in vitro* experiments under simulated gastrointestinal conditions indicate that the dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is not stable (and not bioavailable) under both gastric and duodenal conditions;
  - the sequence of IAPV-specific dsRNA proposed has been found by bioinformatic analysis (BLAST analysis) to have no 100 % sequence homologies greater than 20 base pairs to human genes, indicating that the material would be unable to silence expression of human genes or to otherwise interact with the human genome;
- IAPV-specific RNA occurs naturally in bees as a result of IAPV infection.

In line with the above, it is concluded that orally ingested dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV would not induce pharmacological, toxicological or microbiological effects in the consumer and consequently the establishment of an ADI for this substance was not considered appropriate.

2.2. Residues assessment

2.2.1. Pharmacokinetics in target species

For substances for use in honey bees there is no formal requirement for pharmacokinetic studies in the target species. However, a study was conducted using a northern blotting assay to investigate the occurrence of short interfering RNA in bees after treatment with the intended commercial formulation and in untreated bees that may potentially have been naturally infected with IAPV. The assay was based on the hybridization of a fluorescence probe which hybridizes with sequences of the proposed RNA fragments in bees' RNA extract. Sequences that hybridize with the probe were present in treated and, albeit at lower levels, in bees from untreated control groups, which seems to underscore the natural occurrence of such dsRNAs in response to natural IAPV infection. Short interfering RNAs were detected in bees over 3 weeks after the last feeding with the intended commercial formulation.

2.2.2. Residue depletion studies

A study was done in honey to demonstrate the absence of residues of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV in treated hives. Bees were given three consecutive feeds of 10 mg of the intended commercial formulation/hive in 0.5 l of 66 % (w/v) sucrose solution. Honey samples (obtained from unripened honey) were tested for residues using two different analytical methods, a LC-UV-MS method and a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR).

The LC-UV-MS method could not differentiate between naturally occurring and nucleotide components specific to the intended product; untreated controls and test samples contained comparable amounts of nucleotides which indicated that nucleotides were present in all honey samples at levels greater than those potentially derived from the intended product. The (qualitative) RT-PCR method could not detect any specific residues of the pharmacologically active agent in honey from hives up to its sensitivity limit (limit of detection) of 1 µg/ml honey. The RT-PCR is highly specific for dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV derived nucleotides.

A further study was conducted to test the stability and time-course of degradation of the intended commercial formulation and its pharmacologically active agent under hive ambient conditions at two different concentrations of sucrose solution (66 % w/v and 50 % w/v, i.e. bee-feed). Analysis was performed by gel electrophoresis and nucleotide band sizes on agarose gels for samples taken at different time points after mixing within sucrose and were (qualitatively) compared.

The results indicate that the dsRNA is of limited stability in sucrose solution under ambient conditions. Depending on experimental conditions and sucrose content, partial degradation was already seen after 24 to 96 hours and after 2 weeks (the longest period investigated) the pharmacologically active material seemed to be completely degraded. It was assumed that this degradation was mostly accomplished by the bacterial fauna of the hive.

The residue study had been designed as a clinical trial to investigate efficacy and safety for the bees and is not considered a typical residue study in honey. Though treatment conditions corresponded largely to intended label instructions, sampling of solely unripened honey and storage of samples at room temperature are conditions that would normally not be used. The overall results, however, support the argument that dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is not sufficiently stable in honey matrix to be present in measurable quantities in commercial honey. This observation is consistent with results obtained in stability tests in sucrose.

Overall conclusions on the residues

The residue assessment is primarily based on published literature in relation to physico-chemical/physiological characteristics of (ds)RNAs in general and two studies with the intended commercial formulation on its stability in sucrose and residues in honey following treatment of bees. The level of detail contained in the documentation relating to these studies and their results was limited. Nevertheless, the available evidence indicates that consumers of honey would be unlikely to be exposed to dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV or any of its metabolites at significant levels:

- residues in honey from treated hives (at a dose of 3 x 10 mg of IAPV specific dsRNAwithin 1 week) could not be detected with RT-PCR at concentrations as low as 1 µg/ml immediately after the last administration;
- the pharmacologically active material is degraded relatively quickly in sucrose solution inside the hive, and therefore unconsumed bee feed (and also honey matrix) will most probably not retain the active ingredient over significant periods of time;
- bees will be treated only during months when no collection of honey is taking place (and in the absence of the honey super), which represents an additional safety precaution;
- in general, dsRNA is likely to be quickly degraded under acidic (pH less than 4) conditions and thus dsRNA is likely to be degraded in the human gastrointestinal tract and also in honey, since honey commonly has a pH lower than 4;
- unmodified and unprotected dsRNA and siRNAs can be expected to be rapidly degraded by ubiquitous RNAses in the environment or serum RNAses upon ingestion/absorption.

As consumer exposure to dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is not expected at significant levels, and as exposure to residues of dsRNA is not expected to represent a consumer safety concern, the identification of a marker residue was

not considered necessary. Similarly the establishment of a ratio of marker to total residues ratio was not considered necessary.

2.2.3. Monitoring or exposure data

No monitoring or exposure data other than that described elsewhere in this report are available.

2.2.4. Analytical method for monitoring of residues

A residue analytical method based on reverse transcriptase polymerase chain reaction (RT-PCR) with primers specific for dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV (IR primer, CP primer) was provided. The claimed RT-PCR limit of detection for IR dsRNA in 5 % honey was approximately 0.05 µg/ml (=50µg/l), which is equivalent to 1 µg/ml (=1 mg/l) of dsRNA in 100 % honey. The assay is essentially a qualitative assay and thus not suitable as residue monitoring method. However, as no maximum residue limits are proposed, the absence of a validated analytical method can be accepted.

2.2.5. Findings of EU or international scientific bodies

No evaluations by other international committees were available.

3. Risk management considerations

3.1. Potential effects on the microorganisms used for industrial food processing

The pharmacologically active material is not intended for dairy cattle and therefore potential effects in dairy products were not investigated. In addition, microbiological effects are not expected for this type of substance.

3.2. Other relevant risk management considerations for the establishment of maximum residue limits

Double stranded RNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is intended for feeding of bees only during months when no collection of honey for human consumption is taking place.

3.3. Elaboration of MRLs

The CVMP considers that, based on the general data provided relevant to the safety of nucleic acids, and based on the data generated with the proposed pharmacologically active material, maximum residue limits for dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV are not necessary for the protection of human health.

3.4. Considerations on possible extrapolation of MRLs

Article 5 of Regulation (EU) No 470/2009 requires consideration the possibility of extrapolating the recommended maximum residue limits to other species/food commodities. However, there is no established guidance providing scientific grounds for the extrapolation of MRLs from honey to other food

commodities. Furthermore, the substance under consideration has been developed for a very specific use in honey bees and use of the substance in other food producing species is not expected, as a result of which extrapolation of maximum residue limits to other food producing species cannot be supported on the basis of ensuring availability of veterinary medicines.

3.5. Conclusions and recommendation for the establishment of maximum residue limits

Having considered that,

- for nucleic acid, including dsRNA, a standard pharmacological/toxicological approach including the setting of an ADI is not appropriate;
- there is no inherent risk associated with oral ingestion of nucleic acids in general;
- the sequence of IAPV-specific dsRNA has been found by bioinformatic analysis to have no 100 % sequence homologies greater than 20 base pairs to human genes;
- as a result of the size and polar nature of RNA molecules, cell penetration is most likely very limited;
- lAPV-specific dsRNA are naturally occurring substances in bees and also honey as a result of IAPV infection;
- the dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is degraded relatively quickly in sucrose solution (bee feed) inside the hive, and therefore unconsumed bee feed is unlikely to retain the active ingredient over significant periods of time;
- in a limited residue study, residues of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV in honey from treated hives (at a dose of 30 mg of IAPV specific dsRNA) could not be detected with RT-PCR at concentrations as low as 1 µg/ml;
- in general, dsRNA is likely to be quickly degraded under acidic (pH less than 4) conditions and simulated experiments under both a gastric and a duodenal conditions indicate that dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV is degraded in the human gastrointestinal tract and, most likely, also in honey since honey commonly has a pH lower than 4;
- the establishment of a maximum residue limit for double stranded ribonucleic acid (dsRNA) homologous to coat protein (CP) and intergenic region (IR) of the Israel Acute Paralysis Virus (IAPV) in bees is not necessary for the protection of human health;

the Committee recommends the inclusion of dsRNA homologous to viral RNA coding for part of the coat protein and part of the intergenic region of the IAPV in table 1 of the Annex to Regulation (EU) No. 37/2010 as follows:

Pharmacologically active substance	Marker residue	Animal species	MRL	Target tissue	Other provisions	Therapeutic classification
Double stranded ribonucleic acid homologous to viral ribonucleic acid coding for part of the coat protein and part of the intergenic region of the Israel Acute Paralysis Virus	NOT APPLICABLE	Bees	No MRL required	Honey	NO ENTRY	NO ENTRY

4. Background information on the procedure

Submission of the dossier	29 October 2010
Steps taken for assessment of the substance	
Application validated:	9 November 2010
Clock started:	10 November 2010
List of questions adopted:	9 March 2011
Consolidated response to list of questions submitted:	14 January 2012
Clock re-started:	15 January 2012
CVMP opinion adopted:	13 April 2012
Commission request for reconsideration of opinion	9 July 2012
Revised CVMP opinion adopted:	13 September 2012