Toxicology of ryegrass endophyte in livestock

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Abstract

The ryegrass endophyte/plant interaction produces a number of toxic metabolites responsible for a range of toxicoses including ryegrass staggers. Although lolitrem B has long been considered the toxin responsible for ryegrass staggers in farm animals, it is only recently that we have demonstrated that oral administration of the pure toxin causes tremors in mice consistent with the symptoms of ryegrass staggers. Various levels of the toxin were incorporated into the diet of mice and tremor response measured regularly. Mice were very susceptible to the tremorgenic action of lolitrem B with a dose of only 1.1 mg/kg per day being sufficient to induce a tremor response. Analysis of faeces from a dosed mouse showed that approximately 40% of the toxin ingested was excreted unchanged. The profile of tremor response shows that tremors build up over a 24-hour period and then reach a dose dependent plateau suggesting that toxin turnover reaches a steady state. In experiments designed to test the possibility that tremorgens bind to specific receptors in the brain, mice were injected with the ^{14}C-labelled tremorgen, paxilline. Paxilline was chosen as it is similar in structure and possible action to lolitrem B and could be more easily prepared with an isotopic label. Following injection of ^{14}C-paxilline to mice their brains and spinal cord were sectioned and analysed for ^{14}C content. Localised binding was not detected but rather an even distribution of isotope was observed. This is probably due to the lipophilicity of the tremorgens, which distribute randomly throughout the fatty brain matrix, masking any binding to specific regions or receptors. This study showed, however, that only a minute proportion of the administered tremorgen reached the brain and spinal cord, indicating that, if this is the site of action, the receptors involved in the initiation of staggers are extremely sensitive to the tremorgens.

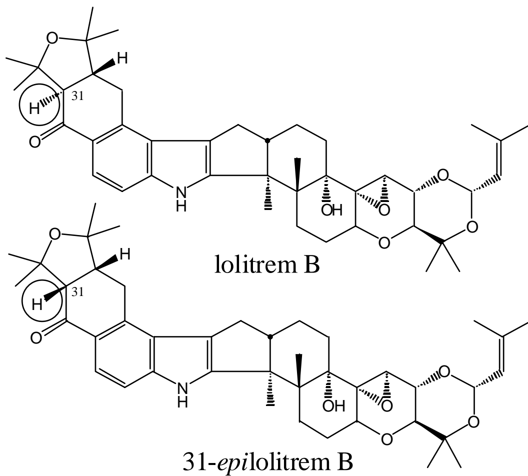
Keywords: endophyte, lolitrem, *Lolium perenne*, mycotoxin, *Neotyphodium lolii*, neurotoxin, oral dosing, ryegrass staggers, tremor, tremorgen

The toxins involved in ryegrass staggers

The toxin thought to be responsible for ryegrass staggers, lolitrem B, was first discovered and isolated in 1984 (Gallagher *et al.* 1984). Lolitrem B is present in endophyte-infected ryegrass in very small quantities. This has made isolation of lolitrem B extremely difficult and has severely hindered attempts to determine the mechanism of action of the tremorgen. Research into the mechanism of action of the toxin is very important since this information is needed if we are to take logical steps to prevent ryegrass staggers. Improved methods for the extraction and purification of lolitrem B have recently been developed which has allowed the isolation of larger quantities of toxin (Miles *et al.* 1994). Although improved, these methods are still very tedious with over 300 kg of seed being extracted for the isolation of only one gram of toxin. However, we now have enough pure toxin to probe the mechanism of action of lolitrem B.

In addition to the isolation of lolitrem B, the large-scale extraction of ryegrass seed allowed the isolation and structural elucidation of fifteen related compounds (Munday-Finch 1997). These compounds not only provided information on the likely pathways of lolitrem biosynthesis but also allowed us to identify a number of structural features that are necessary for the tremorgenic effect. A number of the new compounds were tested for tremorgenicity by injecting them into mice intraperitoneally and then measuring the tremor effect. The degree of tremor was expressed as a tremor score where a score of one represents an intermittent tremor when the animal is stressed through to a score of five which represents convulsions and death (Gallagher & Hawkes 1985; Gallagher & Hawkes 1986). The results of these experiments showed that for tremor to be generated a number of structural and steric requirements must be satisfied. For example, the only difference in structure between lolitrem B and 31-*epi*lolitrem B is the orientation of just one hydrogen atom (H-31) (Figure 1). This small change in structure, however, is enough to influence tremorgenicity, with 31-*epi*lolitrem B showing no effect when injected into mice at 4 mg/kg, a dose at which lolitrem B induces severe tremors (Munday-Finch *et al.* 1996). This is consistent with the involvement of a specific receptor in the initiation of ryegrass staggers.

Figure 1 Structures of lolitrem B and 31-*epi*lolitrem B



Studies of the receptor site

Like other tremor disorders, such as Parkinson's disease, ryegrass staggers is thought to be due to a change in the central nervous system (the brain and/or spinal cord). In a voluntary movement of muscle, an electrical signal is sent from the brain. The electrical signal is then propagated within nerves and transmitted to muscle via a chemical signal, called a neurotransmitter. It is believed that tremor syndromes are due to interference in neurotransmitter release where false chemical signals are generated. These signals are uncontrolled in timing and strength, so that the movement would not be voluntary but a series of involuntary contractions of the muscle, seen as a tremor or tetany. Neurotransmission involves very specific receptor binding sites on nerves and muscle, which act like a lock and key. It has been suggested that tremorgens are recognised as 'keys' by a specific receptor – the 'lock' (Munday-Finch 1997). When the key (lolitrem B) is bound to the lock (specific receptor) uncontrolled interference in neurotransmission may be invoked, resulting in tremor. If we could identify the site at which lolitrem B is acting, we could perhaps prevent its interaction with the receptor and could cure or prevent animals suffering from ryegrass staggers.

Locating and identifying the receptor involved in ryegrass staggers is a very complicated process, since there are many different types of receptor at many different sites. We really need to localise the exact site at which the toxin binds, since this would indicate the position and nature of the receptor. Although there are a number of techniques to tackle this problem, the process is complicated in the case of the tremorgens by their solubility. Lolitrem B is very insoluble in aqueous media, so it is difficult to apply the toxin in a solvent that is compatible with tissue preparations. It is very soluble in organic solvents and in lipid media, so is readily taken up in fatty materials. We did some initial studies in brain slices with lolitrem B that had been radioactively labelled. The label makes the toxin comparatively easy to detect and we hoped that the toxin would be selectively taken up in particular areas of the brain slice and thus indicate the receptor site. In fact, the toxin was taken up throughout the lipid-containing areas of the slice and no selective binding was observed. This confirmed that classic receptor binding studies could not be utilised in the study of tremorgen receptor sites.

We then moved to studies in the whole animal. This is the most relevant model since this is the natural situation and the animal will absorb the toxin and transport it to the target site. It was not possible to use radiolabelled lolitrem B for these experiments since the specific activity of the compound was not sufficient to detect the small quantities involved. As an alternative ^{14}C-labelled paxilline was used, a tremorgen similar in structure to lolitrem B. If a specific receptor site for paxilline could be located then unlabelled lolitrem B could be used to try and displace the labelled paxilline. This would provide information on whether lolitrem B and paxilline bind at the same receptor site. The ^{14}C-labelled compound was injected intraperitoneally into the mouse and at maximum tremor the animal was sacrificed and the brain taken and analysed for ^{14}C content. This was done by accelerated mass spectroscopy (AMS) (Vogel & Turteltaub 1992; Vogel & Turteltaub 1994) which allows for detection of minute amounts of ^{14}C. This experiment showed that the tremorgen was present in the brain but at extremely low levels suggesting that only a fraction of the actual dose of toxin is transported to the brain and its possible binding site.

After finding ^{14}C in the brain, the next step was to try and determine if it was localised to any particular area. The brain is made of a number of discrete areas each of which contain a particular array of receptors. Identification of the area where the tremorgen is binding would give valuable information on the type of receptor involved. ^{14}C-labelled paxilline was injected into the mouse and at peak tremor the animal was sacrificed. This time, however, the spinal cord, cerebellum, cerebrum, hippocampus, medulla and mid-brain were dissected out and each region analysed for ^{14}C content by AMS. This showed that although a specific receptor site is postulated in the initiation of ryegrass staggers, the toxin was deposited evenly throughout the different regions of the brain. Although not revealing any information about receptor sites these experiments

highlighted the minute quantities of tremorgen required at the active site to produce tremors.

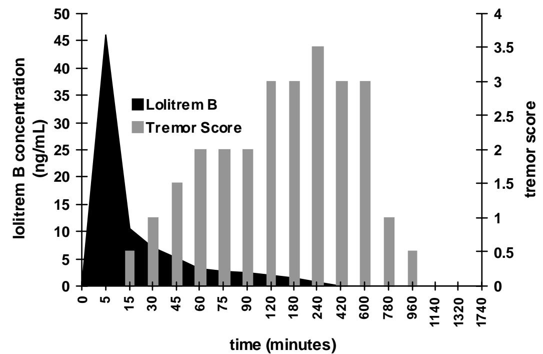
Since an analytical approach toward the identification of the receptor does not look promising, future studies will use a more pharmacological approach. Chemicals which specifically block certain receptor sites are known. The severity of tremor will be compared in animals dosed with the tremorgen alone, and those receiving both the tremorgen and the specific receptor agent or agonist. Difference in the severity of tremor observed after administering an agent will implicate particular receptors in ryegrass staggers. These approaches will be investigated in the near future.

Large animal dosing studies

Large animal dosing studies have focussed on method development. We have established methodologies to detect lolitrem B in animal serum, using ELISA and HPLC. Preliminary studies have been performed where sheep have been dosed intravenously with lolitrem B and the toxin concentration in serum and the severity of tremor followed closely. In these studies, 40–50 kg sheep were given 75 µg of lolitrem B per kg body weight and the lolitrem B levels in serum and the tremor score recorded over a 20-hour period.

The lolitrem B concentration in serum dropped rapidly from a peak of 44 ng/mL to less than 10 ng/mL within 15 minutes post dosing (Figure 2). There was a short lag time between the peak toxin concentration in serum and the detection of tremor, indicating a requirement for delivery to the active site, or conversion to a toxic principle. Tremors were present for 16 hours post dosing with the maximum tremor occurring four hours after the peak lolitrem B concentration in serum. Lolitrem B could no longer be detected in the serum after 7 hours, although the tremor score was still close to the maximum level. This suggests that the toxin may be resident elsewhere in the animal such as the fat, being released back into the blood at very low levels for transport to the active site. Alternatively, once at the active site lolitrem B could have a very long lasting effect. A similar experiment using paxilline showed a profile of toxin concentration consistent with that observed for lolitrem B. The tremor profile, however, was very different with paxilline showing a quicker onset of tremor, but a much shorter period of action. This meant for paxilline the profile of toxin concentration in blood was co-incident with the profile of tremor so that in contrast to lolitrem B, paxilline could be detected throughout the entire period of tremor. (Hawkes *et al.* 1995). It is not known why this difference in tremor profile occurs but it is most likely due to either a difference in the way the two toxins are transported and stored or possibly a requirement for metabolism of lolitrem B before its tremorgenic action is expressed. There is also structural evidence that suggests that the two toxins, although having similar modes of action may bind at different receptor sites (Munday-Finch *et al.* 1997; Munday-Finch 1997).

Figure 2 Serum lolitrem B concentration and tremor score vs time after intravenous dosing of lolitrem B to sheep (75 µg/kg).



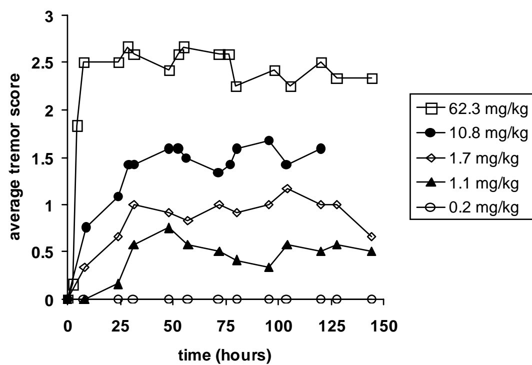
Oral dosing of lolitrem B to mice

We are also investigating the absorption, metabolism and excretion of lolitrem B. This provides an alternative approach toward the problem of ryegrass staggers, since if we can determine how lolitrem B is detoxified, we may be able to speed up detoxification or use this information in the selection of animals resistant to ryegrass staggers. For this, the toxin must be administered orally in order to replicate the normal route of toxin intake. Lolitrem B causes tremors when injected into animals but oral dosing has never previously been possible due to the difficulty of obtaining the toxin. The recent isolation of larger quantities of lolitrem B now allows oral dosing experiments to be performed. Lolitrem B at various levels was incorporated into the diet of mice. Mice were fed this diet for six days and food intake and degree of tremor measured regularly. The degree of tremor was expressed as a tremor score, as previously described.

The results of the dosing experiments (Figure 3) show a dose dependent response where at each dose level tremor builds up and then plateaus. This plateau shows that, rather than a cumulative effect over time, a steady state is reached where the toxin is presumably binding and dissociating at a constant rate. Once the mice were transferred to a control diet the severity of tremor slowly diminished until the mice appeared

normal. The time taken for the tremor to disappear was dependent on dose but in all cases it was within 48 hours of receiving the control diet. For a tremor to be observed a 25 g mouse required just 27.5 µg of lolitrem B per day (1.1 mg/kg per day). Analysis of faeces from a mouse fed the diet containing lolitrem B showed that a significant proportion (approximately 40%) of the toxin is excreted by the animal in an unmetabolised state. This result is in accord with the earlier observation that only very small amounts of toxin are present in the brain of a tremoring animal, and confirms that only minute quantities of toxin are required at the target site to induce tremors.

Figure 3 Tremor score vs time after the oral dosing of mice with various levels of lolitrem B.



Another interesting observation from these dosing experiments was that the mice were able to detect lolitrem B in their food at a level of 100 ppm or above. At these toxin concentrations, an inverse correlation was noted between toxin level and food intake which in turn correlated with a decrease in bodyweight throughout the experiment. Mice receiving control diet or a diet containing toxin levels of 10 ppm or below showed an increase in bodyweight during the experiment. In contrast, those receiving a diet containing toxin levels of 100 ppm and above showed a decrease in food intake and in bodyweight. When mice with low food intakes were switched to a control diet, they readily accepted the food, suggesting that it was not an effect of the lolitrem B which they had already eaten but rather that they could detect the presence of the toxin. This means that lolitrem B has an unpleasant taste or perhaps causes a reaction in the mouth such as a tingle on the tongue. This is of note, and will be investigated further as it may indicate a local action over and above any effect on the central nervous system.

This experiment is the first to dose pure lolitrem B to an animal orally and it has conclusively proved that the toxin can produce tremors by this route. Mice were used in this experiment, however, and these may show a different response to that of sheep. We therefore plan to use this approach of lacing food with lolitrem B to investigate the effect of the toxin on sheep.

ACKNOWLEDGEMENT

We thank Peter Roberts from Geological & Nuclear Sciences for the AMS measurements.

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