Review

Understanding triclabendazole resistance

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Abstract

Triclabendazole (TCBZ) has been the drug of choice to treat liver fluke infections in livestock for >20 years, due to its high activity against both adult and juvenile flukes. More recently, it has been used successfully to treat human cases of fascioliasis. Resistance to TCBZ first appeared in the field in Australia in the mid-1990s. Since then, resistance has been reported from a number of countries throughout Europe: Ireland, Scotland, Wales, Spain and The Netherlands. The heavy reliance on a single drug puts treatment strategies for fascioliasis at risk. Should resistance develop further, the prospect is an alarming one. This review will present an overview of progress in understanding the mechanism of resistance to TCBZ, examining possible changes in the target molecule, in drug influx/efflux mechanisms and in the metabolism of TCBZ by the fluke. The review will also consider ways to deal with resistance, covering drug-oriented options such as: the use of alternative drugs, drug combinations and the search for new compounds.
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Keywords: Triclabendazole; Fasciola hepatica; Resistance; β-Tubulin isotype; Molecular modeling; P-glycoprotein; Drug metabolism; Proteomics

Contents

Introduction

Triclabendazole (TCBZ) has been the drug of choice for treating liver fluke infections in livestock for over 20 years.

More recently, it has been used successfully to treat human cases of fascioliasis. Resistance to TCBZ first appeared in farm animals in Australia in the mid-1990s (Overend and Bowen, 1995) and since then has been reported in a number of European countries (Ireland, the UK, The Netherlands and Spain) (Fairweather, 2005). To date, no confirmed cases of TCBZ resistance have been documented in humans although the

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occurrence of such cases is likely to be a function of the level of resistance in the animals that provide a reservoir of fluke infections. Should resistance develop further in livestock, the heavy reliance on TCBZ to maintain productivity and animal health puts future treatment strategies at risk. This is particularly worrying given the recent rise in the prevalence and spread of the disease. Climate change has been blamed for this phenomenon, providing warm and moist conditions that favor the survival of the snail host (Mitchell, 2002). The aim of this short review is to summarize work being carried out to elucidate the mechanism of resistance. Different, yet parallel, approaches (molecular, morphological, pharmacological and proteomic) are being used by several laboratories to obtain a more complete picture of resistance. This is because any fasciolicide will interact with a number of different systems within the fluke and exert a variety of effects. An effect on one tissue or biochemical system is likely to have a knock-on effect on other systems and so it is difficult to determine whether fasciolicidal action is due to a single effect or a combination of effects. It is to be hoped that the review will illustrate the value of obtaining a more comprehensive view of drug action and resistance.

Mechanism of action

To understand how resistance to TCBZ may develop, it is necessary to understand the mechanism of drug action. TCBZ is a benzimidazole derivative and, by analogy with what is known about other benzimidazole drugs, it would be anticipated that TCBZ might bind to the β-tubulin molecule and so disrupt microtubule-based processes. Evidence in support of this idea has come from morphological studies on the tegument, vitellaria and testis, following treatment with the active sulphoxide metabolite (TCBZ.SO). For example, there is inhibition of mitosis in the vitelline and spermatogenic cells (Stitt and Fairweather, 1992, 1996); disruption of transport processes in the tegument (the outer layer of a trematode), which leads to progressively severe damage of the tegumental surface, culminating in the total loss of the tegument (Stitt and Fairweather, 1993, 1994). Loss of tubulin immunostaining in the tegumental syncytium has also been observed (Robinson et al., 2002; McConville et al., 2006) (Figs. 1, 2). The results suggest that the microtubules have disappeared which, in turn, would prevent the movement of secretory bodies from the cell bodies to the tegumental surface. This process is vital for the maintenance of the integrity of the surface membrane and its disruption would explain the severe morphological changes seen.

Mechanism of resistance

Investigations into the mechanism of resistance to TCBZ have used the Sligo isolate of *Fasciola hepatica*. This isolate has been shown to be resistant to the action of TCBZ *in vivo*, at both the adult and juvenile stages (Coles and Stafford, 2001; McCoy et al., 2005). Flukes from this isolate also resist the action of TCBZ.SO *in vitro*, even at abnormally high concentrations (Robinson et al., 2002).
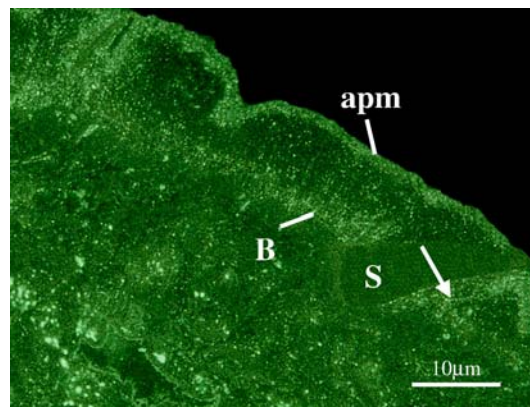

Fig. 1. Control. TCBZ-S flukes. There are strong bands of tubulin-IR beneath the apical plasma membrane (apm) and above the basal membrane (B). The syncytium in the vicinity of the spines (S) also displays strong fluorescence (arrow).

Mechanisms involved in the development of resistance to other anthelmintics can result from changes in the target molecule, in drug uptake/efflux mechanisms and in drug metabolism (Ouellette, 2001). With regard to changes in the target molecule, the target is presumed to be β-tubulin, but tubulin staining is not abolished by TCBZ.SO in the resistant isolate (Robinson et al., 2002; McConville et al., 2006) (Fig. 3). However, in nematodes benzimidazole resistance has been linked to selection of a β-tubulin isotype with a phenylalanine to tyrosine substitution at position 167 or at position 200 (Kwa et al., 1994; Prichard, 2001; Wolstenholme et al., 2004).

It is possible that TCBZ resistance may arise in the liver fluke from a similar β-tubulin isotype variant selection mechanism. Many eukaryotic species encode more than one β-tubulin isotype. Predicted polypeptide sequence comparisons reveal small variable regions between them, most notably at the C-terminus. To date, several α- and β-tubulin isotypes that are expressed in adult fluke have been identified and their coding regions have been fully sequenced (Ryan, unpublished observations). Comparison of the β-tubulin sequences from susceptible and resistant fluke isolates indicate that they contain the same amino acids at the positions implicated in nematode benzimidazole resistance. However, some amino acid differences have been noted at other positions but whether these amino acid changes are relevant to the resistant phenotype or are due to normal allelic variation in the genes encoding these isotypes remains to be determined and many more sequences from individual TCBZ-susceptible (TCBZ-S) and -resistant (TCBZ-R) flukes will need to be obtained. Studies are underway in both adult and juvenile fluke to identify the drug-sensitive isotypes by localizing the sites of expression of the various α- and β-tubulin isotypes, and thus determining which isotypes are expressed in areas that are severely disrupted following TCBZ treatment.

At the molecular level, structural studies have shown that the residues that are variable in benzimidazole-resistant organisms (6, 50, 134, 165, 167, 198, 200 and 257) are brought together to form a cluster during the folding of the β-tubulin protein (Downing, 2000). These studies also indicated that the cluster of "sensitive" residues was not on the surface of the molecule (Fig.
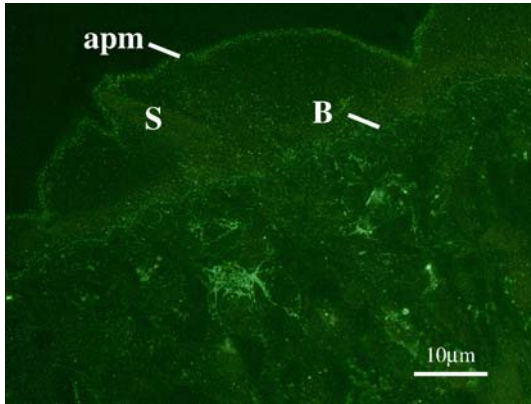
Fig. 2. TCBZ-S fluke, treated with TCBZ.SO (15 μg/ml, 24 h). General lack of tubulin-IR in the syncytium, though bands of weak fluorescence are present below the apical plasma membrane (apm) and above the basal lamina (B). S, spine.

4), raising the question of how could the drug access this region? Molecular modeling studies using β-tubulin sequences from the liver fluke and the nematode *Haemonchus contortus* have been used to propose a solution (Robinson et al., 2004a). By analogy to the bacterial tubulin homologue FtsZ (Nogales et al., 1998) the angle between the N-terminal, intermediate and C-terminal domains of β-tubulin was relaxed by 11°. This increased the surface area of the potential benzimidazole binding cleft sufficiently for albendazole to be "docked" in this region. Mammalian and liver fluke tubulins presented a smaller region for binding, commensurate with the restricted effects of benzimidazole in these organisms. It was proposed that the resistance-conferring mutations at residues 200 and 167 were effective as they allowed the formation of hydrogen bonds "closing off" the binding pocket. The model also suggests that benzimidazoles act not by causing the de-polymerization of microtubules, but by locking the β-tubulin moieties in the "open" conformation and thus interfering with the formation of heterodimers with α-tubulins prior to microtubule formation.

The entry of TCBZ into the fluke has been shown to occur mainly by diffusion across the tegumental syncytium rather than by oral ingestion (Mottier et al., 2006). The diffusion of both
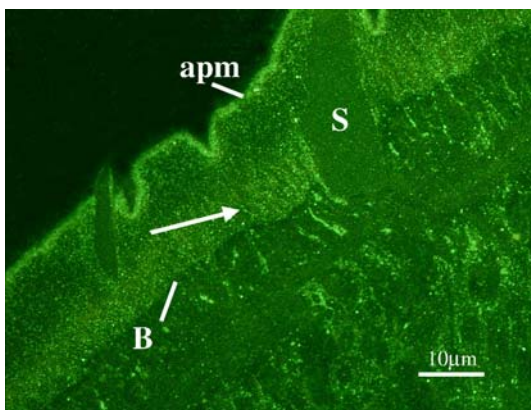

Fig. 4. Ribbon diagram of porcine β-tubulin (1 jff) showing residues associated with benzimidazole resistance. Those residues identified by site-directed mutation are highlighted and numbered in red (H6, Y50, Q134, N165, F167, E198, Y200 and M257) and those derived from photoaffinity labeling experiments are shown in orange (L65 and F81). The three β-tubulin domains are colored as follows: N-terminal—purple, intermediate—green and C-terminal—blue (modified from Robinson et al., 2004a). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

TCBZ and TCBZ.SO into TCBZ-R (Sligo) flukes is significantly lower than in TCBZ-S (Cullompton) flukes (Alvarez et al., 2005; Mottier et al., in press) (Fig. 5). Interestingly, this is not true for the related benzimidazole, albendazole whose uptake is similar in both TCBZ-S and TCBZ-R fluke (Mottier et al., in press). The results suggest that the mechanism is specific to TCBZ and that P-glycoprotein-linked drug efflux pumps could potentially be involved in the resistance mechanism. Overexpression of Pgp has been linked to resistance in nematodes to different classes of anthelmintics (Kerboeuf et al., 2003; Wolstenholme et al., 2004). Experiments with Pgp inhibitors have shown that it is possible to "reverse" the condition of the flukes, from resistant to susceptible. For example, co-incubation

Fig. 3. TCBZ-R fluke treated with TCBZ.SO (15 μg/ml, 24 h). Tubulin-IR is present as a band running beneath the apical plasma membrane (apm) and also throughout the syncytium (arrow). S, spine; B basal lamina.
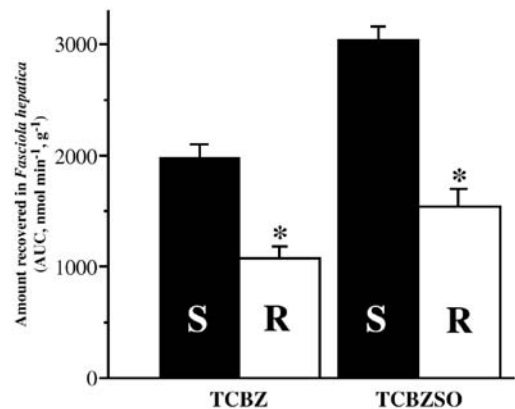

Fig. 5. Uptake of TCBZ and TCBZ.SO by TCBZ-susceptible (S) and –resistant (R) flukes. *$P < 0.05$ (modified from Mottier et al., in press).

with ivermectin decreased the efflux of TCBZ and TCBZ.SO in TCBZ-R flukes such that the drug was present at levels comparable to those in TCBZ-S flukes (Mottier et al., in press) (Fig. 6).

In contrast, ivermectin had no impact on the uptake of albendazole in either TCBZ-S or -R flukes (Mottier et al., in press). The consequence of Pgp inhibition in TCBZ-R fluke has been demonstrated in a separate morphological study with another Pgp inhibitor, R(+)-verapamil. Co-incubation of R (+)-verapamil plus TCBZ.SO led to severe disruption of the tegument of TCBZ-R flukes (Figs. 7, 8), whereas treatment with TCBZ.SO on its own (even at a high concentration) caused minimal changes to the tegumental surface (Savage, unpublished observations). The disruption to the resistant fluke was comparable to that observed in susceptible flukes following treatment with TCBZ.SO (Stitt and Fairweather, 1993). While a change in efflux pump activity may simply represent a non-specific mechanism, nevertheless, it is likely to play a significant role in the development of resistance.

The identification and localization of the Pgp-linked efflux pumps have yet to be determined. Studies using a laser micro-dissection protocol have provided small quantities of specific fluke tissues for Pgp localization. Tegument, gut and reproductive structures have been isolated and probed with a Pgp-specific primer. The results obtained to date are inconclusive and many more specimens need to be examined.

With regard to a role for altered drug metabolism in TCBZ resistance, the sulphoxidation of TCBZ to TCBZ.SO and TCBZ. SO to the sulphone metabolite (TCBZ.SO2) are both greater in TCBZ-R than -S flukes (Alvarez et al., 2005; Robinson et al., 2004b, respectively) (Fig. 9). Indeed, TCBZ-R flukes have a 39% greater capacity to metabolize the parent drug (Alvarez et al., 2005). Use of inhibitors has shown that the flavin-mono-oxygenase (FMO) enzyme system is the main pathway for the metabolism of TCBZ, and it is more important than the cytochrome P450 enzyme system (Alvarez et al., 2005). Moreover, methimazole (MTZ, an FMO inhibitor) had a significantly greater inhibitory impact on TCBZ sulphoxidation in TCBZ-R than -S flukes (43% as against 34%) (Fig. 9). By comparison, the
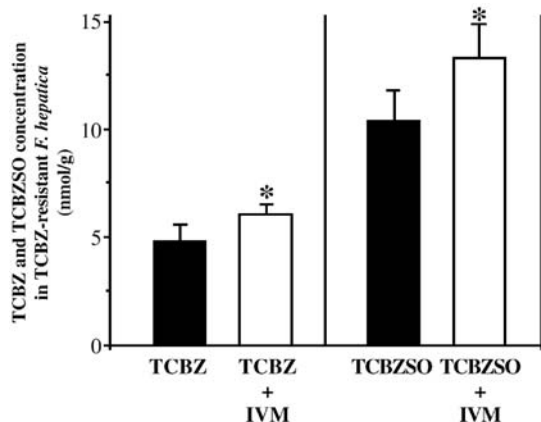

Fig. 7. TCBZ-R fluke treated with TCBZ.SO (15 µg/ml) and verapamil (1×10^{-4} M) for 24 h. The surface tegument has been removed exposing the underlying basal lamina. (OS) Oral sucker, (G) gonopore, (VS) ventral sucker. Scale bar, 100 µm.

cytochrome P450 inhibitor, piperonyl butoxide reduced TCBZ. SO formation to a lesser extent and the inhibition was equal (at 12%) in the two isolates (Alvarez et al., 2005) (Fig. 9).

Proteomics and TCBZ resistance

The first published experimental parasite proteomics study showed that it was possible to identify major proteins from *F. hepatica* by mass spectrometry without a completed genome programme (Jefferies et al., 2001). Global proteomics, with the support of an EST project (www.sanger.ac.uk/Projects/S_mansoni/), have identified a number of soluble proteins over-produced during drug exposure in a TCBZ-R-resistant (Sligo) fluke isolate compared to a TCBZ-S (Cullompton) isolate (Fig. 10). For example, HSP-70 was only over-produced in resistant flukes following TCBZ.SO exposure in culture. These proteins protect cells from cellular stress by binding to partially denatured proteins and preventing aggregation, suggesting that there are also successful downstream drug response mechanisms in TCBZ-R flukes. A proteomic-based assay for the major phase II detoxification system, glutathione S-transferase (GST), in adult *F. hepatica* has been developed using two affinity matrices and MS-MS QTOF peptide sequencing, and will provide a sub-proteomics approach to investigate GST superfamily involvement in TCBZ metabolism and resistance.

Fig. 6. Decreased efflux of TCBZ and TCBZ.SO in TCBZ-resistant flukes following co-incubation with ivermectin. *$P < 0.05$ (modified from Mottier et al., in press).
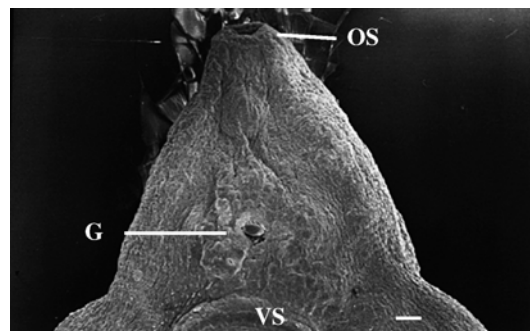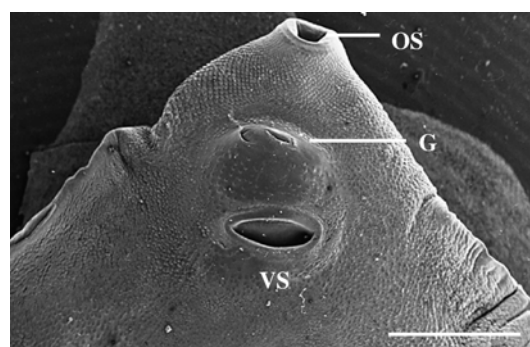

Fig. 8. TCBZ-R fluke treated with TCBZ.SO (15 µg/ml) for 24 h. The surface tegument shows no disruption. (OS) Oral sucker, (G) gonopore, (VS) ventral sucker. Scale bar, 1 mm.
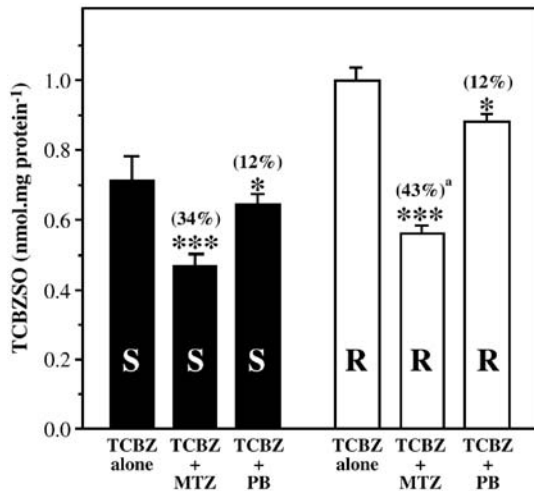
Fig. 9. Metabolism of TCBZ in TCBZ-susceptible (S) and -resistant (R) fluke microsomes and effects of methimazole (MTZ) and piperonyl butoxide (PB) on the formation of TCBZ.SO. Values in parentheses express percentage reduction in sulphoxidative activity by the inhibitors. Values significantly different compared to TCBZ alone at (*) $P<0.05$ and (***) $P<0.001$. [a]Significant differences from that in TCBZ+MTZ-treated TCBZ-S fluke at $P<0.05$ (modified from Alvarez et al., 2005).

Development of resistance to anthelmintics in parasites can lead to a reduction in fitness, but this has not been proven to be the case for a TCBZ-R (Oberon) isolate. It was faster to (egg) hatch, faster to produce cercariae, produced more cercariae, was more infectious for rats and was faster to patency than a TCBZ-S (Fairhurst) isolate (Walker et al., 2006). The maintenance of fecundity would prevent any reversion to drug susceptibility, an observation supported by field data (Borgsteede et al., 2005). The more rapid patency of TCBZ-R flukes in rats has been confirmed in sheep (comparison between the Sligo (TCBZ-R) and Cullompton (TCBZ-S) isolates: McCoy, unpublished

observations). Such data may need to be taken into account in the design of control programs against fluke infections.

Conclusions

It is clear that our understanding of the mechanism of resistance to TCBZ remains far from complete. At a more basic level, the identity of the target molecule for TCBZ action has yet to be fully established. If it is tubulin, then the binding site needs to be characterized so that the significance of any mutations in tubulin isotypes can be determined. This would open the way for the development of molecular tests for assessing the true extent of TCBZ resistance in the field. Some progress in the use of this technology has been made with nematode infections (von Samson-Himmelstjerna, 2006). As regards the mechanism of TCBZ resistance, work to date indicates that altered drug uptake and metabolism may be more important than any changes to the presumed target (namely, tubulin). Reduced drug uptake, followed by faster drug metabolism, would severely limit the amount of TCBZ.SO reaching its target. Much remains to be learned about the identity and expression of efflux pumps and the role of metabolic pathways. It is important to obtain such information, as it opens up the possibility of modulating drug activity in the host by the use of appropriate inhibitors (Alvarez et al., 2006). In turn, this would help to maintain the efficacy of TCBZ in the face of developing resistance.

What the review has shown is the value of avoiding the pitfall of adopting a single approach to examine a particular parameter. Sadly, too many *in vitro* studies have been carried out with drug concentrations too high to have any real bearing on drug action *in vivo*. Even when there appears to be an outstanding case for a single action, drug action is more likely to result from a number of interdependent processes and we should not jump into conclusions that may prove to be false. Research into mecha-
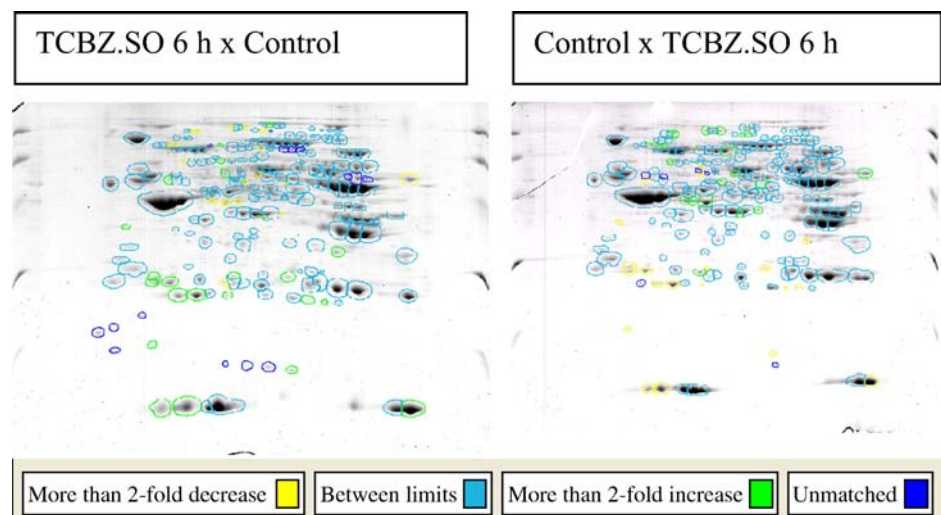

Fig. 10. Comparative proteomics of the soluble proteins of TCBZ-R (Sligo) and TCBZ-S (Cullompton) isolates by global 2DE. Flukes were cultured for up to 6 h with 0–50 μg/ml of TCBZ.SO, and 1 mg of the *F. hepatica* protein was precipitated with 20% TCA/acetone, solubilized in IEF buffer (40 mM Tris–Cl pH 9.6, 7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes, 50 mM DTT) for 1 h and centrifuged at 20,000×*g*. Proteins were resolved by 2DE using 17 cm pH 3–10NL BIO-RAD strips and 12% SDS-PAGE, with gel staining with Coomassie Blue. Gels were analyzed using Phoretix Progenesis PG220, version 2005 (1742.7) (Nonlinear Dynamics Ltd.), with three gels from each isolate being used to build average gels. Spots were cut from the gels, digested with trypsin and peptides sequenced by MS-MS Q-TOF and proteins identified via an in-house *F. hepatica* database.

nisms of drug action and resistance should be evidence driven, not from a pre-conceived view of what the action might be, and that can only be achieved by a more enlightened and holistic approach to experimentation.

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