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Research paper

A GAB Aergic anxiolytic-*like* trait of thymoquinone, possibly *via* diazepam-co-agonistic pathway

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Abstract

This study evaluated the possible anxiolytic potential of thymoquinone (TQ), a promising component of Nigella sativa oil in male Swiss mice under unstressed and stressed conditions. A short-term physical stress (STPS) was produced by using an electric shaker. A new apparatus named as 'swing apparatus' was selected to carry out this study. TQ was tested at doses of 5, 10 and 20 mg/kg. In order to understand possible action mechanism, a GABA receptor agonist and an antagonist were used in this study. As agonist diazepam (DZP), while antagonist flumazenil (FZL) was used at 2 and 2.5 mg/kg doses. Treatments were given intraperitoneally. The brain GABA levels were determined by UV-spectrophotometric method. The results suggest that, TQ dose-dependently exerted a significant ($p<0.05$) anxiolytic effect in rodents, which was confirmed by the calming effect of the experimental animals in comparison to the vehicles' NC, DZP and FZL groups. An augmentation of the movements of the animals was observed in STPS applied groups. GABA levels in the mouse brain were also found to link with the behavioral activity, where TQ co-treated with DZP augmented the calming effect by decreasing GABA levels, which was reversed by the FZL pre- and post-treated groups, demonstrating possible co-agonistic and antagonistic effects with DZP and FZL, respectively. Therefore, this study suggests an involvement of GABAergic, possibly DZP-co-agonistic anxiolytic-*like* effect of TQ.

Keywords: *Anxiety; GABA; Thymoquinone; Swing Apparatus.*

1. Introduction

Anxiety disorders are one of the neuropsychiatric diseases with a high cost of treatment. Anxiety causes a considerable amount of muscle tension, leading to both muscle cramping and spasms. Moreover, it causes considerable excess in adrenaline, thus the exciting effect in the nervous system. Generally, anxiety is the activation of the fight-flight system, known to cause dehydration via sweating and urination. Moreover, in anxiety the body experiences severe stress, where it uses the nutrients in the muscles and bones, like magnesium, which is a known element for proper nerve functioning. In fact, anxiety is not only a leading cause of spasm, but also several other neurological disorders such as generalized anxiety disorder (GAD), social anxiety disorder (SAD), obsessive-compulsive disorder (OCD), panic disorder (PD) and post-traumatic stress disorder (PTSD) (Griebel and Holmes 2013).
Benzodiazepines (BDZs), such as chlordiazepoxide and diazepam (DZP) are the most commonly used drugs in the treatment of anxiety disorders, mainly GAD and PD. Despite the wide use of these medicines, BDZs may cause some dangerous side effects, notably, an impairment in the cognition and coordination, ataxia, sedation and dependence (Dell'osso and Lader 2013). Thus, a search of new, safe and effective anxiolytic drugs is crucial. Natural products derived from plants have been gained an attention by these days. It is due to their promising biological activities (Islam et al. 2016a, b).
Thymoquinone (2-isopropyl-5-methylbenzo-1,4-quinone) (TQ), the vastly studied bioactive constituent of the black cumin (*Nigella sativa*) is evident to have various promising biological effects such as antioxidant, anti-inflammatory, antimicrobial, anticancer, immunomodulatory, antidiabetic and antifibrosis. It also has beneficial effects in oral hygene, metabolic disorders, reproductive and respiratory tract disorders, bone formation and bone decay. Furthermore, TQ has neuro-, gastro-, cardio-, hepato- and nephroprotective capacity. In some recent studies, it has been suggested that, TQ has antinociceptive, anxiolytic-like and anticonvulsant effects in rodents. TQ-mediated beneficial effects on memory and learning improvement are also reported (Islam et al. 2016c).
However, economy in laboratory research by reducing the number of experimental animals with reliable through output always is desirable. This study is shot out to evaluate a possible mechanism of action of TQ anxiolytic effect in Swiss mice by proposing an easy and rapid test protocol using a new and hopeful neuropharmacological study tool, swing apparatus. Additionally, the brain GABA levels were also quantified by using a spectrophotometric analysis.

2. Materials and methods

2.1. Reagents and chemicals

Corn oil was used as a vehicle (negative control; NC) for TQ, while normal saline (0.9% NaCl solution) for the standards, diazepam (DZP) and flumazenil (FZL). All the necessary reagents and chemicals were purchased from (Sigma-Aldrich Chemical Co., St. Louis, MO, USA.).

2.2. Experimental animals

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Male adult Swiss mice (*Mus musculus*) of approximately 2 months old, weighing between 25 and 30 g, were provided by the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b). The animals were kept in a controlled environment (temperature: 26 ± 2 °C; humidity 50 ± 5%; 12 h dark/light cycle; food Purina® pellets and filtered water: ad libitum). All of them were then acclimated for four days prior to the tests commenced. Experimental protocol was approved by the Ethics Committee in Animal Experimentation of Southern University Bangladesh (No. 99903/16).

2.3. Sample preparation

TQ was dissolved in above mentioned vehicle to attain doses at 5, 10 and 20 mg/kg, while DZP and FZL at 2 and 2.5 mg/kg, respectively.

2.4. Study design by using swing apparatus

The description such as design, work principle and mode of operation was already described in a previous study (Islam et al. 2014). In this study, total, forty five male Swiss mice were used (five in each group). At first, the animals were treated as unstressed manner, where, in the first zone, 25 mice were randomly divided into 5 groups like- NC (normal saline: 10 ml/kg); DZP (2 mg/kg) and three groups for TQ (5, 10 and 20 mg/kg). The treatments were given intraperitoneally (i.p.). After 10 minutes, each animal was placed inside the swing box and number of swings was counted manually for 3 minutes. The swing box was cleaned before placing a new animal with 70% ethanol solution.

In the second zone, 15 mice were treated with FZL (2.5 mg/kg, i.p.). After 15 minutes, two groups were isolated as FZL + DZP (2.5 mg/kg + 2 mg/kg, i.p.) and FZL + TQ (2.5 mg/kg + 20 mg/kg, i.p.). The animals were then placed individually in the swing box for 3 minutes after a 10 minute interval.

Finally, 5 mice were treated with DZP (2 mg/kg, i.p.) prior to an administration of TQ (200 mg/kg, i.p.) for further confirmation of the possible anxiolytic effect of TQ. In this occasion, DZP was administered 10 minutes prior to the TQ treatment. After 10 minutes, the number of swings of each animal was counted and then followed by an administration of FZL (2.5 mg/kg, i.p.) and counting again the number of swings after 15 minutes as above.

For stressed manner, unstressed animals were immediately placed in an acrylic box and subjected for a short-term physical stress (STPS) on an electronic shaker for 30 seconds (90 shakes/min). In this case, mice treated as unstressed groups (already recorded their swings), after a 5 minute cutoff time were treated to STPS.

2.5. GABA quantification in mice brain

The process was followed by a rapid removal of mice brain, weighing and transferring to ice-cold trichloroacetic acid solution to obtain a 10% w/v constitution, which was then homogenized and centrifuged at 10,000 X g for 10 min at 0 ^{O}C. then, 0.1 mL of brain tissue extract was added to 0.2 mL of 0.15 M ninhydrin solution in a 0.5 M carbonate-bicarbonate buffer (pH 9.95) and incubated in a water bath at 60 ^{O}C for 30 min. after cooling, the mixture was treated with 5 mL of copper tartrate reagent (0.16% di-sodium carbonate, 0.03% copper sulfate and 0.03% tartaric acid) (Lowe et al., 1958). Absorbance was measured at 360 nm in a spectrophotometer (Lu et al. 2010). The results were expressed as brain GABA levels (µg/g of wet brain tissue).

2.6. Statistical analysis

The results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using one-way ANOVA for multiple comparisons, followed by *t*-Student–Newman–Keuls as a post-hoc test by using GraphPad Prism (version: 6.0) Software (GraphPad San Diego, California, USA. Copyright © 1994-1999) considering $p<0.05$ at 95% confidence level.

3. Results

According to the Table 1 it is clear that, TQ significantly ($p<0.05$) and dose-dependently reduced the number of swings in unstressed mice, where the highest reduction was observed in TQ 20 mg/kg group. The standard DZP at 2 mg/kg also reduced the number of swings significantly ($p<0.05$) as compared to the NCs and FZL (2.5 mg/kg) groups. The effect of TQ was found more prominent at the two higher doses (10 and 20 mg/kg) than the DZP (2 mg/kg) group. Both DZP (2 mg/kg) and TQ (20 mg/kg) treated after 15 minutes of FZL (2.5 mg/kg) administration, significantly ($p<0.05$) reduced the number of swings in the experimental animals in comparison to the NCs and FZL groups. The reduction in the number of swings was also observed in the DZP (2 mg/kg) + TQ (20 mg/kg) and DZP (2 mg/kg) + TQ (20 mg/kg) + FZL (2.5 mg/kg) groups as compared to the DZP (2 mg/kg), FZL (2.5 mg/kg) and FZL (2.5 mg/kg) + DZP (2 mg/kg) groups.

Table 1: Number of Swings after the Treatments of Test Sample and Controls in Unstressed Animals

Treatments (i.p.)	Number of swings in 3 minutes
NC1 (saline, 10 ml/kg)	26.60 ± 2.70
NC2 (saline + corn oil: 1:1, 10 ml/kg)	25.20 ± 2.95
DZP (2 mg/kg)	20.60 ± 2.96^{a,b,d}
TQ (5 mg/kg)	22.00 ± 2.74^{a,b,d}
TQ (10 mg/kg)	18.40 ± 1.82^{a,b,c,d}
TQ (20 mg/kg)	13.40 ± 2.19^{a,b,c,d}
FZL (2.5 mg/kg)	25.00 ± 2.55
FZL (2.5 mg/kg) + DZP (2 mg/kg)	22.20 ± 2.95^{a,b,d}
FZL (2.5 mg/kg) + TQ (20 mg/kg)	20.40 ± 1.67^{a,b,d}
DZP (2 mg/kg) + TQ (20 mg/kg)	15.80 ± 2.49^{a,b,c,d}
DZP (2 mg/kg) + TQ (20 mg/kg) + FZL (2.5 mg/kg)	16.80 ± 2.39^{a,b,c,d}

Values are mean ± SD (n = 5); NC: negative control (vehicle); DZP: diazepam; FZL: flumazenil; TQ: thymoquinone; $^{a}p<0.05$ compared to the NC1 (saline), $^{b}p<0.05$ compared to the NC2 (saline + corn oil); $^{c}p<0.05$ compared to the DZP; $^{d}p<0.05$ compared to the FZL (ANOVA followed by *t*-Student-Neuman-Keuls as a post hoc test).

Table 2 suggests a similar motion in an activity with an augmented number of swings in each group. However, in this occasion, the group co-treated with TQ (20 mg/kg) and DZP (2 mg/kg) was found to reduce the number of swings by 5.49% and 25.22% in comparison to the TQ (20 mg/kg) and DZP (2 mg/kg) groups, respectively. This group, when treated with FZL, again increased in number of swings above DZP (2 mg/kg) + TQ (20 mg/kg), but below FZL (2.5 mg/kg).

Table 2: Number of Swings after the Treatments of Test Sample and Controls in Stressed Animals

Treatments (i.p.)	Number of swings in 3 minutes
NC1 (saline, 10 ml/kg)	28.40 ± 2.96
NC2 (saline + corn oil: 1:1, 10 ml/kg)	27.20 ± 1.82
DZP (2 mg/kg)	23.00 ± 2.55^{a,b,d}
TQ (5 mg/kg)	22.40 ± 1.67^{a,b,d}
TQ (10 mg/kg)	20.60 ± 2.39^{a,b,c,d}
TQ (20 mg/kg)	18.20 ± 3.21^{a,b,c,d}
FZL (2.5 mg/kg)	28.40 ± 3.53
FZL (2.5 mg/kg) + DZP (2 mg/kg)	25.60 ± 2.49^{a,b,d}
FZL (2.5 mg/kg) + TQ (20 mg/kg)	24.40 ± 2.10^{a,b,d}
DZP (2 mg/kg) + TQ (20 mg/kg)	17.20 ± 2.95^{a,b,c,d}
DZP (2 mg/kg) + TQ (20 mg/kg) + FZL (2.5 mg/kg)	19.40 ± 2.96^{a,b,c,d}

Values are mean ± SD (n = 5); NC: negative control (vehicle); DZP: diazepam; FZL: flumazenil; TQ: thymoquinone; $^{a}p<0.05$ compared to the NC1 (saline), $^{b}p<0.05$ compared to the NC2 (saline + corn oil); $^{c}p<0.05$ compared to the DZP; $^{d}p<0.05$ compared to the FZL (ANOVA followed by *t*-Student-Neuman-Keuls as a post hoc test).

Table 3: Effect of Treatments on Brain GABA Levels in Unstressed and Stressed Animals

Treatments (i.p.)	Brain GABA levels (µg/g of wet brain tissue extract)	
	Unstressed	Stressed
NC1 (saline, 10 ml/kg)	376 ± 5.49	367 ± 2.19
NC2 (saline + corn oil: 1:1, 10 ml/kg)	381 ± 4.73	373 ± 2.13
DZP (2 mg/kg)	451 ± 2.38^{a,b,d}	421 ± 3.08^{a,b,d}
TQ (5 mg/kg)	398 ± 4.00^{a,b,d}	363 ± 4.76^{a,b,d}
TQ (10 mg/kg)	430 ± 3.82^{a,b,c,d}	407 ± 3.59^{a,b,c,d}
TQ (20 mg/kg)	486 ± 3.19^{a,b,c,d}	454 ± 5.59^{a,b,c,d}
FZL (2.5 mg/kg)	379 ± 3.54	354 ± 3.34
FZL (2.5 mg/kg) + DZP (2 mg/kg)	459 ± 3.95^{a,b,d}	438 ± 3.35^{a,b,d}
FZL (2.5 mg/kg) + TQ (20 mg/kg)	450 ± 4.00^{a,b,d}	431 ± 4.31^{a,b,d}
DZP (2 mg/kg) + TQ (20 mg/kg)	476 ± 5.63^{a,b,c,d}	440 ± 4.00^{a,b,c,d}
DZP (2 mg/kg) + TQ (20 mg/kg) + FZL (2.5 mg/kg)	415 ± 2.19^{a,b,c,d}	394 ± 3.39^{a,b,c,d}

Values are mean ± SD (n = 5); NC: negative control (vehicle); DZP: diazepam; FZL: flumazenil; TQ: thymoquinone; ^{a}p<0.05 compared to the NC1 (saline), ^{b}p<0.05 compared to the NC2 (saline + corn oil); ^{c}p<0.05 compared to the DZP; ^{d}p<0.05 compared to the FZL (ANOVA followed by *t*-Student-Neuman-Keuls as a post hoc test).

Figure 1 is the demonstration for the without antagonistic treatments of the unstressed and stressed animals. Not only, DZP (2 mg/kg), but also TQ (at all the test doses) significantly reduced the number of swings in the experimental animals in comparison to the NCs groups. However, TQ at 10 and 20 mg/kg were found to decrease in a number of swings better than TQ lowest dose (5 mg/kg) and DZP (2 mg/kg). An increased in a number of swings was observed in all the stressed groups.

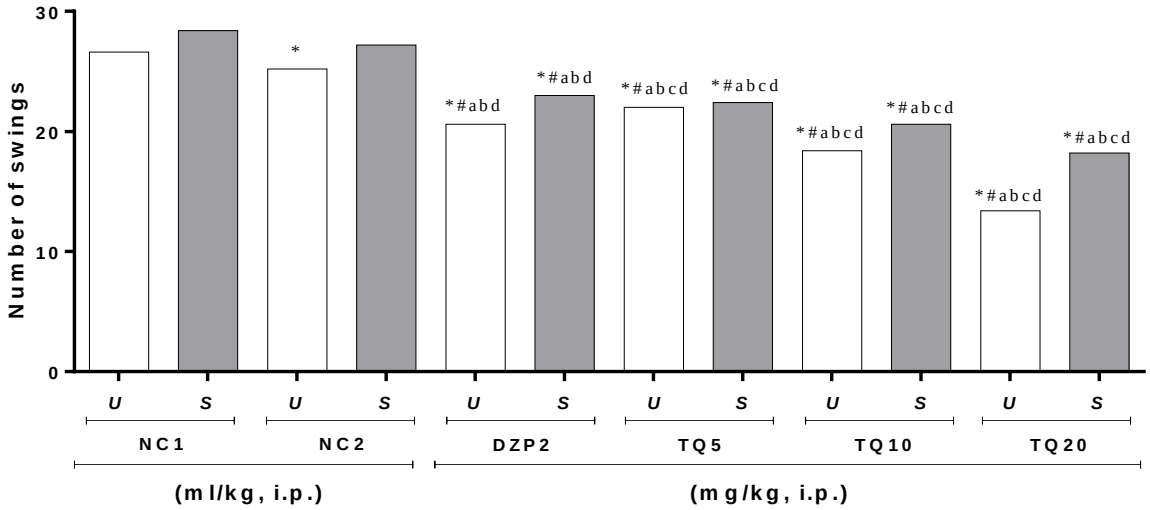


Fig. 1: Number of Swings in the Unstressed (U) and Stressed (S) Groups in Absence of an Antagonist. [NC: Negative Control (Vehicle); DZP: Diazepam; TQ: Thymoquinone; Mean Swings (N = 5) are Plotted Against Treatments; */#Compared to the Stressed/Unstressed NC Groups; ^{A}p<0.05 Compared to the NC1 (Saline), ^{B}p<0.05 Compared to the NC2 (Saline + Corn Oil); ^{C}p<0.05 Compared to the DZP; ^{D}p<0.05 Compared to the FZL (ANOVA followed by *t*-Student-Neuman-Keuls as a post hoc test).].

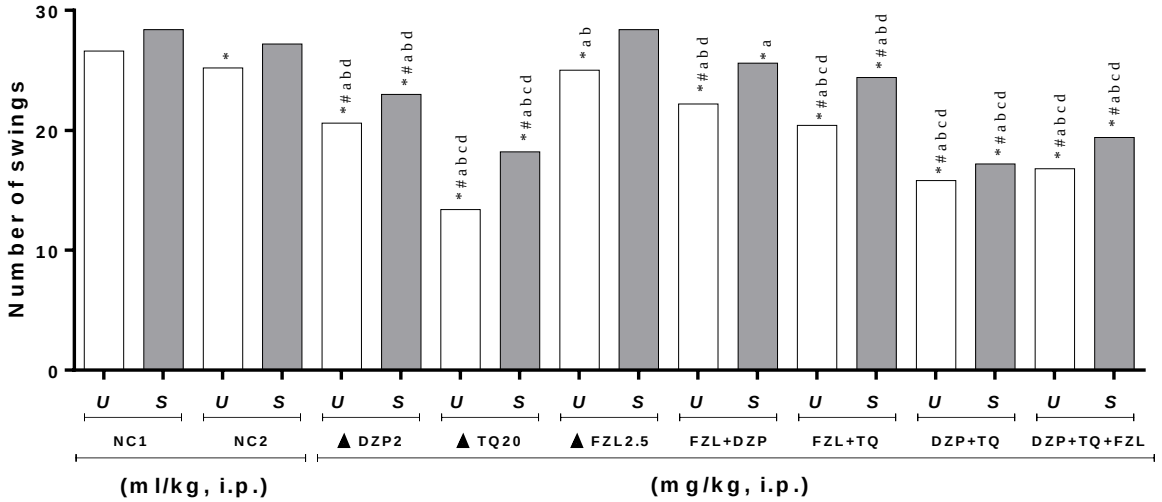


Fig. 2: Number of Swings in the Unstressed (U) and Stressed (S) Groups in the Presence of an Antagonist. [NC: Negative Control (Vehicle); DZP: Diazepam; FZL: Flumazenil; TQ: Thymoquinone; Mean Swings (N = 5) are Plotted Against Treatments; Δ Used in Combined Treatment; */#Compared to the Stressed/Unstressed NC Groups; ^{A}p<0.05 Compared to the NC1 (Saline), ^{B}p<0.05 Compared to the NC2 (Saline + Corn Oil); ^{C}p<0.05 Compared to the DZP; ^{D}p<0.05 Compared to the FZL (ANOVA followed by *t*-Student-Neuman-Keuls as a post hoc test).].

Figure 2 depicts a comparison between the groups treated with or without FZL (2.5 mg/kg) is saying that, TQ (20 mg/kg), DZP (2 mg/kg) + TQ (20 mg/kg) and DZP (2 mg/kg) + TQ (20 mg/kg) + FZL (2.5 mg/kg) groups significantly ($p<0.05$) reduced the number of swings in the experimental animals as compared to the NCs, DZP and FZL groups. Although, a more reduction in a number of swings was observed in TQ (20 mg/kg) group, but TQ (20 mg/kg) co-treated with DZP and DZP + FZL was found to reduce the number of swings in animals than those of the DZP and FZL alone treated groups.

4. Discussion

Diazepam, the most commonly prescribed one of the BDZs for the treatment of anxiety, is a positive allosteric modulator of gamma-aminobutyric acid type A receptor ($GABA_A$), a ligand-gated chloride-selective ion channel that is activated by GABA, the major inhibitory neurotransmitter within the brain. Binding of BDZ with this receptor complex promotes binding of GABA, which increases the total transfer of chloride ions (Cl^-) across the neuronal cell membrane. This increased Cl^--influx hyperpolarizes the neuron's membrane potential. As a result, the difference between the resting and threshold potentials is increased, and firing is less likely. The $GABA_A$ receptor is a heteromer composed of five subunits (two αs, two βs, and one γ; α2β2γ). For each subunit, many subtypes exist (α1–6, β1–3, and γ1–3). $GABA_A$ receptors containing the α1 subunit mediate the sedative, anterograde amnesic, and partially the anticonvulsant effect of DZP. $GABA_A$ receptors containing α2 mediate the anxiolytic actions and, to a large degree, the myorelaxant effects (Tan et al. 2014). On the other hand, FZL is a $GABA_A$ receptor antagonist, which antagonizes central nervous system (CNS)-acting drugs affecting GABAergic neurons via BDZ receptor (Rye et al. 2012).

In this present study, both DZP and TQ are evident to exert a calming effect to the rodents, while FZL reversed the situation. The FZL increased in number of swings in comparison to the TQ and DZP treatments. Both DZP (2 mg/kg) and TQ (20 mg/kg) reduced the number of swings in the FZL pre-treated animals. It seems a re-calming effect by the TQ and DZP. A more reduction in the number of swings was observed in TQ (20 mg/kg) unstressed group. However, a reduction in number of swings in DZP + TQ and FZL + TQ as compared with DZP and DZP + TQ + FZL, respectively, may be due to its agonistic effect with DZP while antagonistic with FZL. This can be supported by the calming effect with DZP + TQ and the further increased in movements with the administration of FZL.

A study conducted by Gilhotra and Dhingra (2011) also suggested that, TQ dose-dependently reduced the brain GABA levels, thus increased in locomotor activity in rodents. This study supports their findings. They were also demonstrated a possible GABAergic anxiolytic-like effect of TQ. This study, conducted by using an agonist (DZP) and antagonist (FZL) of the BDZ-receptor suggesting that, TQ showed a DZP-like effect. An increased in number of swings of the unstressed rodents may be due to the reduction in GABA in their brain, thus the increased of the movements of the animals in the swing box. Furthermore, a reduction from the number of swings was observed in the DZP pre-treated group (DZP + TQ) as compared to the DZP alone treated group. This may be due to the co-agonistic effect of TQ with DZP.

5. Conclusion

Accumulated data suggest that, the TQ dose-dependently exerted an anxiolytic-like effect in Swiss mice. A more calming effect was observed in unstressed animals with TQ two highest doses (10 and 20 mg/kg). More movements into the swing box by the STPS-induced animals may be linked to the decreased in GABA levels in their brain. TQ exerted calming effect to the experimental animals with DZP, while FZL reversed the situation, demonstrating possible co-agonistic and antagonistic effects with DZP and FZL, respectively. Both DZP and FZL are evident to act via $GABA_A$ergic pathway, thus the anxiolytic-like effect of TQ may be plugged in the same pathway. Finally, this study might conclude that, TQ mediated anxiolytic-like effect in Swiss mice may be via a GABAergic co-agonistic pathway. Thus, TQ may be a good target in anxiety, either alone or in a combination with other BDZs agonist, especially with DZP. However, further researches are welcomed to explore the exact mechanism of TQ.

6. Conflict of interest

I have no conflict of interest from any single point of view.

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