INTERACTION OF ARTEMISININ AND TETRACYCLINE OR ERYTHROMYCIN AGAINST *PLASMODIUM FALCIPARUM* IN VITRO

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Summary :

Antimalarial activities of tetracycline (TC) and erythromycin (EM), alone or in combination with artemisinin (Qinghaosu, QHS), were studied using chloroquine (CQ)-sensitive (D6) and -resistant (W2) strains of *Plasmodium falciparum* in vitro. The antimalarial potency of TC (IC₅₀ = 9862 nM for the CQ-sensitive parasite, 32414 nM for the CQ-resistant one) or EM (IC₅₀ = 45787 nM for the CQ-sensitive parasite, 33397 nM for the CQ-resistant one) was much less than that of QHS (IC₅₀ ranging from 25 to 40 nM). The CQ-resistant falciparum parasite displayed a cross-resistance to TC, while both the drug-sensitive and -resistant parasites exhibited similar responses to EM. However, antimalarial potency of EM appeared to be less than that of TC against the drug-sensitive parasite. When TC was combined with QHS, an additive interaction was observed against the CQ-sensitive falciparum parasite, while synergism was found with the CQ-resistant parasite. When EM was tested in combination with QHS, a potentiating interaction occurred with both the CQ-sensitive and -resistant falciparum parasite. The above results indicated that the QHS combination with either TC or EM may be a promising antimalarial preparation with low recrudescence compared to artemisinin used alone in clinical practice.

KEY WORDS : artemisinin, tetracycline, erythromycin, *Plasmodium falciparum*, antimalarials, antibiotics, drug combination.

Résumé : INTERACTION DE L'ARTÉMISININE ET DE LA TETRACYCLINE OU L'ÉRYTHROMYCINE SUR *PLASMODIUM FALCIPARUM* IN VITRO

Les activités antimalariques de la tétracycline (TC) et de l'érythromycine (EM) seules ou associées avec l'artémisinine (Qinghaosu, QHS) ont été évaluées in vitro sur deux souches de *Plasmodium falciparum*, une souche chloroquino-sensible (CQ-S) D6, et une chloroquino-résistante (CQ-R), la souche W2. Les propriétés antimalariques observées avec TC (IC₅₀ = 9862 nM CQ-S, 32414 nM CQ-R) ou EM (IC₅₀ = 45787 nM CQ-S, 33397 nM CQ-R) respectivement sont beaucoup plus faibles que celles observées avec QHS (IC₅₀ = 25 à 40 nM). La souche CQ-R montre une résistance croisée avec TC alors que les deux souches ont la même sensibilité vis-à-vis de EM. Toutefois les propriétés antimalariques de EM sont plus faibles que celles de TC vis-à-vis de la souche CQ-S. Lorsque TC est associé avec QHS, un effet additif est observé vis-à-vis de la souche CQ-S; avec la souche CQ-R, il se produit une synergie. L'association EM plus QHS provoque une synergie sur les deux souches étudiées. Ces résultats montrent que l'artémisinine associée avec soit la tétracycline soit l'érythromycine pourraient se révéler être des associations antimalariques provoquant peu de rechutes en comparaison à ce qui est observé avec l'artémisinine lors de son utilisation clinique.

MOTS CLÉS : artémisinine, tétracycline, érythromycine, *Plasmodium falciparum*, antimalariques, antibiotiques, association thérapeutique.

INTRODUCTION

Artemisinin (Qinghaosu, QHS), isolated from a Chinese medicinal herb, *Artemisia annua*, is a new antimalarial drug characterized by a rapid onset of therapeutic effects against this infection (Klayman, 1985). Since chloroquine (CQ) resistant falciparum malaria has spread rapidly world-wide, QHS presents a serious challenge to the commonly-used drug chloroquine (Jiang *et al.*, 1982; Anon 1982a). Three derivatives of QHS, artemether, artesunate and arteether are currently undergoing clinical trials. In spite of their potency and low toxicity, recrudescence (return of the infection) is intolerably high in patients

receiving QHS or QHS-like drugs (Li *et al.*, 1984). The recrudescence probably results from the short half-life of QHS and its derivatives in plasma (Anon, 1982b). Recently, in order to overcome the recrudescence, work has been directed towards studying a combination of QHS or its derivatives with other antimalarial compounds. Some combinations of QHS with antimalarial drugs, for instance, QHS + CQ, or QHS + pyrimethamine, antagonize each other (Chawira and Warhurst, 1987), while others are potentiating and found very promising. The potentiating action of QHS and mefloquine or tetracycline (TC) both *in vitro* and *in vivo* was reported (Chawira and Warhurst 1987; Chawira *et al.*, 1987). Previously, we demonstrated a potentiation of QHS and bisbenzylisoquinolines such as tetrandrine or berbamine against falciparum parasite *in vitro* (Ye *et al.*, 1989; Ye *et al.*, 1993).

In this present study, we explored an interaction of QHS and erythromycin (EM), as well as QHS and TC against *Plasmodium falciparum* in vitro. Although the combination of EM and other antimalarial drugs have

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been extensively investigated (Khan *et al.*, 1991; Gingras and Jensen 1993; Gingras and Jensen 1992), its combination with QHS, to our knowledge, has not yet been reported. Although a potentiating synergism in the combination of QHS and TC was shown using NF54 (CQ-sensitive) and K1 (CQ-resistant) strains of falciparum parasite (Chawira and Warhurst, 1987), different responses among the various parasitic isolates to an antibiotics or its combination were reported (Khan *et al.*, 1991; Gingras and Jensen, 1992; Gershon and Howells, 1984; Reacher *et al.*, 1981; Phillips *et al.*, 1984; Raichowdhuri and Gajanana, 1984). Therefore, West Africa strain (D6, a CQ-sensitive) and Indochina strain (W2, CQ-resistant) were used in our experiments to see if there are any difference between different strains of *P. falciparum* parasites in interaction between QHS and TC.

MATERIALS AND METHODS

CULTURES AND TESTS :

Two strains of *Plasmodium falciparum* were used in these experiments : one is the CQ-sensitive West Africa (D6) strain and the other is the CQ-resistant Indochina (W2) strain (Martin *et al.*, 1987). Both of them, kindly donated by Dr. Robert Miller, Walter Reed Army Institute of Research, were cultured in the complete medium of RPMI 1640 according to the candle jar method (Jensen and Trager, 1977).

Artemisinin (QHS) is a gift from Dr D.L. Klayman of the Walter Reed Army Institute of Research. A stock solution of QHS (10^{-3} M) was prepared by dissolving it in dimethylsulfoxide (DMSO) because of its insolubility in water. Before adding to the culture, this stock solution was diluted with RPMI 1640 medium without serum to yield a solution with drug concentration of 10^{-4} M. After the drug solution was sterilized by filtering it using a $0.22 \mu\text{m}$ cellulose acetate membrane, further serial dilutions of the drug were prepared in the complete medium of RPMI 1640 containing 10% pooled human A+ serum, 25 mM HEPES, and 25 mM NaHCO_3 . Preliminary experiments showed that final DMSO concentrations of 0.2% or less had no detectable effect on parasite growth. TC hydrochloride and EM, purchased from Sigma Chemicals, were respectively dissolved in RPMI 1640 medium to produce 10^{-2} M solution, and sterilized and diluted in a similar manner to QHS.

For a given experiment, 4-day-old Petri dish cultures with a 5-10% parasitemia were diluted with medium containing 25% non-parasitized human erythrocyte type A+ to obtain a culture with a final hematocrit of 1.5% and a parasitemia of 0.5-1.0%.

Antimalarial activity was assessed using the method of Desjardins and co-workers (1979), except that tritiated adenosine was substituted for tritiated hypoxanthine as a precursor for parasite nucleic acids (Ye *et al.*, 1987). Briefly, the final volume (250 μl) in each well of a 96-well microtiter plate consisted of : (1) 25 μl of complete medium with or without drug or drug combination; and (2) 200 μl of the parasitized cultures or non-parasitized human erythrocytes as a control. After incubation of the plates in a candle jar for 24 hours at 37°C , 25 μl of $[2,8\text{-}^3\text{H}]$ adenosine (0.5 μCi) with a specific activity of 59.20 Ci/mMole was added to each well. The plates were then incubated at 37°C for an additional 18 hours. At the end of the incubation, the contents in each well were harvested onto fiberglass filter disks using a Bellco semi-automated cell harvester. The filters were rinsed with distilled water six times and each disk was placed in a scintillation vial with 5.0 ml Cytoscinttm liquid scintillation fluid. Radioactivity was counted using a Packard Tri-Carb Model 1900 CA scintillation spectrometer.

DRUGS' INTERACTION :

In order to examine any interaction between the two drugs, the compounds were mixed in fixed ratios as described by Martin and colleagues (1987). Three concentrations of TC or EM, i.e., 1×10^{-4} , 2×10^{-4} and 3×10^{-4} M, were mixed with QHS at concentrations of 3×10^{-7} , 2×10^{-7} and 1×10^{-7} M, respectively. Ratios of TC or EM/QHS in concentration were, respectively, 1000:3, 1000:1 and 3000:1, which were designated as Combination 1, 2, and 3. A series of dilutions (continuous one to one dilution) were made respectively from Combination 1, 2, and 3. Antimalarial efficacies of the Combination 1, 2, and 3, as well as QHS, TC, or EM alone were assessed using Desjardins' method. Based on inhibitory percentage-concentrations of the drug combinations, IC_{50} values of an individual drug in the Combination 1, 2 and 3 were calculated using probit analysis. Sum of fractional inhibitory concentration (SFIC) of the drug combination was calculated based on its IC_{50} value ($\text{SFIC} = \text{IC}_{50}$ of one drug in the combination/ IC_{50} of the corresponding drug alone + IC_{50} of the other drug in the combination/ IC_{50} of the corresponding drug alone) and then isobolograms were constructed as described by Berenbaum (1978). Usually, the interaction of two drugs in combination exhibits three possibilities : antagonism, addition, and synergism. This is illustrated by the following example: $1+1 < 2$ (antagonism), $1+1=2$ (addition), and $1+1 > 2$ (potentiation or synergism).

Duplicate wells were prepared for each drug concentration and experiments were repeated a total of three times.

RESULTS

As compared to QHS, antimalarial potency of TC or EM is very low (See Table I and III). It seems that CQ-resistant strain of *P. falciparum* exerts cross resistance to TC, because IC₅₀ of TC for the resistant parasite is significantly higher ($p < 0.05$) than its IC₅₀ for the sensitive one. However, responses of both the sensitive and resistant falciparum parasites to EM were similar, e.g., IC₅₀ values: 45787 vs 33397 nM (See Table III). EM is less potent than TC in antimalarial activity against the drug sensitive parasite based on comparison of their IC₅₀ values: 45787 vs 9862 nM (See Tables I and III), while, with the CQ-resistant parasite, the two antibiotics appeared to be similarly potent (See Tables I and III).

When TC or EM was combined with QHS, IC₅₀ values of QHS in the combination 1, 2, and 3 are significantly lower than that of QHS alone, regardless of which strain of falciparum parasite was used. And decrease of QHS's IC₅₀ value was observed with increase of TC or EM dose in the combination. An additive interaction between TC and QHS was found in the drug sensitive parasite because SFIC values in the combination of TC and QHS are close to 1.0, while a synergistic action in the combination occurred in the drug resistant parasite with SFIC values significantly less than 1.0 (See Table II), which was confirmed by isobolograms in Fig. 1, namely, the isobologram of the drug combination against the CQ-sensitive parasite is very close to the control line (a diagonal line in Fig. 1), displaying addition, and the isobologram for the CQ-resistance is concave, indicating synergism.

However, when EM was combined with QHS, interaction between EM and QHS against both the sensitive and the resistant strains of falciparum parasite was observed to be synergistic. As shown in Table IV, all the SFIC values in the combination of EM and QHS are less than 1.0. In addition, the shape of isobolograms for both strains of falciparum parasites in Fig. 2 are concave, indicating the potentiating interaction occurred in the combination.

DISCUSSION

Many studies on antimalarial activity of antibiotics, especially TC and EM, have been done, but neither TC nor EM alone can be developed into a single antimalarial drug because of its weak efficacy and slow onset of antimalarial action. Our data in this study also demonstrated that TC or EM alone cannot be used as a single antimala-

rial drug because their antimalarial potency is too low as compared to that of QHS. There is from several hundred to over one thousand fold difference between TC or EM and QHS in IC₅₀ values. Probably, their slow and weak antimalarial action may result from their slow uptake and low accumulation in the parasitized red cells (Geary *et al.*, 1988). Therefore, it is unlikely that TC or EM itself could be developed into a single antimalarial drug.

However, a combination of TC or EM and other antimalarials is a practical way to develop a new antimalarial preparation for treating and preventing the CQ-resistant falciparum malaria because the antibiotics were not only able to increase antimalarial efficacy of other drugs (Gingras and Jensen, 1993; Gershon and Howells, 1984; Gershon and Howells, 1986; Noeypatimanond *et al.*, 1983; Watt *et al.*, 1992), but also delay emergence of resistance of falciparum parasite to an individual drug in the combination (Puri and Dutta, 1989). Owing to these advantages of the combination of the antibiotics and other antimalarials, various combinations of TC or EM with other antimalarial drugs such as quinine, CQ, and amodiaquine have been investigated. Up to now, the combination of quinine and TC is a standard regimen for treatment of the resistant falciparum malaria (Watt *et al.*, 1992; Looareesuwan *et al.*, 1992). However, attenuation of the parasite sensitivity to the combination of quinine and TC evoked development of a combination of EM and other antimalarials (Looareesuwan *et al.*, 1992). In addition, TC cannot be used for both children under 8 and pregnant women (White, 1983). Therefore, there is an urgent need for an alternative combination like EM and other antimalarials. EM was shown to have antimalarial activity *in vitro* (*P. falciparum*) (McColm and McHardy, 1984; Geary and Jensen, 1983) and *in vivo* (*P. berghei* and *P. knowlesi*) (Warhurst *et al.*, 1976; Warhurst *et al.*, 1983) and a potentiating synergism was manifested in its combination with CQ (Gershon and Howells, 1984; Warhurst *et al.*, 1976). However, the combination of EM and CQ or quinine failed to treat falciparum malaria in clinical trials (Phillips *et al.*, 1984; Pang *et al.*, 1985; Brandling-Bennett *et al.*, 1988). It seems that the combination of EM with 4-aminoquinolines should not be developed into an antimalarial preparation based on current data available.

Owing to the failure of the EM combination with 4-aminoquinolines in clinical trials and the attenuation of the TC combination with quinine to the CQ-resistant falciparum malaria, a combination of QHS or its derivatives such as artemether and artesunate with other antimalarials including antibiotics have been explored (Jiang *et al.*, 1982; Li *et al.*, 1984; Shwe *et*

Single Drug		Drug Combination ^b		
TC (1-100 μ M)	QHS (0.001-0.1 μ M)	TC (300 μ M) Q HS (0.1 μ M)	TC (200 μ M) QHS (0.2 μ M)	TC (100 μ M) QHS (0.3 μ M)
S 9862 $\pm$ 3777	37.3 $\pm$ 8.7	9441 $\pm$ 456(TC) 3.2 $\pm$ 1.5(QHS)	6996 $\pm$ 2669(TC) 7.0 $\pm$ 2.7(QHS)	4609 $\pm$ 542(TC) 13.9 $\pm$ 1.6(QHS)
R 32414 $\pm$ 4017	40.2 $\pm$ 8.2	14754 $\pm$ 5162(TC) 4.9 $\pm$ 1.7(QHS)	12378 $\pm$ 4261(TC) 12.4 $\pm$ 4.3(QHS)	5659 $\pm$ 3123(TC) 17.0 $\pm$ 9.4(QHS)

Table I. – IC₅₀(nM) of Tetracycline and Artemisinin for Each Drug Alone and in Combination^a

a. The data in the table above are the mean values $\pm$ S.D. (nM) from three experiments; TC and QHS represent tetracycline and artemisinin, respectively; S and R represent CQ-sensitive and resistant strains of *P. falciparum*.

b. Ratios of TC/QHS in the drug combinations are 3000:1, 1000:1, and 1000:3, respectively.

Parasite ^b	SFIC ^c		
	300 μ M(TC) 0.1 μ M(QHS)	200 μ M(TC) 0.2 μ M(QHS)	100 μ M(TC) 0.3 μ M(QHS)
S strain	1.03 $\pm$ 0.18	0.92 $\pm$ 0.15	0.90 $\pm$ 0.16
R strain	0.57 $\pm$ 0.15	0.69 $\pm$ 0.19	0.60 $\pm$ 0.22

Table II. – Effect of Combination of Tetracycline and Artemisinin on *P. falciparum*^a

a. The data in the table above are the mean values $\pm$ S.D from three experiments.

b. S and R strains represent CQ-sensitive and resistant strains of *P. falciparum*.

c. SFIC represents sum of fractional inhibitory concentration. SFIC is equal to one in cases of additive effects of the drugs, higher than one in cases of antagonism, and lower than one in synergistic action.

	Single Drug		Drug Combination ^b		
	EM (1-100 μ M)	QHS (0.001-0.1 μ M)	EM (300 μ M) QHS (0.1 μ M)	EM (200 μ M) QHS (0.2 μ M)	EM (100 μ M) QHS (0.3 μ M)
S	45787 $\pm$ 28248	25.1 $\pm$ 7.2	17160 $\pm$ 8234(EM) 5.7 $\pm$ 2.7(QHS)	12164 $\pm$ 6092(EM) 12.2 $\pm$ 4.1(QHS)	4955 $\pm$ 3907(EM) 14.9 $\pm$ 1.2(QHS)
R	33397 $\pm$ 9639	26.2 $\pm$ 1.1	17163 $\pm$ 718(EM) 5.7 $\pm$ 0.2(QHS)	12165 $\pm$ 5065(EM) 12.2 $\pm$ 5.1(QHS)	4616 $\pm$ 675(EM) 13.9 $\pm$ 2.0(QHS)

Table III. – IC₅₀ (nM) of Erythromycin and Artemisinin for Each Drug Alone and in Combination^a

a. The data in the table above are the mean values $\pm$ S.D. (nM) from three experiments; EM and QHS represent erythromycin and artemisinin, respectively; S and R represent CQ-sensitive and resistant strain of *P. falciparum*.

b. Ratios of EM/QHS in the drug combinations are 3000:1, 1000:1, and 1000:3, respectively.

Parasite ^b	SFIC ^c		
	300 μ M(EM) 0.1 μ M(QHS)	200 μ M(EM) 0.2 μ M(QHS)	100 μ M(EM) 0.3 μ M(QHS)
S strain	0.69 $\pm$ 0.17	0.82 $\pm$ 0.10	0.69 $\pm$ 0.16
R strain	0.76 $\pm$ 0.11	0.83 $\pm$ 0.09	0.67 $\pm$ 0.10

Table IV. – Effect of Combination of Erythromycin and Artemisinin on *P. falciparum*^a.

a. The data in the table above are the mean values $\pm$ S.D from three experiments. EM and QHS represent erythromycin and artemisinin respectively.

b. S and R strains represent CQ-sensitive and resistant strains of *P. falciparum*.

c. SFIC represents sum of fractional inhibitory concentration. SFIC is equal to one in cases of additive effects of the drugs, higher than one in cases of antagonism, and lower than one in synergistic action.

al., 1988; Shwe *et al.*, 1989; Win *et al.*, 1992; Looreesuwan *et al.*, 1992). Although QHS is a new antimalarial with rapid onset and relatively low toxicity, its clinical application has been limited because of its high recrudescence, probably due to short half life in the human. In order to circumvent its recrudescence, a combination of QHS with other antimalarial drugs may be a practical solution. Some experiments *in vitro* and *in vivo* in laboratories have demonstrated that the combinations of QHS with other antimalarial drugs such as mefloquine, tetrandrine, berbamine, and TC may be promising candidates for new antimalarial preparations which may attenuate recrudescence of QHS. Actually, the combinations of QHS and mefloquine or QHS and TC significantly decrease the recrudescence as compared to QHS used alone in clinical trials (Jiang *et al.*, 1982; Li *et al.*, 1984; Sy *et al.*, 1993). Probably, this is because potentiating interaction in those combinations can enhance antimalarial efficacy of QHS so much that a residual parasite could not escape being attacked by QHS, and the drugs above mentioned in the combination with QHS have a relatively longer half-life in the human, which may compensate for short half-life of QHS causing the recrudescence.

Using D6 and W2 strains of falciparum parasite, synergistic or additive interaction occurred in the present study when QHS was combined with TC (See Table II and Fig. 1). The data obtained from these experiments confirmed therapeutic effectiveness of the combination of QHS and TC as a new antimalarial regimen for treatment of patients infected with either the CQ-sensitive or resistant falciparum parasite. Although QHS exhibits relatively low toxicity, it can produce bone marrow depression or cardiovascular adverse effects (Anon, 1982c). Another advantage of the drug combination is to be able to decrease the dose of QHS, which itself would lead to less side effects. As shown in Table I, the dose of QHS in combination with TC is significantly lower than that of QHS alone based on a comparison of their IC₅₀ values. Therefore, the combination of QHS with TC can not only increase total antimalarial efficacy so that the recrudescence may be overcome, but also lead to possible lower toxicity of QHS. Therefore, it is necessary to conduct in various worldwide locations clinical trials on the QHS combination with TC on a large scale in order to evaluate the therapeutic importance of the combination.

In these experiments, we also revealed potentiating synergism in the combination of EM and QHS against both the drug sensitive and resistant strains of parasite, indicating EM might be useful in combination with QHS as an alternative antimalarial preparation, since QHS is quite different from 4-aminoquinolines

in chemical structure and in antimalarial mechanism (Klayman, 1985; Ye *et al.*, 1983; Lee *et al.*, 1988). Unlike TC, the drug resistant parasite in our experiments did not possess a cross resistance to EM, although the antimalarial potency of EM against the drug sensitive parasite is less than TC. However, some data obtained from another laboratory demonstrated higher antimalarial potency of EM than TC (McColm and McHardy, 1984). In addition, EM has properties of being inexpensive, readily available, and not having deleterious effects during odontogenesis, so that it can be used safely in the treatment of pregnant women and young children. Therefore, this combination warrants clinical trial as well as a further investigation using different isolates of falciparum parasite.

Mechanistic experiments revealed that TC and EM exert their antimalarial effects by inhibiting protein synthesis in microorganisms, and especially, they are potent inhibitors on protein synthesis in mitochondrion (Blum *et al.*, 1984; Divo *et al.*, 1985; Prapunwattana *et al.*, 1988; Kiatfuengfoo *et al.*, 1989). Similarly, QHS was found to have remarkable attack on mitochondrion in the parasite (Jiang *et al.*, 1985). Also, protein synthesis in the parasite was proven to be probably the earliest and main target of antimalarial action by QHS (Gu *et al.*, 1983). Usually, synergism occurs when the two drugs in a combination attack different chain of the same biochemical pathway. Presumably, because of possible similarity in mechanism by which TC or EM, and QHS exert their antimalarial activity, synergistic effects were found in the combination of TC or EM and QHS against the CQ-resistant falciparum parasite and in the combination of EM and QHS against sensitive one. However, we cannot explain why the combination of TC and QHS was found to be an additive interaction against the CQ-sensitive parasite. Usually, an additive interaction manifests itself when the targets of the two drugs in a combination are independent of each other in biochemical pathways. If this is true, it is implied that some biochemical pathways, especially those related to protein synthesis in mitochondria, have been changed when the parasite is developed into the resistant one.

Owing to the fact that the CQ-resistant strain of *P. falciparum* exhibits a cross-resistance to TC in this investigation, this makes it more difficult to explain the reason why the drug resistant parasite is refractory to respond to TC, but the combination of TC and QHS possess synergism against the drug resistant parasite. However, a study using some isolates of falciparum parasite other than D6 or W2 strain revealed that TC had similar antimalarial potency against both the sensitive and the resistant strains of *P. falciparum*

Fig. 1 – Isobolograms of interaction between artemisinin and tetracycline against CQ-sensitive (S) and CQ-resistant (R) strains of *Plasmodium falciparum* in vitro.

Fig. 2 – Isobolograms of interaction between artemisinin and erythromycin against CQ-sensitive (S) and CQ-resistant (R) strains of *Plasmodium falciparum* in vitro.

(Chawira and Warhurst, 1987). The fact that various isolates of the drug-resistant falciparum parasite have different responses to an individual drug leads to a consideration that the mechanism by which different isolates of the parasite are developed into the resistance to CQ is somehow different.

In sum, the QHS combination with TC is a promising candidate as an alternative antimalarial preparation for treatment of malarial infections, especially the CQ-resistant falciparum malaria. Also, the QHS combination with EM needs further investigation both in laboratories and in clinical trials.

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