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Phosphorylation of Wheat Germ Initiation Factors and Ribosomal Proteins[1]

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ABSTRACT

The ability of the wheat germ initiation factors and ribosomes to serve as substrates for a wheat germ protein kinase (Yan and Tao 1982 J Biol Chem 257: 7037–7043) has been investigated. The wheat germ kinase catalyzes the phosphorylation of the 42,000 dalton subunit of eukaryotic initiation factor (eIF)-2 and the 107,000 dalton subunit of eIF-3. Other initiation factors, eIF-4B and eIF-4A, and elongation factors, EF-1 and EF-2, are not phosphorylated by the kinase. Quantitative analysis indicates that the kinase catalyzes the incorporation of about 0.5 to 0.6 mole of phosphate per mole of the 42,000 dalton subunit of eIF-2 and about 6 moles of phosphate per mole of the 107,000 dalton subunit of eIF-3. Three proteins (M_r = 38,000, 14,800, and 12,600) of the 60S ribosomal subunit are phosphorylated by the kinase, but none of the 40S ribosomal proteins are substrates of the kinase. No effects of phosphorylation on the activities of eIF-2, eIF-3, or 60S ribosomal subunits could be demonstrated *in vitro*.

Protein phosphorylation has been shown to play a role in the regulation of protein synthesis. In reticulocyte lysates, both initiation factors and the ribosomal proteins serve as substrates for protein kinases (9, 17). Two protein kinases isolated from rabbit reticulocytes, one regulated by hemin (HCR) and the other by double stranded RNA (DSI), catalyze the phosphorylation of eIF-2[2]. Both of these kinases, which are cyclic AMP-independent, phosphorylate the α subunit of eIF-2. While the hemin-regulated enzyme is specific for eIF-2, the dsRNA-dependent protein kinase also phosphorylates histones (9). The phosphorylation of the α subunit of eIF-2 is correlated with inhibition of protein synthesis; however, the exact mechanism of this inhibition is still under investigation (6, 9, 12).

Rychlik *et al.* (11) isolated from wheat germ a protein kinase (M_r = 20,000) that phosphorylates two polypeptides (M_r = 32,000 and 76,000) present in preparations of unwashed wheat germ ribosomes. Salt-washed ribosomes are no longer a substrate for the kinase, indicating the polypeptides phosphorylated are not ribosomal proteins. This kinase also selectively inhibits the translation of brome mosaic virus (BMV) RNAs 1 and 2, but not BMV RNA 4. The mechanism of this inhibition is not clear; however, poly(U)-directed polyphenylalanine synthesis is not inhibited, indicating elongation is not affected. A second wheat germ kinase, partially purified by Ranu (10), appears to phosphorylate the 38,000-D subunit of eIF-2 from both wheat germ and rabbit reticulocytes. This kinase also inhibits translation. The physical properties of this kinase have not been reported.

A third wheat germ kinase has been purified to homogeneity by Yan and Tao (19) and shown to have a M_r of 32,000. This kinase appears to be physically similar to the wheat germ kinase (M_r = 31,000) isolated by Davies and Polya (5). The kinase isolated by Yan and Tao (19) preferentially phosphorylates a 48,000-D polypeptide found in wheat germ (20). In this study, we have determined the ability of wheat germ initiation factors and ribosomal proteins to serve as substrates for the wheat germ protein kinase, purified by Yan and Tao (19). Our results show that eIF-2, eIF-3, and three 60S ribosomal proteins are phosphorylated *in vitro* by this kinase.

MATERIALS AND METHODS

Preparation of Wheat Germ Protein Kinase, Ribosomes, and Ribosomal Subunits. Homogeneous preparations of the wheat germ kinase were obtained as described by Yan and Tao (19). Ribosomes were isolated from extracts of wheat germ prepared in buffer containing 0.12 M KCl (18) and were washed once (1× salt washed) or twice (2× salt washed) with buffer containing 0.6 M KCl and 5 mM Mg^{2+}. Ribosomal subunits were obtained by suspending the 1× salt-washed ribosomes in buffer containing 0.5 M KCl and 5 mM Mg^{2+} and centrifuging through a sucrose density gradient as previously described (14).

Preparation of Wheat Germ Initiation Factors. The 0 to 40% and 40 to 60% $(NH_4)_2SO_4$ fractions were prepared from the 0.12 M KCl postribosomal supernatant of wheat germ extracts as previously described (4, 18). Fraction 2A obtained from the 0 to 40% $(NH_4)_2SO_4$ fraction (4) was used as a source of initiation factor eIF-4B. Highly purified eIF-3 was obtained from the 0 to 40% $(NH_4)_2SO_4$ fraction by a modification of the procedure of Checkley *et al.* (4). Highly purified eIF-2 was isolated from the 40 to 60% $(NH_4)_2SO_4$ fraction by a modification of the procedure of Lax *et al.* (8). Highly purified eIF-4A was also obtained from the 40 to 60% $(NH_4)_2SO_4$ fraction (J. M. Ravel *et al.*, unpublished procedure).

Protein Determinations. Protein concentrations were determined by the procedure of Bradford (3) with crystalline BSA as a standard and also by the procedure of Bensadoun and Weinstein (2).

Phosphorylation of Initiation Factors and Ribosomes. The reaction mixture contained in 25 μl: 24 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid-KOH (Hepes), pH 7.6, 2.4 mM

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[2] Abbreviation: eIF, eukaryotic initiation factor.

DTT, 5 mM Mg-acetate, 100 mM KCl, 0.2 mM [γ-^{32}P]ATP (400–800 cpm/pmol), and initiation factors, ribosomes, and kinase as indicated. After incubation at 25°C for 10 min, the entire reaction mixture was denatured by heating with SDS. Reaction mixtures containing initiation factors were electrophoresed in a 5.6% (16) or a 12.5% polyacrylamide gel (1) in the presence of SDS. Reaction mixtures containing ribosomes were electrophoresed in a 10% polyacrylamide gel (7) in the presence of SDS.

The gels were stained with Coomassie brilliant blue in acetic acid and methanol, dried and exposed to x-ray film. The amounts of protein or polypeptide in individual bands in the stained gels were quantified by scanning with a Zeineh Soft-Laser densitometer, integrating the area under each peak, and comparing these areas to those of a calibration curve of crystalline BSA electrophoresed and scanned under the same conditions.

The amounts of ^{32}P incorporated were determined by excising the protein band from the dried gel, solubilizing in 0.5 ml of Tissue Solubilizer (Research Products International Corp.), and counting in a liquid scintillation counter. The actual amounts of ^{32}P incorporated may be underestimated due to hydrolysis of phosphoramide linkages during the acid staining procedure.

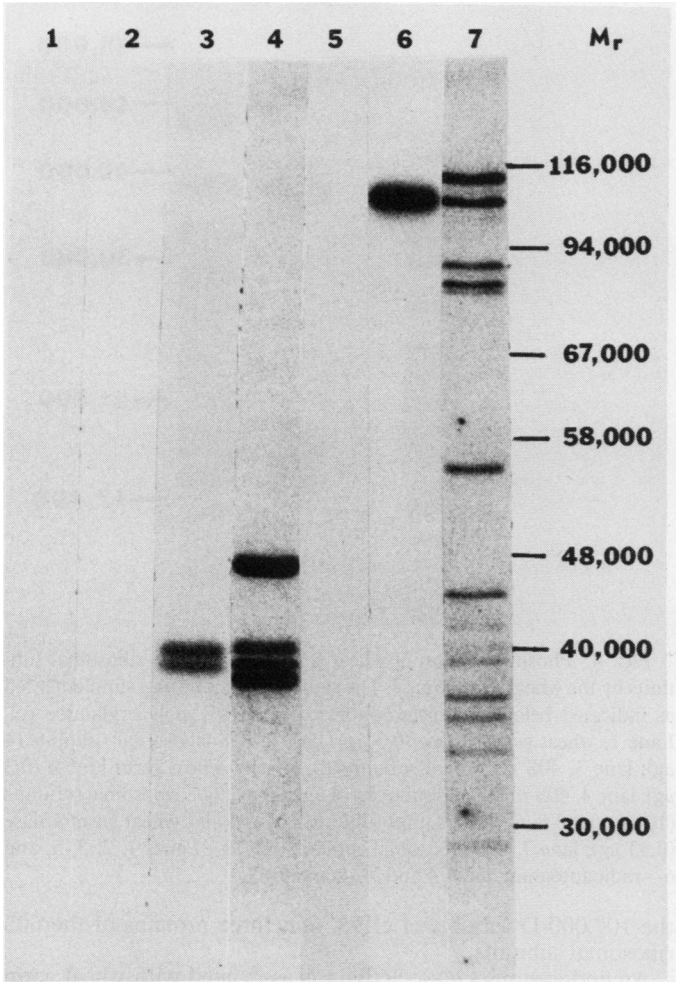

FIG. 1. Phosphorylation of wheat germ eIF-2 and eIF-3 by the wheat germ kinase. The standard reaction was supplemented with kinase and/or initiation factors as indicated below. The samples were electrophoresed in a 12.5% polyacrylamide gel. Lane 1, wheat germ kinase (0.3 μg); lane 2, eIF-2 (5 μg); lane 3, eIF-2 (5 μg) and wheat germ kinase (0.3 μg); lane 4, eIF-2 (5 μg); lane 5, eIF-3 (5 μg); lane 6, eIF-3 (5 μg) and wheat germ kinase (0.3 μg), lane 7, eIF-3 (5 μg). Lanes 1, 2, 3, 5, and 6— radioautogram; lanes 4 and 7—stained gel.

RESULTS

Phosphorylation of Initiation Factors. The ability of the various initiation factors purified from wheat germ extracts to serve as substrates for the wheat germ kinase was investigated. Among the various initiation factors examined, eIF-2 and eIF-3 were found to be phosphorylated to a significant degree by the wheat germ kinase (Fig. 1). In contrast, initiation factors eIF-4A and eIF-4B and elongation factors EF-1 and EF-2 were poor substrates for the kinase (data not shown).

The phosphorylation of eIF-2 by the wheat germ kinase occurred primarily on the 42,000-D subunit. The time course of phosphorylation of the 42,000-D subunit of eIF-2 by the wheat germ kinase is shown in Figure 2A. The phosphorylation leveled off after about 1 h of incubation. The 42,000-D subunit of eIF-2 was phosphorylated to the extent of 0.5 to 0.6 mol of phosphate/mol of the 42,000-D subunit of eIF-2.

Studies with eIF-3 showed that the wheat germ kinase catalyzed the phosphorylation of the 107,000-D subunit. The time course of phosphorylation of the 107,000-D subunit of eIF-3 by the wheat germ kinase is shown in Figure 2B. The phosphorylation occurred relatively rapidly and reached saturation after 20 min of incubation (Fig. 2B). The 107,000-D subunit was phosphorylated to the extent of 6 mol of phosphate/mol of 107,000-D polypeptide.

Phosphorylation of Ribosomal Proteins. The data presented in Figure 3 show that 1× salt-washed 80S ribosomes contained endogenous kinase activity. Incubation of the 1× salt-washed ribosomes with [γ-^{32}P]ATP in the absence of exogenous kinase resulted in the labeling of three proteins with apparent mol wt of 38,000, 14,800, and 12,600. The labeling of these components was enhanced by the addition of purified wheat germ kinase. The endogenous kinase could be removed by a second high salt wash of the 80S ribosomes, indicating that it is loosely associated with the ribosomes.

The 40S and 60S ribosomal subunits were used as substrates for the kinase to obtain the data shown in Figure 4. None of the 40S ribosomal subunit proteins were phosphorylated by the wheat germ kinase (Fig. 4). In contrast, the 60S ribosomal subunit contained the three proteins which were labeled by the wheat germ kinase. These appeared to be the same proteins which were phosphorylated in the 80S subunits either by the endogenous kinase or the purified wheat germ kinase, as judged by mol wt (Figs. 3 and 4).

Effects of Phosphorylation on the Activity of eIF-2, eIF-3, and 60S Ribosomal Subunits. The addition of high concentrations of wheat germ kinase (150 μg/ml) did not inhibit poly(U)-directed polyphenylalanine synthesis, but did inhibit about 20 to 50% yeast mRNA-directed polypeptide synthesis in *in vitro* systems from wheat germ (Table I). However, the inhibition was not overcome by the addition of more eIF-2, eIF-3, or 80S ribosomes (Table II).

In addition, it was found that phosphorylation of the 42,000-D subunit of eIF-2 did not affect its ability to form a ternary complex with Met-tRNA$_f^{Met}$ and GTP (Table III). Phosphorylation of the 107,000-D subunit of eIF-3 did not affect the ability of eIF-3 to support polypeptide synthesis (Table IV), and had no effect on the ability of eIF-3 to bind to 40S ribosomal subunits (S. Lauer, E. Burks, J. Ravel, unpublished data). Phosphorylation of the 60S ribosomal subunits did not decrease to a significant extent their ability to support polypeptide synthesis.

DISCUSSION

This study shows that eIF-2, eIF-3, and 60S ribosomal subunits from wheat germ are phosphorylated by the wheat germ kinase recently purified by Yan and Tao (19). Wheat germ kinase catalyzes the phosphorylation of the 42,000-D subunit of eIF-2,

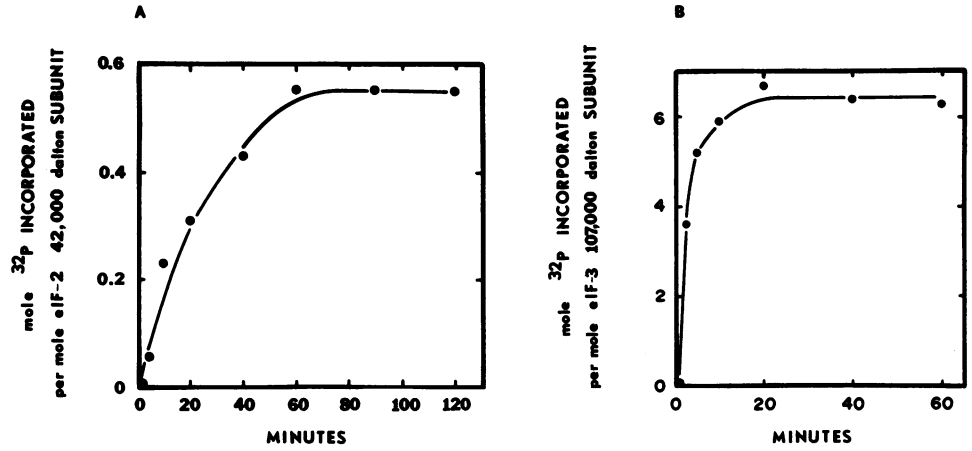

FIG. 2. A, Time course of phosphorylation of the 42,000-D subunit of eIF-2 by the wheat germ kinase. eIF-2 (100 μg) was phosphorylated at 25°C in a reaction mixture (0.5 ml) containing 0.3 μg of the wheat germ kinase. At the times indicated, an aliquot of 50 μl was withdrawn and electrophoresed in a 5.6% polyacrylamide gel. The ^{32}P incorporated into the 42,000-D subunit was determined as described in "Materials and Methods". B, Time course of phosphorylation of the 107,000-D subunit of eIF-3 by the wheat germ kinase. The phosphorylation of eIF-3 (100 μg) was carried out as described above.

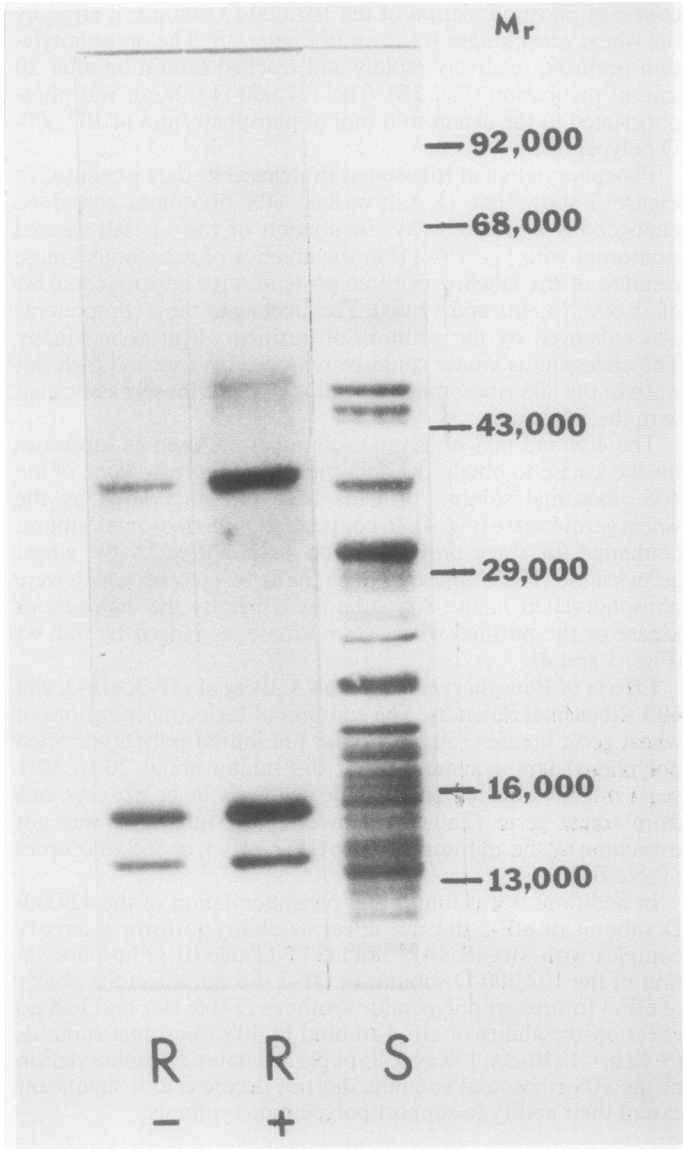

FIG. 3. Phosphorylation of 80S ribosomal proteins in the presence and absence of the wheat germ kinase. The standard reaction mixture was supplemented wih 25 μg of 1× salt-washed ribosomes without added kinase (−) and with 0.3 μg of wheat germ kinase (+). R, radioautogram; S, stained gel.

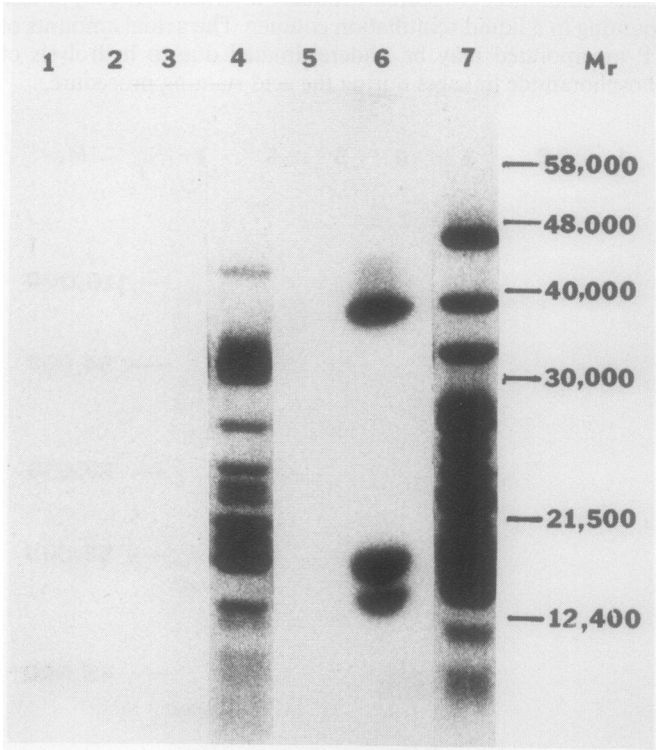

FIG. 4. Phosphorylation of wheat germ 40S and 60S ribosomal subunits by the wheat germ kinase. The standard reaction was supplemented as indicated below and electrophoresed in a 10% polyacrylamide gel. Lane 1, wheat germ kinase (0.3 μg); lane 2, 40S ribosomal subunits (4 μg); lane 3, 40S ribosomal subunits (4 μg) and wheat germ kinase (0.3 μg); lane 4, 40S ribosomal subunits (4 μg); lane 5, 60S ribosomal subunits (10 μg); lane 6, 60S ribosomal subunits (10 μg) and wheat germ kinase (0.33 μg); lane 7, 60S ribosomal subunits (10 μg). Lanes 1, 2, 3, 5, and 6—radioautogram; lanes 4 and 7—stained gel.

the 107,000-D subunit of eIF-3, and three proteins of the 60S ribosomal subunits.

An endogenous kinase activity is associated with wheat germ ribosomes as evidenced by the ability of the ribosomes to catalyze autophosphorylation in the absence of an exogenous kinase. The autophosphorylation reaction results in the labeling of three ribosomal proteins with mol wt of 38,000, 14,800, and 12,600. These three polypeptides are also substrates of the purified wheat germ kinase, suggesting the possibility that the two activities may be due to the same enzyme. The three polypeptides phosphorylated by the wheat germ kinase are localized on the 60S ribosomal

Table I. *Effect of Wheat Germ Kinase on Poly(U)-Directed Poly-Phenylalanine Synthesis and on Yeast mRNA-Directed Polypeptide Synthesis*

Poly(U)-directed phenylalanine incorporation into polyphenylalanine and yeast mRNA-directed incorporation of leucine into polypeptide were measured as previously described (15).

Kinase	[^{14}C]Phenylalanine Incorporated	[^{14}C]Leucine Incorporated
μg	*pmol*	
—[a]	124	40
0.63	124	26
1.25	122	22
2.5	120	18

[a] No kinase added.

Table II. *Effects of Initiation Factors and Ribosomes on the Inhibition of Polypeptide Synthesis by the Wheat Germ Protein Kinase*

Yeast mRNA-directed corporation of leucine into polypeptide was measured as previously described (4) in the presence of 2 μg of eIF-3 and the absence and presence of 3 μg of kinase and other additions as indicated.

Additions	[^{14}C]Leucine Incorporated	
	Without kinase	With kinase
	pmol	
None	28	17
eIF-2, 3 μg	26	16
eIF-3, 4 μg	30	16
2× washed 80S ribosomes, 67 pmol	35	17

Table III. *Effect of Phosphorylation on the Ability of eIF-2 To Form a Ternary Complex with Met-tRNA and GTP*

Met-tRNA binding was determined as previously described (8), except the eIF-2 was preincubated (with 0.2 mM ATP) with or without wheat germ kinase (1 μg) for 10 min at 25°C, as described in "Materials and Methods".

eIF-2	[^{35}S]Met-tRNA Bound	
	Preincubation without kinase	Preincubation with kinase
μg	*pmol*	
1.5	0.7	0.7
3.0	3.1	2.9

Table IV. *Effect of Phosphorylation on the Ability of eIF-3 To Support Polypeptide Synthesis*

Incorporation of leucine into polypeptide was measured as previously described (4), except the eIF-3 was preincubated (with 0.2 mM ATP) with or without wheat germ kinase (1.5 μg) for 30 min at 25°C, as described in "Materials and Methods".

eIF-3	[^{14}C]Leucine Incorporated	
	Preincubation without kinase	Preincubation with kinase
μg	*pmol*	
0.9	30	25
1.8	41	38

subunit. Isolated 40S ribosomal subunits are not phosphorylated by the wheat germ kinase. Moreover, neither of the purified ribosomal subunits exhibits any autophosphorylation activity, indicating that the endogenous kinase activity is lost during the purification of the subunits.

The significance of phosphorylation of the wheat germ initiation factors and ribosomal proteins remains unknown. The 42,000-D subunit of eIF-2 is phosphorylated to the extent of about 0.5 to 0.6 mol of phosphate/mol of subunit and the 107,000-D subunit of eIF-3 accepts as much as 6 mol of phosphate/mol of subunit. This is a significant amount of phosphorylation and could conceivably lead to the modification of their activities.

No effects of phosphorylation on the activity of initiation factors, eIF-2 and eIF-3, or 60S ribosomes could be demonstrated *in vitro*. The kinase may function *in vivo* in a manner that cannot be duplicated in an *in vitro* system. A recent study by Scharf and Nover (13) shows that several ribosomal proteins are phosphorylated in cell cultures of tomato when grown in the presence of ^{32}P-orthophosphate. One of the phosphoproteins corresponds to S6, whereas three others are found to be localized on the large ribosomal subunit. The mol wt of these latter three phosphoproteins derived from the 60S subunits are very similar to those proteins phosphorylated by the wheat germ kinase. Thus, it is tempting to speculate that the reaction catalyzed by the wheat germ kinase may be of physiological significance but the effects cannot be demonstrated in an *in vitro* system.

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