Recent developments on the use of urease inhibitors in the tropics

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Abstract

Urea has become the most widely used form of N fertilizer in the world, particularly in the tropics. Its efficiency, however, is decreased by losses of N through ammonia volatilization when the urea is not incorporated into the soil. High temperatures and high biological activity at the soil surface promote rapid hydrolysis of urea to ammonia and carbonate species by the soil enzyme urease, leading to large ammonia losses. These conditions have generated interest in materials that can inhibit the urease enzyme, slowing urea hydrolysis and allowing the urea to move away from the soil surface to where it is not as susceptible to ammonia loss. The phosphoryl di- and triamides, which are structural analogs of urea, meet the requirements for effective soil urease inhibition to varying degrees depending on the conditions of their use. Until the discovery of these compounds, there was little hope that urease inhibition could be achieved either economically or in an environmentally acceptable way. Included in this group is N-(n-butyl) thiophosphoric triamide (NBTPT), which is that most widely tested proinhibitor or precursor of the actual inhibitor N-(n-butyl) phosphoric triamide. Recent research in tropical rice systems indicates that urease inhibitors such as N-(n-butyl) phosphoric triamide and cyclohexylphosphoric triamide can play an important role in increasing urea efficiency. In some experiments where urease inhibition was only partially successful, better results were obtained when the phosphoromnides were used in conjunction with an algicide, to restrict ammonia loss, and nitrification inhibitors, to reduce loss of N by denitrification. Further research on tropical soils in different environments is required to determine the most suitable combination of inhibitors to reduce N loss and increase the efficiency of fertilizer N use.

Introduction

Urea has become the most used N fertilizer in the world, accounting for approximately 40% of the total synthesized N supply, or about 30 million tons of N [5]. Its market share is increasing because it is the least expensive form of solid N fertilizer available and its high nutrient content (46% N) offers transportation advantages over other sources. In tropical agriculture, it accounts for about 79% of the total fertilizer N use. Although urea has cost advantages over other products, it also has an important disadvantage in that considerable losses of N can occur if the urea is not incorporated into the soil soon after application. The loss occurs through ammonia volatilization after the

urea is hydrolyzed at the soil surface by reaction with the enzyme urease.

Soil pH, moisture, cation exchange capacity, type and quantity of cations present, and wind speed also affect the extent of ammonia volatilization [30]. Carbonate or bicarbonate species are also formed as a result of urea hydrolysis, providing buffering capacity at a high pH at the site where $NH₃$ is released, further facilitating the loss of $NH₃$ and increasing $NH₃$ toxicity to germinating seeds. To reduce ammonia volatilization losses and thereby increase the efficiency of the urea applications, compounds have been tested for their ability to inhibit the urease enzyme. Decreasing ammonia toxicity to germinating seeds and increasing nitrate accumulation are also benefits of urease inhibition [42]. This paper reviews recent developments

Urease inhibitors:

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O - \overset{O}{P} - NH_2
$$
 phenyl phosphorodiamidate
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$$
\overset{O}{NH_2}
$$
\n
$$
R - N - \overset{II}{P} - NH_2
$$
phosphoryl triamide structure
\n
$$
\overset{I}{H} NH_2
$$

Thio proinhibitors of the above phosphoryl triamides:

$$
R - N - P
$$

R - N - P - NH₂ thiophosphoric triangle structure
H NH₂

"R" is any saturated (alkyl) group

Fig. 1. Urease inhibitors and proinhibitors which are, or produce, structural analogs of urea.

in urease inhibitors to improve the efficiency of urea fertilizer applications, particularly regarding their use in tropical agriculture.

Urease is an extremely powerful enzyme produced by practically all microbial and plant species, which may indicate that it is extremely advantageous for organisms to be able to utilize N excreted from animals as urea. Considering that primitive life forms predate higher forms of life, it may indicate that the gene to produce urease originated with the development of life in a primordial sea, in which urea may have been a major N-containing compound. Urease activity of soils is highly related to biological activity, which depends on organic substrates or other energy sources (including sunlight), temperature, and moisture. Urease is apparently protected from protease enzymes by its association with organic-mineral complexes in soils and therefore can persist in the abiotic soil fractions. Although urease activity can be temporarily increased by transitory occurrences that affect biological activity, its inherent (minimum) soil activity is dependent on the amount and types of clays and organic material present in surface soils [4, 43].

Because of the wide distribution and high specific activity of urease, urea hydrolysis in soils normally occurs at a faster rate than do microbially mediated N transformations; often hydrolysis occurs within 3- 7 days of urea application to soils unless moisture is limiting. The distribution of the urea can greatly affect the hydrolysis rate in that the greater the access of urea to urease, the more rapid the hydrolysis.

Urease inhibitors are expected to be most beneficial on soils when (i) incorporation of urea is difficult, (ii) there is little opportunity for the urea to move into the soil with infiltrating water, and (iii) the soil surface has a high urease activity due to the lack of cultivation or the accumulation of organic material.

Given the ubiquitous and the high specific activity of soil urease, and the ability of soils to precipitate and adsorb, urease inhibition is difficult to achieve. In addition, a urease inhibitor must meet other requirements as well:

- 1. The inhibitor must move with the urea, which is not absorbed to any extent by soils, or the urea will still be hydrolyzed near the soil surface as it moves out of the zone of inhibition.
- 2. It must be reasonably stable in soils, but be ultimately degraded to benign residues.
- 3. It must be safe for workers exposed to the compound and be environmentally acceptable.
- 4. It must be inexpensive enough to compete with alternative N management options and must cost less than the fertilizer N conserved.
- 5. For ease of application, it must be compatible with urea production methods and be stable in admixtures with urea.
- 6. The demand for the inhibitor must be large enough to warrant development, testing, production, and marketing of the material.
- 7. The N saved from ammonia volatilization must not be subject to loss by other mechanisms to such a degree that increased plant uptake is not achieved.

Research on urease inhibition dates back more than 50 years [21], and a large number of compounds with a wide range of characteristics have been tested and patented as urease inhibitors [31, 42]. Some inhibitors apparently coordinate with the sulfhydryl group at the active site of the enzyme, whereas others chelate with the essential nickel atom [40]. Despite the considerable effort to identify urease inhibitors for application with fertilizer urea, most of the compounds tested were ineffective for use in soils. The only compounds that have proved to be viable for soil application are the structural analogs of urea (the phosphoryl di- and triamides), and possibly hydroquinone and 2,5-dimethyl p-benzoquinone [52], which must be used at much higher concentrations than the phosphoryl amides. This paper concentrates on the recent research results with the structural analogs of urea.

Phosphoramides

The bond angles and lengths of the amide groups of the phosphoryl di- and triamides (Fig. 1) are similar to those of urea [40], and yet these amides are not substrates of urease. This characteristic apparently enables the inhibitor to react with the active site of the urease and occupy the site for long periods. Whether it degrades, leaving a residue attached to the active site, has not been established. The first structural analog found to be a potent urease inhibitor was phenyl phosphorodiamidate (PPDA), which was identified by East German scientists through screening over 14,000 compounds [40]. An electron-withdrawing group, such as phenol or trichloroethyl [6], is necessary for the diamide to achieve inhibition. Substitutions, such as C1 on the phenol ring, can improve inhibition if they do not cause stearic hindrance by being located adjacent to the ester group [40]. In contrast, a triamide must contain a saturated or alkyl group to have appreciable inhibition. The more commonly tested compounds have contained either n-butyl or cyclohexyl as the 'R' group (Fig. 1). Compounds with other alkyl groups show the same urease inhibitory characteristics, although the solubility and mobility of the compound can be reduced as the group becomes larger (IFDC, unpublished reports). Phosphoryl triamide itself (where 'R' is a proton rather than an alkyl group) is also a urease inhibitor, but it is not as potent as the other compounds [35, 48].

The most widely tested triamide, N-(n*butyl)thiophosphoric* triamide (NBTPT), is not a urease inhibitor, nor are the other thiophosphoric triamides. Rather they are precursors or proinhibitors of their oxygen analogs, which are the actual inhibitors. Numerous *in vitro* tests of the thio compounds in a purified state have shown their total ineffectiveness. Their effectiveness as inhibitors is dependent on their conversion to an oxygen analog upon contact with soil or other material. This has been shown conclusively by separate groups using various means [22, 39].

The factors that affect the rate of NBTPT conversion to its oxygen analog N-(n-butyl) phosphoric triamide (NBPTO) have not been elucidated. In unpublished work at IFDC, the pure oxygen analog was identified after reaction of an aqueous solution of NBTPT with activated charcoal. Separation of the analogs was achieved with a Sephadex chromatographic column, and the isolated and dried oxygen analog was identified by high-performance liquid chromotography.

Formation of the oxygen analog can be accurately determined in soil extracts or in floodwater using an

Fig. 2. Development of urease inhibition from peffusing 25 ml of 9.21 mM solution of NBTPT through 15 g of soil; 500 μ l aliquots of peffusate used in *in vitro* system (IFDC, unpubl.).

in vitro Jack bean urease assay in a pH-stat system [13]. Briefly, this method requires an accurately reproducible amount of Jack bean urease, which is reacted in solution with an aliquot containing the active inhibitor. An aliquot of urea solution is then added to the partially inhibited urease solution. Following the urea addition, urea hydrolysis produces ammonia at a constant rate; the ammonia is titrated with acid to maintain the pH of the solution at 6.7. If the quantities of urease and inhibitor correspond to achieve partial, but not complete, inhibition of the urease, the rate of acid addition with the pH-stat system is inversely proportional to the quantity of active inhibitor added. Standard curves can be developed with PPDA or the oxygen analogs of the triamides.

Using this system, we found that detectable amounts of the oxygen analog developed within a few minutes of NBTPT addition to the four aerobic soils tested (Fig. 2). Formation of the oxygen analog was also rapid when NBTPT was added to pure bentonite clay. We also tested the development of the active form in the floodwater of rice *(Oryza sativa* L.) soils from the United States, Malaysia, Indonesia, Thailand, and Egypt. The U.S. soils developed appreciable quantities of the active inhibitor within 2 h; however, it took 2 days to achieve the same concentration of active inhibitor in the floodwater of the tropical soils. These soils were preincubated under flooded conditions for 3 weeks, and algal growth was vigorous in all soils. The reasons for the differences between these groups of soils have not been elucidated.

Phenyl phosphorodiamidate has potency similar to that of N-(n-butyl) phosphoric triamide *in vitro* with Jack bean urease (IFDC, unpublished reports). How-

Table 1. Effects of urease inhibitors on total plant recovery, N loss, and grain yield^a

	Total plant recovery		Loss		Grain yield (g/pot)	
Treatment	TUM	IFDC	TUM	IFDC	TUM	IFDC
	(% of applied 15 N)					
Control	\blacksquare	-		۰	25.5 C	15.1 D
Urea alone	29.7 D ^b	28.0 C	49.9 D	49.2 C	34.8 B	37.0 C
PPDA	45.3 C	38.1 B	27.8 C	30.8 B	40.4 AB	41.6 ABC
$PPDA + algebra$	54.0 B	41.8 B	20.9 B	30.2 B	47.3 A	43.5 AB
NBTPT	65.5 A	52.5 A	9.6 A	17.2 A	48.6 A	46.9 A

^aGreenhouse experiments at the Technical University of Munich (TUM) [14] and IFDC [11].

bData within a column followed by the same letter are not significantly different at the 5% level by Duncan's multiple range test.

ever, PPDA is rapidly degraded in the floodwaters of soils, mainly by base hydrolysis of the ester bond [16]. Degradation in aerobic soils is even faster than in solution and occurs mainly through the mechanism of base hydrolysis, as evidenced by the almost quantitative extraction of the degradation product, phenol, from the soil. Half-lives of PPDA in both sterilized and unsterilized soils were from 2 to 6 h [11]. Soil pH had little effect, whereas the presence of clays and organic matter generally increased degradation rates [11,381. The potency of PPDA is attested by the fact that it functions in soil despite its rapid degradation (for example, see [381).

Urease inhibition in flooded soils

The phosphoroamides PPDA and NBTPT have shown promise for limiting the hydrolysis of urea in laboratory experiments [3, 12, 17, 38, 48] and for increasing N uptake and grain yield in greenhouse studies [14, 15, 54].

In greenhouse experiments at the Technical University of Munich and IFDC, the inhibitors were added at 1% of the weight of urea; electric fans circulated the air above the floodwater to promote ammonia volatilization. Improvement in recovery of applied N was due solely to increased plant uptake, which had to be enhanced by 14% for the increase to be significant $(p < 0.05$ [Table 1]).

In field experiments, however, application of these compounds seldom resulted in significantly $(p < 0.05)$ increased grain yields [8, 9, 17, 25, 28, 44, 45, 51] even though plant uptake of applied N was often

^a All experiments are with delayed applications of urea, 15-25 days after transplanting.

bRate of inhibitor on basis of percentage weight of the urea.

 $c_{n,s}$. - not significant; $*$ - significant at 95% confidence interval.

increased to the same extent as in the greenhouse studies (Table 2). The promising results with NBTPT in greenhouse experiments were not duplicated in field trials in southeast Asia (IFDC, unpublished reports). In 20 field studies with delayed applications of urea, addition of NBTPT (0.5 % of the weight of urea) slowed urea hydrolysis (based on the rate of disappearance of urea from the floodwater) in several experiments and increased uptake of applied N, but did not increase grain yield. When urea is applied 15-25 days after

transplanting, as in these experiments, the benefits of a urease inhibitor should have been maximized; the rice plants are well developed at this stage, and delaying ammonia loss by a few days would have resulted in increased uptake of applied N by the rice.

Numerous reasons have been given for the lack of success with these two compounds including (i) microbial decomposition, (ii) degradation of PPDA due to high pH or temperature [11, 16, 38], (iii) the requirement for NBTPT to be converted to the oxygen analog before it is effective [13, 20, 22, 23, 39], (iv) volatilization or nitrification and denitrification of the conserved N $[17, 29, 44, 51]$, and (v) the fact that the saving of 9% to 15% of the applied N is often not sufficient to significantly ($p < 0.05$) increase grain yield in field experiments.

Direct measurements of ammonia volatilization in field studies showed that PPDA slowed ammonia loss for only 2 to 4 days; after that time, ammonia loss was quite high [24, 5l]. Delaying ammonia loss for such a short time may not be beneficial because the relative loss from the control and inhibited systems will depend on the pH and temperature of the floodwater and wind speed when the ammoniacal-N concentration of the floodwater is high [51].

The main reason for the lack of success of PPDA in flooded soils seems to be its rapid hydrolysis [16] under the alkaline conditions generated in the floodwater by photosynthetic algae [41]. Floodwater pH values in excess of 10 have been recorded in rice fields in Australia, China, and Malaysia [50]. High floodwater temperatures seem to be less important than pH in the decomposition of PPDA, because PPDA was found to be active for at least 7 days when the pH of the floodwater was controlled by the addition of the algicide terbutryn [26] or simazine [11]. In the presence of algicide, PPDA significantly (p <0.05) reduced ammonia loss, increased N uptake by rice, and increased grain yield in greenhouse experiments (Table 1). Likewise, in a flooded rice field in Thailand, addition of terbutryn with PPDA significantly ($p < 0.05$) slowed the hydrolysis of urea for more than 7 days, reduced ammonia loss from 15% to 7.3% of the applied N, and increased grain yield from 3.6 to 4.03 t ha⁻¹ [46].

The success or failure of NBTPT in flooded soils seems to be related to the urease activity of the soil and the capacity of the soil to convert NBTPT to the oxygen analog NBPTO. Thus, in soils with high urease activity, most of the urea is hydrolyzed before appreciable conversion of NBTPT occurs, and in strictly anaerobic soil, NBTPT fails to inhibit urease activity (IFDC, unpublished reports; [35, 36, 37, 55]). Inhibition of urease activity was more successful when NBTPT was added to soils of low urease activity, but the hydrolysis was merely slowed and not completely stopped.

In laboratory experiments, the capacity of NBTPT to inhibit urease activity was enhanced by aerobic conditions (in contrast to anaerobic conditions [36]) created by bubbling oxygen into the floodwater [37] or by the growth of photosynthetic algae, which add oxygen to the flooded system [35]. Complete inhibition of urease activity was not achieved in any of these experiments. More effective inhibition was achieved by adding PPDA and NBTPT in combination [37]. It appeared that PPDA initially inhibited urease activity and during this period part of the NBTPT was converted to the active oxygen analog; then as the concentration of PPDA declined, the concentration of NBPTO became high enough to inhibit urea hydrolysis.

The use of both inhibitors together also worked successfully on flooded rice in Thailand when terbutryn was applied to prolong the effectiveness of PPDA [46]. The combined treatment decreased ammonia loss from 15% to 3% of the applied N and increased grain yield from 3.6 to 4.1 t ha^{-1} [46]. Obviously, it would have been more satisfactory to apply a phosphoric triamide rather than the mixture of inhibitors; however, at the time of conducting the experiment, a phosphoric triamide was not available. It appears that inclusion of an inhibitor with immediate inhibitory activity rather than use of NBTPT alone will be essential to achieving the necessary effect in tropical flooded rice systems.

In greenhouse experiments with flooded soils, the NBTPO inhibited urease activity to a greater extent than did NBTPT in 6 of 10 temperate U.S. soils when the urea and phosphoroamides were incorporated into the soils. However, both analogs performed well when they were applied to the floodwater without mixing (IFDC, unpubl, data).

Application of NBPTO to a flooded rice field in Thailand resulted in much lower ammoniacal-N concentrations in the floodwater than in the control treatment following the application of urea [27]. Ammonia loss was significantly (p <0.05) decreased from 14.4% to 7.6% of the applied N, but grain yield was not increased by this treatment alone. Increased grain yield was not obtained unless an algicide or nitrification inhibitor was added to decrease N loss by ammonia volatilization and denitrification [27]. These results confirm the observations of Phongpan and Byrnes [44] and Byrnes and Amberger [12] that denitrification of conserved N was responsible for the lack of inhibitor response on uptake or yield when it was demonstrated that urea hydrolysis had been slowed.

Keerthisinghe and Freney [35], in laboratory studies, found that cyclohexylphosphoric triamide (CHPT) was even more effective as a urease inhibitor than was NBPTO, and this was confirmed in a field study in Thailand [27]. CHPT maintained the ammoniacal-N concentration of the floodwater at a lower level than did NBPTO ($<$ 2 compared to 5 g N m⁻³), decreased ammonia loss to a greater extent (50% versus 90%), and resulted in greater uptake of applied N by rice (15.7% versus 10.5%). Again significant increases in grain yield were only obtained in the presence of algicide or algicide plus nitrification inhibitor to further conserve applied N [27].

Urease inhibitors in upland soil systems

The loss of N from surface-applied urea can be an important loss mechanism, particularly on plantation and long season crops that allow development of high urease activities at the soil surface. Warm, moist tropical soils, particularly those that receive additions of organic matter from leaf litter or support algal growth on the soil surface, develop very high urease activities, and ammonia loss can be extremely high and variable. Soil moisture, urease activity, pH, and cation exchange capacity, rainfall, wind speed, temperature, and humidity affect the magnitude of loss [30, 47]. Despite the high potential for ammonia loss following application of urea, few studies have evaluated urease inhibitors in upland systems in the tropics. The growing importance of no-tillage or limited-tillage production practices in maize *(Zea mays* L.) has increased the interest in the use of urease inhibitors. Most of the work in the United States has been with surface applications of urea and urea ammonium nitrate (UAN) solutions (approximately 50% of the N in the form of urea).

Laboratory testing under aerobic soil conditions indicated the superiority of the triamides, both thio and oxygen analogs, over PPDA [2, 3, 7, 49]. The promising results with NBTPT have prompted numerous field studies. Tests were conducted with NBTPT rather than other analogs because the performance of NBTPT on aerobic soils was generally better than that of other compounds. This has been attributed to the greater stability of NBTPT, particularly in acidic soils [23, 34]. In 21 field experiments from 1989, mostly in the Midwest of the United States, NBTPT increased average grain yields of maize by 750 kg ha^{-1} (9%)

Fig. 3. Effect of temperature and NBTPT concentration on cumulative ammonia volatilization losses from urea applied on a soil surface (adapted from [18]).

at an average fertilization rate of 100 kg N ha^{-1} [32, 33]. The yields with NBTPT were equivalent to those resulting from 80 kg N ha⁻¹ of additional urea-N without NBTPT. The average increase for 78 trials over 5 years at all rates of N was 270 kg of grain per hectare (4%). In southern Illinois, maize yields were increased by an average of 525 kg ha^{-1} (9%) for 13 experiments with broadcast urea and 750 kg ha^{-1} (14%) for 9 experiments in which urea was band placed on the soil surface [53]. Use of NBTPT on ryegrass pasture in Northern lreland improved urea performance by about 12%, making it comparable to calcium ammonium nitrate [56].

In most of these field studies, NBTPT was applied at 0.25% of the weight of urea. Urea hydrolysis and ammonia volatilization are sensitive to temperature, as is the effectiveness of NBTPT (Fig. 3). These results indicate that a higher concentration of NBTPT will be needed for the warmer tropical soils than for the temperate soils to achieve similar effects on N losses [18].

The marketing of NBTPT in admixture with urea in the United States, principally for use with maize, is tentatively to be at the rate of approximately 0.15% of the weight of urea, and the product is to be sold under the trade name of Agrotain. A second product will likely be marketed, which will also contain a nitrification inhibitor, dicyandiamide (DCD), with the NBTPT (A. Sutton, IMC-Agrico, pers. commun.). Market availability of the products, slated for 1995, will greatly assist the widespread evaluation of the materials for their economic and environmental benefits.

Additional considerations

In aerobic soils, both the phosphoric and the thiiophosphoric analogs containing the cyclohexyl moiety performed slightly better than did the n-butyl compounds at low (0.01% by weight) concentrations [20]. The oxygen analogs of the triamides are readily synthesized; because of this capacity for immediate inhibition, it is logical that mixtures of the oxygen and thio compounds may find a niche in markets with flooded rice or on soils that convert the thio compound to its active form too slowly to be effective. However, there may be economic or technical constraints that make the use of the oxygen analog or mixtures less viable than NBTPT.

Several years ago, IFDC researchers became concerned about the stability of NBTPT in combination with urea because the effectiveness of urea-NBTPT mixtures used for research decreased with time. They found that such mixtures produced by melt prilling and by spraying a methanol solution of NBTPT onto urea can degrade fairly rapidly if not stored at cold. temperatures $(-4 \degree C)$. Degradation occurs through acid hydrolysis (deamination) followed by a much slower secondary step, which evolves H_2S . The main findings from this work were as follows:

- 1. Larger crystals of NBTPT degraded more rapidly than did smaller crystals which suggests a selfcatalysis in that any initial degradation gives rise to an acidic residue that causes more rapid acid hydrolysis.
- 2. Removal of $NH₃$ from the product increased the degradation rate. Therefore, the top layers of open piles tended to degrade more rapidly than did those lower in the pile.
- 3. Moisture in the urea and purity of the urea or NBTPT had little effect on stability. The dark color of the urea-NBTPT admixture is not an indication of degradation of the NBTPT.
- 4. During storage, NBTPT can recrystallize out of urea in white, needle-shaped crystals.
- 5. Laboratory-scale preparations of urea-NBTPT showed highly variable degradation rates and different patterns of degradation, even when they were prepared in the same way. The major factor(s) affecting degradation of NBTPT in urea were never determined.
- 6. Despite the problems observed in laboratory-scale preparations, the materials produced by melt granulation in a commercial-sized plant were extremely stable. Thus, the problem of NBTPT degradation is

apparently of more concern to researchers intending to repeat experiments over seasons than for commercialization.

The conversion of NBTPT (or other thio phosphoryl triamides) to the active oxygen analog will likely be dependent on a number of as yet undetermined biotic and abiotic soil properties. The rate of conversion along with the relative absorption and degradation rates of the two compounds will determine the compound's effectiveness. The experience with flooded soils indicates that these rates can be affected not only by intrinsic but also by transient soil factors, such as algal growth or soil mixing. This area of research will be important in further attempts to use the thio proinhibitors in flooded soil systems.

These very active triamide inhibitors and proinhibitors have mobility very similar to that of urea in soils [19]. Thus, they facilitate movement of urea away from the soil surface prior to hydrolysis, slow nitrification, and cause less increase in pH at the microsite of the fertilizer granule. Despite a recent assertion [1], these compounds, as far as is currently known, have no toxic or detrimental effects, nor do their degradation products (A. Sutton, IMC-Agrico, pers. commun.). Prior to their use in tropical systems, further research on the forms and amounts needed will be required. Commercialization of NBTPT in the United States may lead to its availability in tropical countries as the requirements and economic considerations are worked through.

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