catecholamines is achieved by inhibition of TH by MT-Me, the concentration of dopamine and norepinepherine decrease in parallel [8]. As seen in Fig. 4, MFMT-Me depletes norepinephrine before dopamine. *In vitro* at least, neither MFMT-Me nor MFMT inhibits dopamine β-hydroxylase at concentrations up to 1 mM. To explain the preferential depletion of norepinephrine by MFMT-Me, we would like to suggest that the ratio between the AADC and TH activities is smaller in noredrenergic neurons than in dopaminergic neurons. Less inhibition of AADC activity is therefore necessary before the decarboxylation of Dopa becomes rate-limiting in the synthesis of norepinephrine. Depletion of catecholamines by MFMT-Me is found with significant AADC remaining—this was not the case with MFMD [3]. A reasonable explanation is that MFMD indiscriminately inhibits AADC whether it is functional in amine synthesis or not, while MFMT which needs activation by TH will inhibit AADC in those sites where TH-activity is the most elevated, i.e. in sites of active amine synthesis. Therefore MFMT should be selective for neuronal AADC and could possibly be used as a marker for catecholaminergic neurons.

In conclusion, MFMT, or better MFMT-Me, has a number of advantages over MFMD as a means to regulate catecholamine synthesis and hopefully to study the pharmacological consequences thereof: selective depletion of catecholamines over indoleamines, selectivity at low doses for the norepinephrine system, sensitivity to factors regulating TH activity. We believe that this represents the first attempt to use an enzyme to generate a suicide inhibitor for an enzyme coming later in the normal catabolic cascade.

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Reversibility of protein synthesis inhibition by quassinoid antineoplastic agents in a rabbit reticulocyte system

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Bruceantin, a quassinoid now in phase II clinical trials, was first isolated from *Brucea antidysenterica* and was shown to have potent antitumor activity in several animal screens [1, 2]. The antitumor activity of bruceantin is presumed to be due to its strong inhibition of eukaryotic protein synthesis, although it does inhibit DNA synthesis to a lesser extent [3]. Detailed studies have shown that bruceantin binds reversibly to the peptidyl transferase center of eukaryotic ribosomes and inhibits peptide bond formation [4]. More recently, the structurally related compound brusatol has been obtained from *Brucea javonica* [5, 6] and has been shown to possess potent antitumor activity [5, 7]. Brusatol inhibits protein synthesis by the same mechanism as bruceantin in both rabbit reticulocytes [8] and P-388 cells [7].

Liao *et al.* [3] originally classified these compounds as irreversible inhibitors of protein synthesis. Their data conflict with the later observations by Fresno *et al.* [4] that quassinoids bind reversibly to the ribosome. Since the question of reversibility of inhibition by potential therapeutic agents is of practical interest, the experiments in this paper were designed to resolve this issue.

Materials and Methods

[^{3}H]Leucine (125 Ci/mmole) was obtained from Amersham-Searle. [^{14}C]Trichodermin (20.4 mCi/mmole, 2.66 mM) was obtained from Research Triangle Institute (Research Triangle Park, NC) on special contract. The [^{14}C]trichodermin was synthesized from trichodermol (supplied by Dr. W. O. Godfredson, Leo Pharmaceutical Products) and [1-^{14}C]acetic anhydride as previously described by Barbacid and Vazquez [9]. Bruceantin and brusatol were prepared as described previously [5, 6]. Both compounds were initially dissolved in ethanol–acetone (1:1). After removal of the acetone under a stream of nitrogen, each drug was diluted to a 200–500 μM stock solution in 2.5% ethanol and stored in small aliquots at −20°. ATP, GTP, and creatine phosphate were obtained from Sigma. Creatine phosphokinase was obtained from Boehringer–Mannheim.

Rabbit reticulocyte lysates were prepared from New Zealand minikin rabbits weighing 1–2 kg as described by Hardesty *et al.* [10]. Dialysis of lysates was carried out against buffer D (5 mM Tris–Cl, pH 7.6; 1 mM 2-mercaptoethanol; 1 mM $MgCl_2$). The 80 S run-off ribosomes were prepared from the lysate essentially as described by Falvey and Staehelin [11].

Endogenous protein synthesis in rabbit reticulocyte lysates was carried out in an assay containing: 2/3 vol. of lysate, 5 mM Tris–Cl (pH 7.6), 78 mM KCl, 1.4 mM $MgCl_2$, 7.8 mM creatine phosphate, 0.3 mg/ml creatine phosphokinase, 15 μM hemin, 0.1 mM ATP, 0.05 mM GTP, 0.1 mM each of nineteen amino acids, 0.6 mM 2-mercaptoethanol, and 50 μCi [^{3}H]leucine (125 Ci/mmole). Incubation was at

30°. Hot trichloroacetic acid precipitable counts were measured as described by Williams *et al.* [12].

Binding of [^{14}C]trichodermin to 80 S rabbit reticulocyte run-off ribosomes was measured using the sedimentation assay essentially as described by Fernandez-Muñoz *et al.* [13, 14]. Each incubation (240 μl) contained 2.1 μM run-off ribosomes, 5.7% sucrose, 45 mM Tris–Cl (pH 7.6), 95 mM KCl, 7.5 mM $Mg(OAc)_2$, 0.07 mM disodium EDTA, 4.25 mM dithiothreitol, 1.0 μM [^{14}C]trichodermin (TCD)* and either 10 μm unlabeled brusatol or an equivalent volume of 2.5% ethanol. The total TCD present in each assay mixture was determined by removing a 50-μl aliquot and counting directly in toluene scintillation fluid. Following equilibration, 150 μl of the remaining sample was then placed in a 175 μl centrifuge tube (Beckman) and centrifuged in an A/100 rotor in a Beckman airfuge at 30 psig (165,000 *g*) for 60 min. The airfuge was specially adapted with a refrigeration unit to maintain 4° throughout the centrifugation. Free TCD was determined by counting a 50 μl aliquot of the supernatant fraction. TCD bound to the run-off ribosomes was calculated as total TCD − free TCD after correction for quenching and counting efficiency as described by Considine *et al.* [15].

Results and Discussion

Preliminary experiments confirmed the original observations of Liao *et al.* [3]. Whole rabbit reticulocytes were exposed to 100 μM brusatol, 100 μM bruceantin, or an equivalent volume of 2.5% ethanol for 10 min at 30°. The cells were then cooled to 4° and washed several times with fresh medium and assayed for their ability to carry out protein synthesis as described by Liao *et al.* [3]. Complete inhibition of protein synthesis was obtained with 100 μM brusatol or bruceantin, and no reversal of the protein synthesis inhibition was observed even after five washes with fresh medium (data not shown). To determine whether the drugs were binding very tightly to some component required for protein synthesis or were merely unable to diffuse out of the cell, the following experiment was performed. Whole rabbit reticulocytes were exposed to 100 μM brutasol, 100 μM bruceantin, or an equivalent volume of 2.5% ethanol for 10 min at 30°. The cells were then rapidly cooled to 4° and a lysate was prepared from both drug-treated and untreated cells as described previously [8]. The lysates were dialyzed against 1000 vol. of buffer D (5 mM Tris–Cl, pH 7.6; 1 mM 2-mercaptoethanol; 1 mM $MgCl_2$) for 3 hr at 4° and assayed for protein synthesis as described in Materials and Methods. Once again, no recovery of protein synthetic activity was seen following this brief dialysis (data not shown). These data suggested that brusatol and/or bruceantin do indeed bind very tightly or irreversibly to some intracellular component required for protein synthesis.

To characterize this inactivation more fully, the time course for both inactivation and recovery of activity in rabbit reticulocyte lysates was examined. Duplication of the results obtained previously with whole cells required a careful choice of incubation conditions. Initially, the experiments were carried out by incubating the lysates with 100 μM brusatol, 100 μM bruceantin, or an equivalent volume of 2.5% ethanol for 10 min at 30°. The lysates were then cooled and either assayed for protein synthesis immediately (Fig. 1A) or dialyzed 3 hr and assayed for protein synthesis (Fig. 1B). Under these conditions, the inhibition of protein synthesis appeared to be completely reversible. However, a close examination of Fig. 1A suggests that no inhibition of protein synthesis had actually taken place during the 10-min preincubation. For both

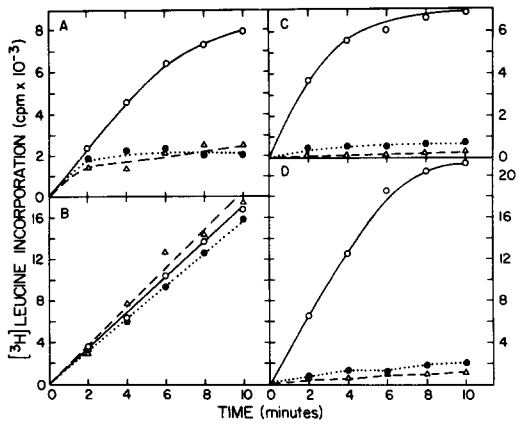

Fig. 1. Effect of preincubation conditions on the apparent reversibility of protein synthesis inhibition by brusatol and bruceantin. Key: (A) Rabbit reticulocyte lysates were preincubated with either 100 μM brusatol (△), 100 μM bruceantin (●), or an equivalent volume of 2.5% ethanol (○) for 10 min at 30°. The samples were immediately cooled to 4° and subsequently assayed for protein synthesis at 30° as described by Williams *et al.* [12]; (B) The conditions were the same as in 1A, except that between preincubation and assay the lysates were dialyzed against 1000 vol. of buffer D for 3.0 hr at 4°; (C) The conditions were the same as in 1A, except that the preincubation mixture contained all of the components required for the protein synthesis assay except the [^{3}H]Leu; and (D) The conditions were the same as 1C, except that between preincubation and assay the lysates were dialyzed against 1000 vol. of buffer D for 3.0 hr at 4°. Each data point represents an average of four independent measurements. The standard error of the mean is less than 5% of the average value for each data point.

brusatol and bruceantin, there was a 2-min lag before the onset of inhibition in Fig. 1A, which is exactly what one would have expected if brusatol or bruceantin had been added at time 0 [3, 8].

Previous studies have shown that brusatol and bruceantin will only bind effectively to free 80 S ribosomes [4, 8] and that effective inhibition only occurs under protein synthesis conditions which allow ribosome cycling [8]. Thus, if the concentration of free 80 S ribosomes in the reticulocyte lysate were very low and if one or more components required for protein synthesis were limiting during preincubation, one might expect the apparent lack of inhibition observed during the preincubation period in Fig. 1A. This hypothesis was tested by adding all of the components required for protein synthesis (except [^{3}H]leucine) during the preincubation period. The corresponding experiment is shown in Fig. 1, panels C and D. Under these conditions, complete inhibition appeared to be achieved during the preincubation period. No lag in inhibition by either brusatol or bruceantin was observed when the lysate was assayed immediately following the preincubation (Fig. 1C). Furthermore, the inhibition was not reversible by a 3-hr dialysis at 4° (Fig. 1D), which agreed with our previous observations. Subsequent experiments confirmed that effective inhibition required the presence of all of the components required for protein synthesis.

To determine whether the interaction between quassinoids and one or more components required for protein synthesis was truly irreversible, or merely an example of very tight binding, lysates which had been inactivated by brusatol as described in Fig. 1B, were dialyzed for extended periods of time at 4° against buffer D. The results are shown in Fig. 2. The inhibition was partially reversible by

* Abbreviations: TCD, trichodermin; and poly-U polyuridylic acid.

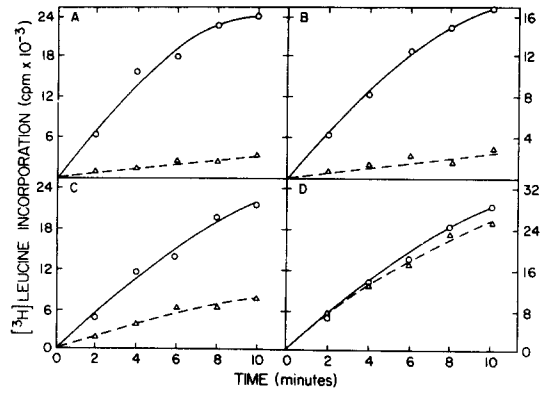

Fig. 2. Reversal of protein synthesis inhibition by brusatol following long dialysis at 4°. Rabbit reticulocyte lysates were preincubated with either 100 μM brusatol (△) or an equivalent volume of 2.5% ethanol (○) for 10 min at 30° as described in Fig. 1C. Each sample was then cooled to 4° and dialyzed for various lengths of time against 1000 vol. of buffer D at 4°. Immediately following dialysis, the samples were assayed for endogenous protein synthesis as described by Williams *et al.* [12]. Key: (A) no dialysis; (B) 3-hr dialysis, one change of buffer; (C) 6-hr dialysis, three changes of buffer; and (D) 18-hr dialysis, five changes of buffer. Each data point represents the average of four independent measurements. The standard error of the mean is less than 6% of the average value for each data point.

6 hr of dialysis and fully reversible by 18 hr of dialysis at 4°. Clearly, while brusatol and bruceantin bind very tightly to some component(s) required for protein synthesis, that binding is slowly reversible at 4°.

Since Fresno *et al.* [4] demonstrated rapid reversibility of binding to ribosomes, it became of particular interest to determine which component(s) was inactivated in the previous assay. We have shown previously that the inhibition observed in a poly U-directed polyphenylalanine-synthesizing system is sufficient to explain the inhibition observed in whole lysates [8]. Since the polyphenylalanine-synthesizing system contains far fewer rabbit reticulocyte components, an experiment was carried out to determine which component(s) was inactivated in that system (Table 1). Either the pH 5 enzyme mixture or the 80 S run-off ribosomes were preincubated with 10 μM bruceantin. These components were then either assayed immediately or dialyzed before being assayed. Both components were diluted 1:10 in the final assay mixture, so the bruceantin concentration in the final assay was approximately 1 μM when either drug-treated pH 5 enzymes or drug-treated ribosomes were included in the assay without prior dialysis. We have shown previously that 1 μM bruceantin inhibits poly-U-directed polyphenylalanine synthesis by 50% [8, 15]. Thus, one would expect approximately 50% inhibition in the final assay even if no inhibition had occurred during the preincubation period. As expected, when drug-treated pH 5 enzymes were added to the assay immediately after preincubation, activity was only 54% of control. Following dialysis to remove bruceantin from the pH 5 enzyme preparation, the polyphenylalanine-synthesizing activity in the final assay mixture was restored. However, when drug-treated ribosomes were added to the assay immediately after preincubation, the activity was only 28% of control, and the activity was only slowly regained during subsequent dialysis. These results indicate that the ribosomes were likely the only component inactivated and that inactivation of the ribosomes was slowly reversible at 4°.

The slow reversibility of binding of these compounds to rabbit reticulocyte ribosomes at 4° did not agree with the rapid reversibility reported by Fresno *et al.* [4]. However, the possibility remained that the binding might be much more rapidly reversible at 30°. Since dialysis at 30° led to very rapid loss of activity, the binding of brusatol to rabbit reticulocyte run-off ribosomes was also assessed by measuring competition with [^{14}C]trichodermin [4] in the rapid sedimentation assay described by Fernandez-Muñoz *et al.* [13, 14]. Fresno *et al.* [4] have shown that bruceantin and trichodermin compete for a single binding site on yeast ribosomes, and we have confirmed these findings with other quassinoids and trichodermin on rabbit reticulocyte ribo-

Table 1. Reversibility of inhibition of polyuridylic acid-directed polyphenylalanine synthesis by bruceantin

Pre-assay treatment*	Polyphenylalanine synthesis†	
	pmoles‡	% of Control
No dialysis§		
Control	342 ± 15	100
pH 5 Enzymes treated with bruceantin	185 ± 8	54
Ribosomes treated with bruceantin	97 ± 4	28
3-hr Dialysis following drug treatment‖		
Control	140 ± 8	100
pH 5 Enzymes treated with bruceantin	138 ± 6	99
Ribosomes treated with bruceantin	45 ± 3	33
6-hr Dialysis following drug treatment¶		
Control	77 ± 4	100
pH 5 Enzymes treated with bruceantin	81 ± 3	105
Ribosomes treated with bruceantin	36 ± 2	47

* The pH 5 enzyme preparation or the run-off ribosomes were preincubated with 10 μM bruceantin or an equivalent volume of 2.5% ethanol for 10 min at 30°.

† Polyphenylalanine synthesis was measured as described by Willingham *et al.* [8].

‡ Each value is the mean ± S.E.M. for three independent measurements.

§ Concentration of bruceantin during the final incubation with treated samples was 1 μM. This would be expected to result in about 50% inhibition of polyphenylalanine synthesis in this assay.

‖ Dialysis against 1000 vol. of buffer D, one change of dialysis buffer.

¶ Dialysis against 1000 vol. of buffer D, two changes of dialysis buffer.

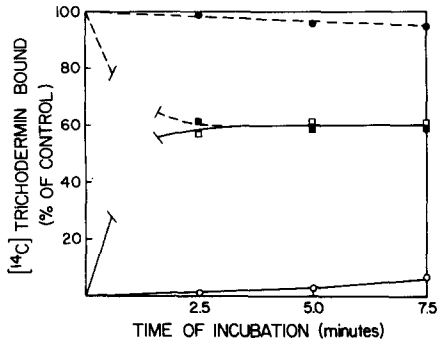

Fig. 3. Time course of competition between brusatol and [^{14}C]trichodermin for binding to run-off ribosomes. The final binding assay mixture contained 1 μM [^{14}C]trichodermin, 2.1 μM run-off ribosomes, and 10 μM brusatol. The binding of [^{14}C]trichodermin to the run-off ribosomes following the indicated times of incubation was determined as described by Considine *et al.* [15]. Key: (●---●) ribosomes were preincubated with [^{14}C]trichodermin for 5 min at 30° and then cooled to 4°. At this point the brusatol was added and the incubation continued for the indicated period of time at 4°. Immediately following the incubation, the amount of [^{14}C]trichodermin bound was determined as described by Considine *et al.* [15]; (■---■) the same as (●) except that the incubation was carried out at 30°; (○——○) ribosomes were preincubated with brusatol for 5 min at 30° and then cooled to 4°. At this point [^{14}C]trichodermin was added and the incubation continued for the indicated period of time at 4°; and (□——□) the same as (○) except that the incubation was carried out at 30°. Each data point represents the average of three independent measurements. The standard error of the mean is less than 8% of the average value for each data point.

somes [15]. The actual experiment was carried out by preincubating the ribosomes with either brusatol or [^{14}C]trichodermin at 30° and then adding the competing ligand and measuring the rate of approach to equilibrium at either 4° or 30° (Fig. 3). The data in Fig. 3 do not yield actual rate measurements, but it is apparent that equilibrium was reached in less than 2.5 min at 30°, while considerably more time was required at 4°.

Thus, from the available data, we conclude that the quassinoid antineoplastics bind very tightly to the ribosomes of rabbit reticulocytes. It is likely that this represents the major site of inactivation of protein synthesis. While this binding was only slowly reversible at 4°, it was very rapidly reversible at more physiological temperatures.

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