target (Fig. 2B) or 180° out of phase with it (Fig. 2D). In both situations the unit continued to modulate in relation to the track-target's velocity, increasing its rate with the leftward movement. All 13 track-related Purkinje cells tested on all of these paradigms showed essentially the same pattern of firing. In the few units examined under conditions in which the movements of the target and chair were completely dissociated, track-target motion relative to earth-fixed surroundings was still the crucial factor. Irregularities in firing which are apparent as noise in the reciprocal interval plots tend to obscure the correlation with target velocity; they may reflect microvariations in the monkey's performance which are beyond the resolution of our oculogram recordings but are nonetheless significant.

That these neural correlates of visual tracking in the primate flocculus are probably not mere epiphenomena but may instead reflect some vital role in the programming of pursuit eye movements is suggested by the deficits which follow lesions in this area. Severe, and relatively specific, impairments of smooth pursuit eye movements are often associated with cerebellar atrophy in man (*8*), and total cerebellectomy in the mature monkey results in the permanent loss of such eye movements (*9*). Deficits in optokinetic responses after bilateral flocculectomy in monkeys have been reported, but, unfortunately, the animals were not examined specifically for pursuit eye movements of the kind under consideration here (*10*).

We hypothesize that Purkinje cell output from the primate flocculus provides oculomotor centers with target velocity information essential for visual tracking and represents an output of the smooth pursuit subsystem. In addition, we have some data which may help explain how the flocculus generates these velocity command signals. Most units in the flocculus showed no evidence of a CS, and we assume that they represent one or another of the various input elements known to influence Purkinje cells. The majority of such units fired vigorously in relation to saccadic eye movements often with both transient and tonic components but no apparent special concern with tracking; others seemed to be driven by vestibular inputs, modulating nicely in phase with chair (that is, head) velocity. We became aware of a class of visually driven units lacking CS's; these were especially sensitive to retinal image slip in the region of the fovea and often were more responsive to ipsilateral target movements. Firing of these units may be the putative error signal that ultimately sustains pursuit eye movements. These units closely resemble those recently described for the monkey's nucleus of the transpeduncular tract (*11*), a part of the accessory optic system; at least in the rabbit, this tract projects to the flocculus (*12*). Signal processing in the pursuit system may require a precise velocity representation of the target (*13*); we propose that this is the function of the Purkinje cells in the primate flocculus. A true neuronal facsimile of the track target's absolute velocity would require the summing of three signals: velocity of the target's retinal image (target motion relative to eye motion), eye velocity (eye motion relative to head motion), and head velocity (head motion relative to earth motion); we know that information concerning the first and last of these reaches the flocculus, and the second might easily be derived from the numerous inputs related to eye movements. A possible complication arises if the system has predictive capabilities, since the tracking waveforms contrived in our study were usually highly periodic (sinusoids and linear ramps).

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6. About one-quarter of the units identified as Purkinje cells showed no consistent modulation with any of the parameters considered in our study, except for rather weak, transient pauses during saccadic movements. Some of these neurons may have been involved in vertical tracking, a possibility we did not investigate.
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Antimitotic Activity of the Potent Tumor Inhibitor Maytansine

Abstract. *Maytansine, at 6×10^{-8}M, irreversibly inhibits cell division in eggs of sea urchins and clams. It causes the disappearance of a mitotic apparatus or prevents one from forming if added at early stages. Maytansine does not affect formation of the mitotic organizing center but does inhibit in vitro polymerization of tubulin. Maytansine and vincristine inhibit in vitro polymerization of tubulin at about the same concentrations, but vincristine is about 100 times less effective as an inhibitor of cleavage in marine eggs.*

Maytansine, a novel ansa macrolide (Fig. 1), isolated from various *Maytenus* species, is an antitumor agent (*1*) that significantly inhibits mouse P-388 lymphocytic leukemia in dosages of micrograms per kilogram of body weight, and is active over a 50- to 100-fold dosage range. Maytansine also shows significant inhibitory activity against the L-1210 mouse leukemia, the Lewis lung carcinoma, and the B-16 melanocarcinoma murine tumor systems. This agent is being tested toxicologically in preparation for clinical trials (*2*).

We report now on the antimitotic effects of maytansine. At a concentration of $6 \times 10^{-8}M$, it totally inhibited cleavage in sea urchin eggs when applied at fertilization (*3*). At $4 \times 10^{-8}M$, 10 to 20 percent of the eggs divided (although cleavage was somewhat irregular). The remaining eggs formed irregular furrows that either did not separate equal-sized blastomeres or they subsequently retracted. When the eggs were treated with $10^{-8}M$ (or less) maytansine, the cleavage time, cleavage pattern, and later development were normal.

The critical time in egg development for inhibition of cleavage by $10^{-7}M$ maytansine was determined by adding the drug at 5-minute intervals, from the time of fertilization to first cleavage. Cleavage was totally inhibited when the drug was added at any time during the first half of the cleavage period; after that, an increasing number of cells went through some form of cleavage. However, even when the drug was added 10 minutes prior to cytokinesis, approximately 40 percent of the eggs did

not cleave, and those that did, looked irregular and did not cleave a second time. When drug was added to unfertilized eggs for at least 1 hour, it could be removed prior to fertilization, with normal cleavage and development following. Drug added immediately after fertilization could be removed by repeated washings in seawater up to 20 minutes after fertilization, with minimal effects on cleavage pattern or rate. If removed between 20 and 30 minutes, irregular cleavages occurred; and if removed 30 minutes or more after fertilization, cleavage (which occurs 60 to 90 minutes after this point in controls) was permanently inhibited. Both the minimum concentration and critical time for total inhibition of cleavage were the same for the eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus*.

We investigated the possibility that the critical process affected by maytansine is DNA synthesis by determining the incorporation of [^{3}H]thymidine (*4*). The rate and amount of DNA synthesis was the same in eggs treated continuously with $10^{-7}M$ maytansine from 10 minutes before fertilization as that in controls, even though cleavage was totally inhibited in treated eggs.

In sea urchin eggs treated with $10^{-7}M$ maytansine before fertilization or starting 10 to 15 minutes after fertilization, fusion of the male and female pronuclei never occurred. A sperm aster (SA) (*5*) did not form in the egg. Since this body is necessary for the transport of the pronuclei to the egg center (*5*), pronuclear fusion does not occur in its absence. Further, since microtubules form an integral part of the SA (*6*), these observations suggested that maytansine may interfere with microtubule formation or tubulin mobilization into the aster.

To investigate the possibility that the drug might prevent the formation of mitotic apparatuses (MA's) as well as SA's, we used clam eggs (from *Spisula solidissima*) because of the ease with which the MA can be seen with a polarization microscope and the rapidity of MA formation [the MA starts to form about 10 minutes after fertilization or activation (*7*)]. Eggs treated with $10^{-7}M$ maytansine in seawater were parthenogenetically activated with KCl-seawater solutions (*7*). Activation of the eggs was normal and rupture of the nuclear membrane occurred on schedule with drug treatment, but an MA did not form. When maytansine was applied as late as 10 minutes after activation, an MA was not obtained; but eggs treated with $10^{-8}M$ maytansine had normal MA's (similar to those of the controls). Examination of eggs activated in the presence of maytansine at concentrations between $10^{-8}M$ and $10^{-7}M$ revealed the following. At $3 \times 10^{-8}M$, the MA was about one-half normal length and width (Fig. 2, a and b); at $6 \times 10^{-8}M$ (a dose that completely blocked polar body formation) the MA was approximately one-third normal length and one-fourth normal width (Fig. 2c); at $10^{-7}M$ a very small birefringent MA was present in the center of the egg and could not be seen without compressing the egg (Fig. 2d). Thus, maytansine regulates the size of the MA in a dose-dependent manner.

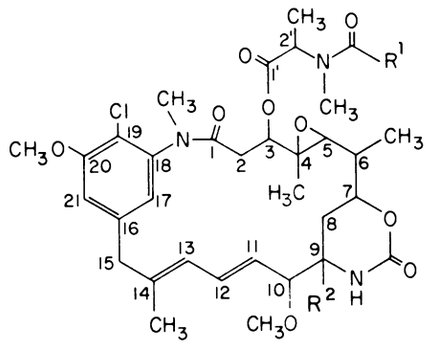

Fig. 1. Maytansine, $R^1 = CH_3$ and $R^2 = OH$; maytanbutine, $R^1 = CH(CH_3)_2$ and $R^2 = OH$; maytanbutine 9-*n*-propyl thioether, $R^1 = CH(CH_3)_2$ and $R^2 = SCH_2CH_2CH_3$.

We studied the question of whether maytansine could affect SA's in sea urchin or MA's in clam eggs once the structure had already formed. We found that SA's in sea urchin eggs and already formed MA's in clam eggs could be made to shrink in a dose-dependent manner by application of maytansine. Although exact measurements have not yet been made, the final size of the MA appears to be the same whether the MA formed in the presence of certain concentrations of maytansine or whether that concentration is added subsequent to MA formation.

Maytansine inhibits the formation of

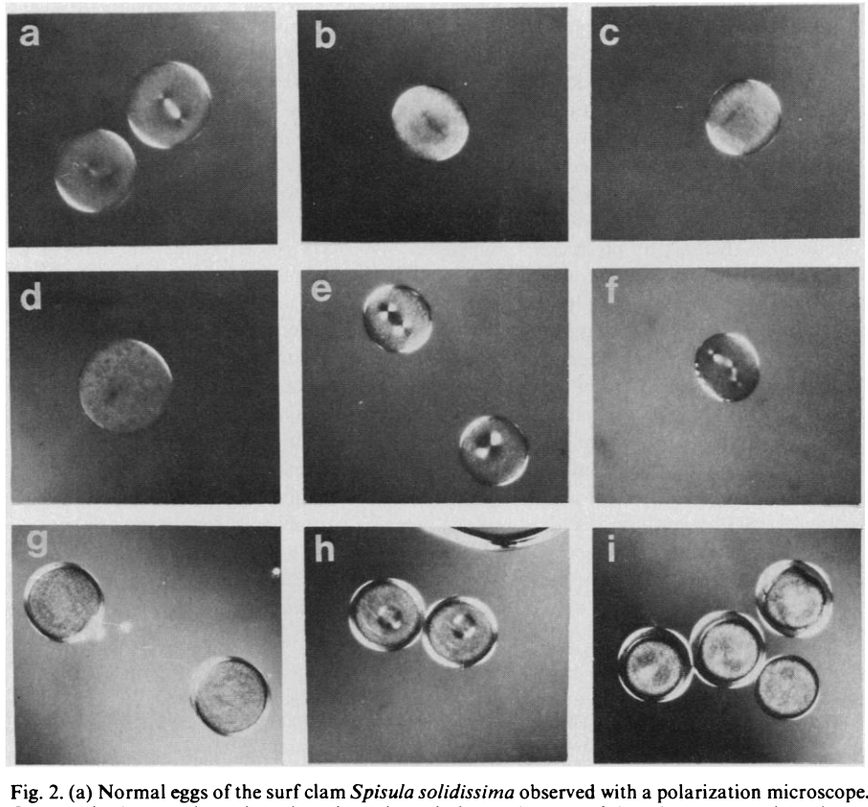

Fig. 2. (a) Normal eggs of the surf clam *Spisula solidissima* observed with a polarization microscope. Contrast in the MA depends on its orientation relative to the axes of the microscope and can be reversed by rotation of the MA axis by 90° or change in angle of a compensator (×300). (b) *Spisula* egg treated with $3 \times 10^{-8}M$ maytansine prior to activation. The MA is reduced in size; the egg will form a first polar body, but it is considerably delayed in time (×300). (c) *Spisula* egg treated with $6 \times 10^{-8}M$ maytansine prior to activation. The first polar body never forms (×300). (d) *Spisula* egg treated with $2 \times 10^{-7}M$ maytansine prior to activation and compressed to visualize very small MA still remaining in the cytoplasm (×300). (e) *Spisula* egg treated with $6 \times 10^{-8}M$ maytansine plus 3 percent HG; compare with (c). The degree of augmentation with glycols decreases with increasing concentration of maytansine (×300). (f) *Spisula* egg in $2 \times 10^{-7}M$ maytansine treated with 3 percent HG to "develop" the MA after 30 minutes. Double MA indicates replication of the MTOC (×300). (g) Eggs of sea urchin *S. purpuratus* treated with $2 \times 10^{-7}M$ maytansine to eliminate SA (×190). (h) Augmented SA in *S. purpuratus* eggs treated with 5 percent HG (×190). (i) *S. purpuratus* eggs treated with 5 percent HG to which $4 \times 10^{-7}M$ maytansine is then added. SA is reduced in size in a dose-dependent manner similar to the phenomenon in MA of clam eggs (×190).

the MA, either by inhibition of a mitotic organizing center (MTOC) (*8*), by interference with tubulin polymerization onto the center, or by some other mechanism. We tested the possibility that it might act on the MTOC by utilizing the long-chain glycol hexylene glycol (HG) since HG and other similar glycols cause augmentation of size and birefringence of SA's and MA's in a manner requiring the presence of an active MTOC (*9*). For these experiments both clam and sea urchin eggs were treated with maytansine before, at the same time as, or after treatment with 3 percent HG in seawater. At concentrations of $10^{-7}M$ maytansine an SA or MA was formed which was intermediate in size between normal and augmented SA's (Fig. 2, g to i) and MA's; at $3 \times 10^{-7}M$ maytansine, even the augmented structures could be caused to disappear. Further, clam eggs activated in maytansine and left for a half hour or so show multiple MA's or asters when augmented with glycols (Fig. 2f), suggesting that replication of the MTOC is not inactivated by the drug. Thus, maytansine does not appear to affect the ability of the MTOC to divide or to act as an organizing center for tubulin assembly. In this respect it acts in a manner similar to that of colchicine (*8*).

We then studied the effects of maytansine on brain tubulin polymerization (*10*), which can be used as a model for MA tubulin (*11*). Rabbit or pig brain tubulin was prepared by a slight modification of the method of Weisenberg (*10*) and purified through one or two cycles of polymerization. In each case polymerized microtubules were washed in buffer by centrifugation, a procedure that yields relatively pure tubulin after one cycle of polymerization, as judged by sodium dodecyl sulfate gel electrophoresis. Microtubules were depolymerized by cooling to 0°C in buffer in which guanosine triphosphate (GTP) was absent, and the resulting tubulin was used in subsequent experiments. Polymerization of tubulin was followed by turbidity increase at 310 nm (*12*) in a Gilford automatic recording spectrophotometer at 37°C. A sample of tubulin containing 1 mM Ca^{2+} and no added GTP was used as a blank, and turbidity was recorded as the difference between sample and blank. Polymerization was initiated by adding GTP to tubulin at 37°C. In initial experiments, maytansine was added to cold tubulin. Total inhibition of polymerization was found at $10^{-5}M$ maytansine in tubulin solutions of 4 to 5 mg/ml. With $5 \times 10^{-6}M$ maytansine the extent of polymerization of tubulin at 5 mg/ml was about 50 percent, as judged by turbidity at plateau levels. Maytansine added to polymerized tubulin caused a rapid decrease in turbidity to levels approximately the same as those attained when the same concentration was added before polymerization was started (Fig. 3). Inhibition of polymerization is about one-half at a mole ratio of 1 part of maytansine to 10 parts of tubulin, and total inhibition occurs at a mole ratio of 1 to 5.

To ascertain whether the mole ratio of maytansine to tubulin required for inhibition of in vitro polymerization could account for its antimitotic effects we assumed that maytansine would equilibrate between seawater and egg cytoplasm. The number of tubulin subunits in an MA the size of that of *S. purpuratus* is about 2×10^8 (*13*). Since the egg diameter is 75 μm, there should be about 1.2×10^7 molecules of maytansine in each egg at $10^{-7}M$ in the seawater if the drug equilibrates, and more if accumulation occurs. This is a ratio of 1 maytansine molecule to 16 tubulin molecules. Since brain tubulin has been shown to be a good model for MA tubulin (*11*), these results suggest that the inhibition of tubulin polymerization by maytansine can account for its in vivo antimitotic effects, given the nature of the calculations. Initial uptake experiments with [^{3}H]maytansine suggest a rapid accumulation of maytansine by eggs although the kinetics are not simple. They nevertheless suggest that enough maytansine enters the egg to support the above analysis.

Because the report of Wolper-Defilippes *et al.* (*14*) (which appeared as our experiments were nearing completion) suggested a relation of maytansine to vincristine in its antimitotic effects, we compared the two drugs for inhibition of tubulin polymerization in vitro and for inhibition of cleavage in vivo. Sea urchin eggs did not cleave in $10^{-5}M$ vincristine but formed irregular shallow furrows. At $5 \times 10^{-6}M$ vincristine cleavage was approximately normal; thus, maytansine is at least 100 times more potent as an antimitotic agent than vincristine. A difference in potency of about 15-fold was obtained with Chinese hamster ovary cells in culture. The molar concentration of vincristine necessary to inhibit polymerization of a given amount of brain tubulin, however, is actually somewhat lower for vincristine, and the kinetics of inhibition are very different. Colchicine inhibits tubulin polymerization in vitro at molar ratios even lower than that of maytansine (*15*) but requires a minimum dose of $10^{-4}M$ or more for inhibition of cleavage in sea urchin eggs (*16*). Why such marked differences of potency for MA inhibition in vivo exist in the face of the ability of all agents to inhibit brain tubulin polymerization in vitro to approximately the same degree is not known, but may be due to differences in uptake. However, another possibility suggests itself since we have found that oxidation of the sulfhydryl groups in tubulin inhibits its polymerization (*17*); conceivably maytansine acts by binding certain key sulfhydryls of tubulin.

The latter suggestion is supported by the readiness with which maytanbutine, a homologous maytansine ester, alkylated *n*-propane thiol to form the 9-*n*-propyl thioether (*18*). Presumably, maytansine may alkylate protein thiols in an analogous manner (*2*). We have also found that geld-

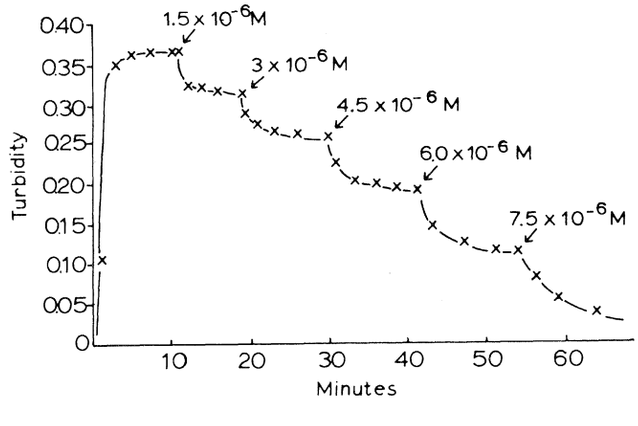

Fig. 3. Change in turbidity at 310 nm (ordinate) as a function of time. Brain tubulin was prepared as described in the text and was resuspended at 4.7 mg/ml ($4.27 \times 10^{-5}M$, assuming a molecular weight of 110,000 for tubulin). Polymerization was followed at 37°C by recording turbidity increase at 310 nm after addition of GTP (*12*) with the use, as a blank, of a sample of the same tubulin that was made 1 mM in $CaCl_2$ and which had no added GTP (thus, polymerization was inhibited). Maytansine was added to sample and blank at the indicated points to give the final concentrations shown. The plateau levels reached were the same as those attained (within about 10 percent) if maytansine was added prior to initiation of polymerization by GTP. The turbidity in $7.5 \times 10^{-6}M$ maytansine eventually reached the baseline. In each case a portion of the sample was checked for birefringence with a polarizing strain detector. Birefringence only disappeared in the $7.5 \times 10^{-6}M$ sample when turbidity (compared to blank) vanished. We have found that birefringence of tubulin solutions always correlates with the presence of microtubules when viewed with the electron microscope. The ratio of tubulin to maytansine for decrease of turbidity by one-half was approximately 10 : 1 in four separate experiments performed at different tubulin concentrations. The ratio for complete depolymerization was approximately 5 : 1.

anamycin, an ansa macrolide closely related to maytansine (*19*) but lacking the carbinolamide functionality involved in –SH alkylation (and which does not show antileukemic activity), is 1000 times less effective in inhibition of sea urchin egg cleavage. However, at $5 \times 10^{-5} M$ geldanamycin affects the MA in a manner analogous to that of maytansine and the effect is reversible.

Why some tumors should be sensitive to maytansine in vivo when it is an antimitotic agent which can also inhibit normal cells is not clear. We know of no evidence that tubulin of tumor cells differs from that of normal cells. However, microtubules have been implicated in certain cell surface related processes in lymphocytes, polymorphonuclear leukocytes, and other cells (*20*); and, since tumor cell surfaces differ from those of normal cells (*21*), it is not unlikely that specificity resides in such cell surface properties.

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Cancer by County: New Resource for Etiologic Clues

Abstract. *Mapping of U.S. cancer mortality by county has revealed patterns of etiologic significance. The patterns for bladder cancer in males point to industrial determinants: some are known (chemical manufacturing) but others (automobile and machinery manufacturing) represent new leads for epidemiologic study. By contrast, the geographic clusters of high rates of stomach cancer in both sexes are consistent with ethnic susceptibility.*

Geographic variation in cancer mortality in the United States has usually been evaluated on a state-by-state basis. The paucity of clues to cancer etiology arising from such surveys can be traced to the heterogeneity of statewide populations. Counties may provide a compromise, as units small enough to be homogeneous for demographic and environmental characteristics that might influence cancer risk, and yet large enough for stable estimates of site-specific cancer mortality. We have made some preliminary observations that illustrate the value of county mortality measurements in providing leads to the origins of cancers.

We obtained age-, race-, and sex-specific numbers of cancer deaths for the 3056 counties of the contiguous United States over a 20-year period, 1950–1969, from the National Center for Health Statistics, Rockville, Maryland. Corresponding county populations were provided by the 1950, 1960, and 1970 censuses (*1*), with intercensal estimates derived by linear interpolation. For 35 cancer sites, we calculated age-standardized mortality rates by race and sex in each county, the standard being the age distribution of the entire U.S. population in 1960. Ninety-five percent confidence intervals were computed using the standard error of the age-standardized rate as determined by the method of Chiang (*2*). Differences between the county and national rates were statistically significant when the 95 percent confidence intervals for these rates did not overlap. Tabulations of cancer mortality rates by county were recently compiled (*3*).

Table 1. Industrial categories in which the percentage of men employed in counties where the bladder cancer risk is high differed significantly ($P < .05$) from the percentage of men employed nationwide. See text for method of selecting high-risk counties. Abbreviations: Exp., expected; Obs., observed.

Industry	Percentage of employed men		
	In the U.S. (Exp.)	In high-risk counties (Obs.)	Obs./Exp.
Agriculture	15.8	4.2	0.3
Mining	2.2	0.3	0.1
Manufacturing	27.0	42.2	1.6
Furniture, lumber, wood	2.7	1.4	0.5
Nonelectrical machinery	2.8	6.3	2.3
Electrical machinery	1.3	2.8	2.2
Motor vehicles	1.9	4.8	2.5

Although population-based mortality data are a crude means of testing hypotheses concerning public health hazards, geographic correlations with environmental measurements can be done quickly and inexpensively, and may be a valuable first step in evaluating possible dangers. In this manner we have assessed cancer mortality patterns among people residing where drinking water is contaminated by asbestos (*4*), where homes are built on radioactive tailings from uranium mines (*5*), and where the chemical industry is highly concentrated (*6*).

The major contribution of the county resource, however, is in hypothesis formulation, namely the detection of geographic clustering that suggests etiologic clues, which can then be pursued by epidemiologic studies of an analytical type. Computer-generated maps were produced to visualize the spatial configuration of cancer mortality by county. We first plotted the distribution for bladder cancer, the tumor most strongly linked to occupational exposures (*7*). In white males there were clusters of elevated mortality in heavily industrialized areas (Fig. 1), a pattern that was not duplicated in females. The clusters in males suggest industrial hazards that should be evaluated.

To further characterize the possible hazards, we selected for correlation analysis a