

# Abundant Expression of the Microtubule-Associated Protein, Ensconsin (E-MAP-115), Alters the Cellular Response to Taxol

Dorota Gruber, Kathleen Faire, and J. Chloë Bulinski\*

*Department of Biological Sciences, Anatomy and Cell Biology and Pathology, Columbia University, New York, New York*

Correlation between expression level of a microtubule-associated protein called ensconsin (E-MAP-115) and degree of Taxol sensitivity in several cultured cell lines prompted us to investigate potential cause-and-effect relationships between ensconsin level and Taxol action. We used human MCF-7 or HeLa cells, which are sensitive to low Taxol concentrations ( $LD_{50}$  of 30–35 and 3.5 nM, respectively) to prepare stably transfected populations of cells expressing heterogeneous levels of ensconsin chimeras, either green fluorescent protein (GFP) conjugated to full-length ensconsin (GFP-Ensc) or to ensconsin's microtubule-binding domain (GFP-EMTB). Both a subjective microscopic assay, i.e., scoring fluorescence of GFP-ensconsin chimeras following Taxol treatment, and a quantitative immunobiochemical assay, i.e., measuring level of GFP-ensconsin chimera in cells surviving treatment with Taxol, showed that cells expressing higher levels of GFP-ensconsin chimera were killed more readily by Taxol concentrations approaching the  $LD_{50}$ . In contrast, in TC-7 cells, which are relatively insensitive to Taxol ( $LD_{50} > 600$  nM), high-level expression of GFP-EMTB conferred no significant susceptibility to killing by Taxol. However, heightening the Taxol sensitivity of GFP-EMTB-TC-7 cells by pre-incubating cells with the p-glycoprotein inhibitor, verapamil, did result in selective killing of cells highly expressing GFP-EMTB. Taken together, results obtained in MCF-7, HeLa, and TC-7 cells suggest that elevated ensconsin level bestowed a selective disadvantage upon Taxol-sensitive cells. To probe potential mechanisms by which ensconsin could alter the Taxol response, we isolated microtubules from HeLa cells that were or were not pretreated with Taxol. In vivo Taxol treatment significantly tightened microtubule-binding of ensconsin, suggesting that Taxol alters ensconsin's microtubule-binding properties and may, in turn, alter the Taxol response of the microtubules. Our data support the hypothesis that Taxol works synergistically or in concert with microtubule-binding proteins in bringing about deleterious effects on the microtubule cytoskeleton. *Cell Motil. Cytoskeleton* 49:115–129, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** paclitaxel; taxanes; carcinoma; Taxol-resistant cells; mitotic arrest

## INTRODUCTION

Taxol (paclitaxel) is an important chemotherapeutic agent that has been used alone or in combination with other drugs, for treating tumors that often respond to nothing else [Rowinsky, 1997]. Taxol acts by binding along microtubules (MTs) and inhibiting their disassembly. High-resolution electron microscopy of tubulin paracrystals [Nogales et al., 1998, 1999] has elucidated the exact binding site of Taxol within  $\beta$ -tubulin, and mutations in Taxol-resistant cell lines have also implicated other regions of  $\beta$ -tubulin in the Taxol interaction [Giannakakou et al., 1997].

For more than two decades, Taxol has been known to be deleterious to cell growth and behavior [Fuchs and Johnson, 1978; Shiff and Horwitz, 1980]. For example, it inhibits cell cycle transit, arresting cells in mitosis, and it

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\*Correspondence to: J. Chloë Bulinski, Department of Biological Sciences, 804A Sherman Fairchild Center, MC 2450, Columbia University, 1212 Amsterdam Avenue, New York, NY 10027-2450. E-mail: jcb4@columbia.edu

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causes the MT array to reorganize into brush-like bundles that derange organellar distribution and function [Shiff and Horwitz, 1980; Herman and Langerin, 1983; Hamm-Alvarez et al., 1996]. When present at substoichiometric concentrations insufficient to alter the level of MT polymer (i.e., the quantity of MTs in the cell), Taxol merely alters the dynamics of MT polymerization/depolymerization subtly [Jordan et al., 1993; Liao et al., 1995]. Yet, even small perturbations in dynamics suffice to manifest dramatic effects on cell division, arresting or delaying mitosis or causing cells to elaborate abnormal spindles [Jordan et al., 1993]. Low concentrations of Taxol have also been shown to inhibit cell motility [Tanaka et al., 1995; Liao et al., 1995].

There is wide variation in the Taxol sensitivity exhibited by different human and primate cell lines. For example, cells of the human cervical carcinoma line, HeLa, are among the most sensitive to Taxol. In the presence of <10 nM Taxol, which causes no visible abnormality such as MT bundling or a significant increase in MT polymer, virtually all HeLa cells arrest in M-phase within 20 hr [Jordan et al., 1993]. Other types of human cells show similar sensitivity, such as HL-60 cells [Rowinsky et al., 1988; Bhalla et al., 1993], while ovarian tumor cells [Liu et al., 1994], and some other human leukemic cell lines [Rowinsky et al., 1988] show much less sensitivity to Taxol cytotoxicity. Understanding the relative resistance/sensitivity of different cell types would help basic scientists better comprehend how the drug works, and would provide clinicians with a rationale and predictive ability concerning the drug's utility in combating various tumors. This is especially important for epithelial cells and for carcinomas, which are epithelial-derived.

MT-associated proteins (MAPs), species that bind along the surface of MTs, are known to be cell type-specific MT components [Mandelkow and Mandelkow, 1995]. Although variation in abundance or function of MAPs could affect responses to Taxol, this topic has not been extensively explored. A MAP called ensconsin (E-MAP-115) is an intriguing MAP to investigate in Taxol-treated cells, since it is particularly abundant in carcinoma-derived cells such as HeLa [Bulinski and Borisy, 1979] and is expressed in many epithelial and carcinoma cells [Masson and Kreis, 1993]. Also, ensconsin has been shown to exhibit altered *in vitro* binding to MTs [Bulinski and Bossler, 1994], and decreased dynamics of binding to MTs *in vivo* in the presence of Taxol [Bulinski et al., 2001]. In this paper, we explore correlations and potential cause-and-effect relationships between the expression of ensconsin and the deleterious effects of Taxol.

## MATERIALS AND METHODS

### Materials

Except as noted, chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Tustin, CA). Tissue culture materials, solutions, and oligonucleotides were purchased from GIBCO Life Sciences (Gaithersburg, MD) or Hyclone (Logan, UT), restriction enzymes from New England Biolabs (Beverly, MA), and immunochemicals from Organon Technika (Malvern, PA).

All cell lines used were originally obtained from American Type Culture Collection (Bethesda, MD), except for the breast carcinoma line, MCF-7, kindly supplied by Dr. Jan Kitajewski (Department of Pathology, Columbia University); the prostatic carcinoma cells, DU-145, LNCaP, and PC-3, provided by Drs. Lisa Drew and Daniel Petrylak (Department of Urology, Columbia University), and the large cell lung carcinoma cells, 103H, donated by Dr. Eberhard Spiess (DKFZ, Heidelberg, Germany). All cells were cultured in DMEM with fetal bovine serum (10%) except for HeLa and L cells, which were grown in DMEM containing bovine calf serum (10%).

### Preparation and Analysis of Endogenous MAP and GFP-Ensconsin Chimeras

Human MCF-7 and HeLa cells, and African green monkey kidney TC-7 cells were stably transfected with constructs encoding GFP-tagged conjugates of either full-length ensconsin (GFP-Enscon) or EMTB, its MT-binding domain (GFP-EMTB), and were characterized as described [Bulinski et al., 1999; Faire et al., 1999]. Briefly, expression of endogenous ensconsin, GFP-Enscon, and GFP-EMTB proteins were monitored by Western blotting with guinea pig anti-EMTB antibodies (prepared against a his-tagged EMTB construct, as outlined in Faire et al. [1999]). For labeling MAP4, actin, and tubulin on Western blots, the antibodies and conditions for their use were as follows: LHB [Nguyen et al., 1997], C4 [Otey et al., 1986], and 3F3 or W<sup>2</sup> [Chapin and Bulinski, 1991]. Western blots were developed either with chromogenic substrate (4-chloro-1-naphthol) or the more sensitive enzyme-linked chemiluminescence (ECL). Expression of GFP-chimeras in living cells was monitored microscopically; scoring for bright (abundant) or dim (less abundant) expression was corroborated by measurement of pixel intensity (using Metamorph software, Universal Imaging, West Chester, PA). These measurements were corroborated by FACS analysis and Western blotting to quantify the amount of expression, as described in Faire et al. [1999]. Anti-actin [Otey et al., 1986] staining was used to normalize for protein assay or gel loading errors; anti-tubulin staining was not used since Taxol-induced MT stabilization has been shown to alter tubulin synthe-

sis and accumulation [Cleveland et al., 1981; Nguyen et al., 1999].

In MCF-7 and HeLa cells treated with Taxol, *P* values were calculated for each drug concentration, comparing raw data for expression of GFP chimera with those of endogenous ensconsin at each drug concentration. In TC-7, in which low endogenous ensconsin content precludes accurate quantification, level of GFP chimera at each drug concentration was compared to the level in the drug-free control. Northern blotting to detect expression of transcripts encoding endogenous MAP was performed as described by Faire and Bulinski [1998], again using actin transcript as a control for total transcript amount. As expected, muscle-derived lines possessed abundant actin mRNA.

### Biochemical Analysis of Ensconsin:MT Binding

Extracts of naive HeLa or GFP-EMTB-HeLa cells were prepared from suspension cultures pretreated with Taxol (1  $\mu$ M, 2 hr), or from cultures treated with vehicle alone (DMSO, 0.1%). Because Taxol induces bundling of MTs into stable arrays, we modified usual procedures in order to obtain extracts that contained Taxol-induced polymers, but lacked nuclei and other organelles. Therefore, cells were sedimented, washed, and lysed via gentle sonication in ice-cold buffer containing 0.1 M PIPES, pH 6.9, and 1 mM each of dithiothreitol, EGTA, and  $MgSO_4$  (PDEM). Next, sonication at low power on ice ( $\leq 20$ w, 10 bursts, each of 30-sec duration) was used to shear Taxol-MT structures, the largest of which were visible in phase microscopy. That this was effective was initially tested via microscopy after each sonication step. To prevent annealing or reassembly of sheared MTs, cell lysates were kept ice-cold and *immediately* centrifuged at low speed (0°C, 8,000g, 5 min) to remove nuclei and debris. Re-sonication and centrifugation (0°C, 48,000g, 20 min), yielded high-speed supernatants used for MT isolation, as described previously [Chapin and Bulinski, 1991]. Briefly, Taxol was added to a final concentration of 10  $\mu$ M, and solutions were warmed to 37°C for 25 min. A 20% sucrose cushion (1/2 volume) was under-layered, and the MTs were sedimented (37°C, 48,000g, 25 min). After washing four times in PDEM, the MT pellets were resuspended in fresh PDEM, divided into eight equal portions in microcentrifuge tubes, and sedimented. Each pellet was resuspended in an equal volume (40–60  $\mu$ l) of PDEM containing the applicable concentration of NaCl (0–1.0M), incubated for 10 min at 37°C, and centrifuged (40,000g, 15min). Supernatant fractions containing unbound MAP were assayed by Western blots probed with anti-EMTB antibody. The amount of ensconsin or GFP-EMTB released at each salt concentration was normalized to that eluted with 1.0 M NaCl, which was set at 100%.

## RESULTS

We probed the expression level of ensconsin (E-MAP-115) in a variety of human, monkey, and rodent cell lines. As shown in Figure 1 (lanes a–e, and g–j), in many cell types, ensconsin was sufficiently abundant to detect on Western blots labeled with peroxidase-conjugated secondary antibodies, using the chromogenic substrate, 4-chloro-1-naphthol. Standard curves generated with bacterially expressed ensconsin allowed us to quantify expression accurately (data not shown); in HeLa, in which ensconsin was most abundant, it amounted to 0.1% of soluble protein, as previously reported [Bulinski and Borisy, 1979]. Ensconsin abundance differed among three human prostate carcinoma lines, DU-145, LNCaP, and PC-3 (Fig. 1, lanes h–j), although in no line was the MAP as abundant as in HeLa (Fig. 1, compare lanes h–j to g). We also detected ensconsin in cells in which it is not abundant (Fig. 1), including PC-12 (Fig. 1, lane d), RMO (Fig. 1, lane e), and TC-7 (Fig. 1, lane f), in which ensconsin constitutes 0.025, 0.02, and  $\leq 0.01\%$ , of soluble protein, respectively. In monkey kidney cells (TC-7; Fig. 1, lane f), we had to use ECL, which is  $\sim 10$  times more sensitive than 4-chloro-1-naphthol, to detect ensconsin.

In some cells, e.g., rat NRK or mouse L cells, we could not detect ensconsin even with the sensitive ECL technique, even though ensconsin's binding partner, tubulin, was roughly constant in level (3–5% of soluble protein) in all cell lines examined. We were concerned that limited inter-species cross-reactivity of our anti-human EMTB antibody might yield artifactually low values for ensconsin abundance. However, the MT-binding domain to which EMTB antibody was elicited is 85% identical between mouse and human [Fabr e-Jonca et al., 1998], and phylogeny and cDNA sequences show that monkey and rat EMTB are even more like human than like mouse EMTB (data not shown). Thus, our antibody should cross-react completely with these species, and the intensities of bands in Figure 1 most likely accurately represent ensconsin's relative abundance in each cell type.

The abundance of ensconsin mRNA transcripts was correlated with protein abundance in each cell line, as shown in sample Northern blots in Figure 2: In particular, the level of protein in HeLa, relative to MCF-7 and TC-7 cells, is correlated with the relative level of RNA transcript found in each line. That steady-state level of protein and transcript are correlated suggests that there is no significant post-transcriptional or post-translational regulation of ensconsin level in cell lines we examined. This result contrasts with another MAP, MAP4, whose transcript vs. protein abundance is not correlated [e.g., West et al., 1991; Chapin et al., 1995].

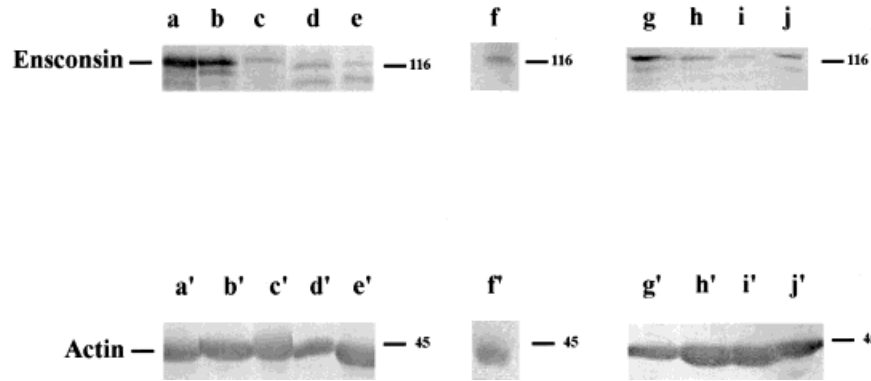


Fig. 1. Expression of enscnoin (E-MAP-115) protein in cell lines. Western blots of soluble protein extracts of (a, g) HeLa human cervical carcinoma; (b) MCF-7, human breast carcinoma; (c) 103H, human large cell lung carcinoma; (d) PC-12, rat pheochromocytoma; (e) RMO, rat myoblast; (f) TC-7, African green monkey kidney epithelial; and human prostate carcinoma cell lines (h) DU-145, (i) PC-3, and (j) LNCaP. Blots were immunolabeled with anti-EMTB antibody, peroxidase-conjugated secondary antibody, and then processed with chromogenic substrate for all lanes except f, which was processed with enzyme-linked chemiluminescence (ECL) (f). In blots of all lanes, the Enscnoin band can be visualized, sometimes accompanied by one or two proteolytic fragments. Lanes a'–j' represent staining with a monoclonal antibody to Actin as a loading control; as expected, actin was

greater in abundance in the sample derived from myogenic cells (RMO, lane e). Analysis with known quantities of recombinant EMTB (lanes not shown) allowed quantification of the enscnoin level in each cell type shown, ranging from 0.1% of soluble protein in HeLa to  $\leq 0.01\%$  of soluble cell protein in TC-7 cells; in several other cell lines tested, no enscnoin band could be detected (see Table I). Note that in the three prostate carcinoma lines, in which enscnoin is intermediate in abundance, the order of relative enscnoin abundance in lines is DU-145  $\geq$  LNCaP  $>$  PC-3. In contrast to enscnoin level, content of MAP4 as a proportion of soluble cell protein was approximately constant in all cell lines (data not shown). Quantities of protein loaded were as follows: a–e, 60  $\mu$ g; f, 10  $\mu$ g; g, 25  $\mu$ g; h–j, 50  $\mu$ g. Migration of molecular mass markers of 116 and 45 kDa are shown at right.

We defined the LD<sub>50</sub> for Taxol, that is, the concentration required to kill 50% of cells, by adding various concentrations of Taxol to culture medium for 3 days and counting the live cells that remained. Cell lines varied widely in their Taxol sensitivity (Table I). For example, HeLa cells, HL-60, DU-145, and U251 cells showed the highest Taxol sensitivity. Other human cells, such as MCF-7, PC-3, and 103H, showed moderate sensitivity, while monkey and rodent lines were markedly insensitive to Taxol (LD<sub>50</sub>  $> 300$  nM). For each cell line, we compared LD<sub>50</sub> for Taxol to enscnoin level quantified from blots (e.g., Fig. 1); enscnoin content and Taxol-sensitivity showed a significant correlation (Table I). Especially obvious in Table I are extreme data points; i.e., cells with exquisite Taxol sensitivity and abundant enscnoin (e.g., HeLa), and cells with  $\sim 100$ -fold lower Taxol sensitivity than HeLa and either very little (TC-7) or no (rodent lines) detectable enscnoin. Cells in Table I fit roughly into two groups, those with abundant enscnoin and those with little or none; Taxol sensitivity was high in the former and low in the latter. An additional interesting correlation was that enscnoin level and drug sensitivity varied analogously among three prostate carcinoma lines, DU-145, LNCaP, and PC-3.

Although correlative data in Table I suggest that enscnoin level and Taxol sensitivity are connected, we asked whether abundance of other MAPs might also correlate with degree of Taxol sensitivity. However,

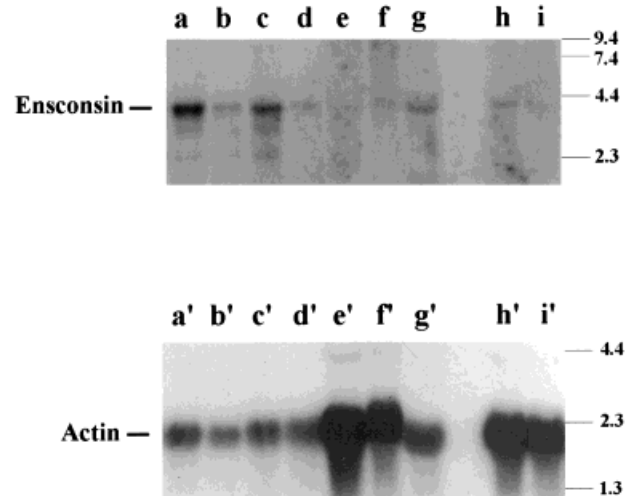


Fig. 2. Expression of enscnoin (E-MAP-115) RNA transcripts in cell lines. Northern blots of total RNA isolated from (a) HeLa, human cervical carcinoma; (b, c) MCF-7, human breast carcinoma; (d) 103H, human large cell lung carcinoma; (e) C2C12, mouse myoblasts; (f) 3T3, mouse fibroblasts; (g) TC-7, African green monkey kidney epithelial; (h) RMO rat myoblasts; (i) L6 rat myoblasts, are shown, probed with Enscnoin cDNA probe, as described in Materials and Methods. Ten micrograms of RNA were loaded for a and b, while 20  $\mu$ g was loaded for all other samples (lanes c–i). Blots were also probed with Actin cDNA probe (lanes a'–i'), as a measure of the quantity of mRNA contained in each sample. As expected, actin mRNA was markedly greater in abundance in samples derived from myogenic cells (lanes e', h', i'). The electrophoretic positions of markers (in kb) is noted at right.

TABLE I. Taxol Sensitivity and Abundance of Ensconsin (E-MAP-115) in Cell Lines\*

Cell type	LD <sub>50</sub> Taxol (nM)	Ensconsin abundance	LD <sub>50</sub> Vinblastine (nM)
HeLa, human cervical carcinoma	3.5	Very abundant <sup>a</sup>	12–15
HL-60, human lymphoblastic cells	3–4	n.d.	n.d.
U251, human glioma	5–6	Abundant <sup>a</sup>	15
DU-145, human prostate carcinoma	4–5	Abundant <sup>a,b</sup>	25–30
LNCaP, human prostate carcinoma	5–6	Abundant <sup>a,b</sup>	25–30
PC-3, human prostate carcinoma	12	Abundant <sup>a,b</sup>	25–30
MCF-7, human breast epithelial	30–35	Abundant <sup>a</sup>	20–25
103H, human large cell lung carcinoma	50–60	Not abundant	30–45
PC-12, rat pheochromocytoma cells	>400	Not abundant	n.d.
RMO, rat myoblasts	>400	Not abundant	30–45
TC-7, African green monkey kidney epithelial	>600	Not abundant <sup>c</sup>	175
L6, rat myoblasts	>400	Not detected	30–50
C <sub>2</sub> C <sub>12</sub> , mouse myoblasts	>400	Not detected	n.d.
3T3, mouse fibroblastic	>400	Not detected	35–50
HeLa, human cervical carcinoma, stably expressing MAP4 anti-sense cDNA	3.5	Very abundant <sup>a</sup>	12–15
L, mouse fibroblastic	>300	Not detected	30–40
L, stably transfected with MAP4 DNA	>300	Not detected	30–40
L, mouse fibroblasts, mock-transfected	>300	Not detected	30–40

\*LD<sub>50</sub> is the concentration of Taxol or vinblastine lethal to >50% of the cells in the population, after a 3-day treatment. All measurements are  $\pm 10\%$ ,  $n \geq 2$  for each cell line. n.d., not determined.

<sup>a</sup>Ensconsin makes up  $\sim 0.02$ – $0.1\%$  of soluble protein in these cells, which range from most abundant in HeLa cells ( $0.1\%$ ) to least abundant in PC-3 cells ( $0.02$ – $0.04\%$ ).

<sup>b</sup>As shown in Figure 1, ensconsin's abundance can be ordered: DU-145 cells  $\geq$  LN-CAP > PC-3 cells.

<sup>c</sup>< $0.01\%$  of soluble protein.

MAP4, the other abundant, ubiquitously expressed MAP, was essentially constant in level in all lines (data not shown). Although several *in vivo* functions of MAP4 mimic effects of Taxol [e.g., Nguyen et al., 1997, 1999], our data provide no evidence that manipulating the level of MAP4, either down (HeLa cells whose MAP4 was anti-sense depleted) or up (L cell MAP4 stable transfectants) changes cells' Taxol sensitivity (Table I).

Differences in sensitivity to Taxol may be due to differences in efficacy of P-glycoprotein, the pump responsible for efflux of Taxol and other hydrophobic drugs from the cytoplasm. However, we found that, although sensitivity to the MT antagonist, vinblastine, another p-glycoprotein substrate, also varied among cells, vinblastine sensitivity did not correlate with ensconsin level (Table I). For example, each of the prostate carcinoma lines, DU-145, LNCaP, and PC-3, which varied in Taxol sensitivity, showed identical vinblastine sensitivity. Comparison of Taxol- and vinblastine-sensitivities in Table I documents many instances in which differing response to Taxol cannot be explained by differing efficacy of drug efflux.

TC-7 are African green monkey kidney cells, which have been reported to exhibit abundant P-glycoprotein activity [Brouty-Boye et al., 1995]. Consistent with this report, Table I shows that TC-7 cells are not very sensitive to either Taxol (LD<sub>50</sub> >600 nM) or vin-

blastine (LD<sub>50</sub> = 175 nM). Thus, even though the low ensconsin abundance and low Taxol sensitivity are well correlated in TC-7 cells, their relative insensitivity to Taxol may be explained by efficient drug efflux.

Previous results implicated ensconsin level in helping to determine the potency of cells' responses to Taxol, either by causing mitotic arrest and abnormalities [Faire et al., 1999], or by binding to MTs more tightly in the presence of Taxol [Bulinski and Bossler, 1994]. Accordingly, we tested the hypothesis that there is a cause-and-effect relationship between ensconsin level and Taxol's efficacy in killing cells, using human and monkey cell lines that stably express chimeras of green fluorescent protein (GFP) conjugated to full-length ensconsin (GFP-Encs) or to its MT-binding domain (GFP-EMTB). Both GFP-Encs and GFP-EMTB chimeras were shown to mimic the *in vivo* MT-binding and dynamic properties of the endogenous MAP [Bulinski et al., 1999, 2001; Faire et al., 1999]. In human MCF-7 cells, which are markedly sensitive to Taxol (LD<sub>50</sub> of Taxol = 30–35 nM), we noted a difference between the Taxol sensitivity of naive cells and that of GFP-Encs- or GFP-EMTB-transfectants. We quantified this effect in populations of MCF-7 cells heterogeneous in their levels of expression of GFP-Encs or GFP-EMTB (Table II); cells exhibiting bright GFP fluorescence (i.e., high level expression) decreased in abundance after Taxol treatment, and fluorescence acti-

**TABLE IIA. Expression of GFP-MAP Chimeras During Drug Treatment of MCF-7 Cells**

	GFP-Ensc-MCF-7 [cells w/bright GFP MTs (%)]	GFP-EMTB-MCF-7 [cells w/bright GFP MTs (%)]
Taxol concentration (nM) <sup>a</sup>		
0	59.0 ± 4.3	67.1 ± 13.2
20	51.0 ± 12.0	44.4 ± 11.7
25	47.0 ± 6.4	37.6 ± 7.29
30	33.9 ± 7.2	38.0 ± 6.8
Vinblastine concentration (nM) <sup>b</sup>		
0	73.97 ± 11.90	87.34 ± 9.98
5	71.64 ± 6.22	79.23 ± 0.98
10	67.87 ± 20.97	73.74 ± 9.08
12.5	66.64 ± 21.96	71.74 ± 1.79
15	48.15 ± 2.92	87.94 ± 5.03
17.5	71.93 ± 14.50	83.42 ± 0.22
20	75.15 ± 2.54	68.92 ± 1.65
22.5	67.62 ± 8.56	67.42 ± 3.99

<sup>a</sup>LD<sub>50</sub> for naive MCF-7 cells treated with Taxol is 30–35 nM, while this table shows that LD<sub>50</sub> for brightly fluorescent (highly expressing) GFP-EMTB-MCF-7 cells treated with Taxol is ~20 nM. For brightly expressing GFP-Ensc cells the LD<sub>50</sub> for Taxol is 20–25 nM.

<sup>b</sup>LD<sub>50</sub> is identical (20–25 nM) for naive MCF-7 cells and for highly expressing GFP-EMTB-MCF-7 or GFP-Ensc MCF-7 cells treated with vinblastine.

vated cell sorter (FACS) analysis verified the presence of brightly and dimly fluorescent populations of cells following each treatment (data not shown). For highly expressing GFP-EMTB-MCF-7 cells, Taxol's LD<sub>50</sub> was ~20 nM; that is, the abundance of brightly fluorescent cells was reduced by half after treatment with 20 nM Taxol. In contrast, for highly expressing GFP-Ensc cells the LD<sub>50</sub> was 20–25 nM. Thus, highly expressing cells were killed more readily than lower expressing cells during treatment with Taxol concentrations close to the LD<sub>50</sub> of the parental line.

Western blots provided a more definitive measure of exogenous (transfected) ensconsin chimera in Taxol-treated MCF-7 transfectants. Figure 3A shows that expression of GFP-Ensc decreased as a proportion of cell protein after treatment of MCF-7 cells with increasing Taxol concentrations. In contrast, levels of two endogenous MAPs, ensconsin and MAP4, were unchanged relative to total protein at all Taxol concentrations (Fig. 3A), as were the levels of free GFP or a GFP-MAP4 construct, showing that Taxol had no effect on expression from the EGFP plasmid, and did not selectively kill cells expressing GFP conjugated to another MAP (data not shown). Together with visual scoring data (Table II)

**TABLE IIB. Expression of GFP-EMTB During Drug Treatment of GFP-EMTB-TC-7 Cells**

	GFP-EMTB-TC-7 [cells w/bright GFP MTs (%)]
Taxol (nM) (+Verapamil) <sup>a</sup>	
0	88.84 ± 9.09
100	79.36 ± 27.41
200	84.22 ± 14.73
250	71.25 ± 19.75
300	48.68 ± 18.75
350	45.96 ± 37.49
400	17.60 ± 13.73
Vinblastine (nM) (+Verapamil) <sup>b</sup>	
0	78.61 ± 14.76
5	78.08 ± 11.50
10	65.82 ± 14.73
20	78.82 ± 10.41
30	67.87 ± 29.53
40	71.08 ± 5.31
50	72.54 ± 16.34

<sup>a</sup>LD<sub>50</sub> for naive TC-7 cells treated with Taxol (with 10 μM verapamil) is 400 nM, while this table shows that LD<sub>50</sub> for brightly fluorescent (highly expressing) GFP-EMTB-TC-7 cells treated with Taxol (with 10 μM verapamil) is 300 nM.

<sup>b</sup>LD<sub>50</sub> for both naive TC-7 cells and highly expressing GFP-EMTB-TC-7 cells treated with vinblastine (with 10 μM verapamil) is 50 nM.

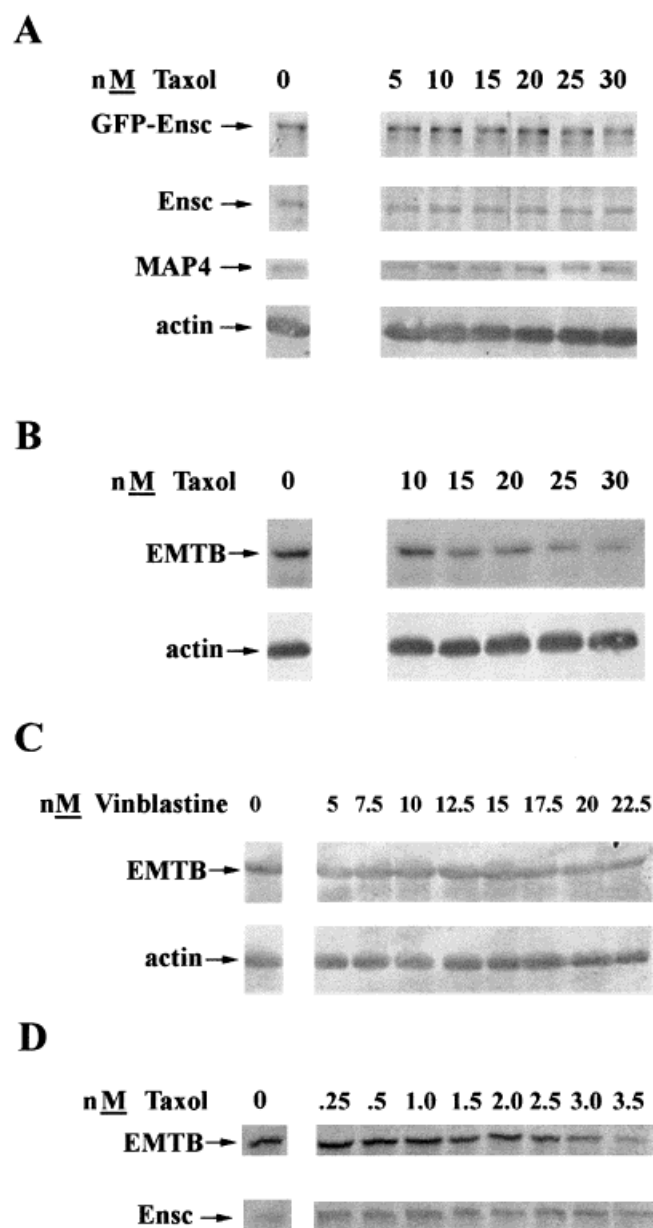
and corroborated by FACS analysis of surviving cells (data not shown), data in Figure 3 show that GFP-Ensc expression decreased because the population of cells with highest GFP-Ensc expression was selectively killed by Taxol.

Taxol treatment of GFP-EMTB-MCF-7 cells yielded a more pronounced decrease in GFP-EMTB expression than was obtained in GFP-Ensc-MCF-7 cells (compare Fig. 3A,B). The higher expression achieved with the smaller construct, GFP-EMTB, likely explains its greater potency. Selective cell killing, leading to a decrease in the level of exogenous ensconsin chimera, was a result specific to Taxol treatment, since vinblastine treatment of MCF-7 transfectants did not decrease expression of GFP-EMTB (Fig. 3C) or GFP-Ensc (data not shown).

Taxol treatment of GFP-EMTB-HeLa cells gave results similar to those obtained for MCF-7 cells (compare Fig. 3B and D); expression of GFP-EMTB decreased after cells had been treated with increasing concentrations of Taxol, while level of endogenous ensconsin protein was unchanged. Abundance of ensconsin and GFP-EMTB transcripts was unchanged by Taxol treatments (data not shown). These data show that cells that survived Taxol treatment differed from those that did not only in their content of GFP-EMTB or GFP-Ensc chimera.

Quantification of our results showed that MCF-7 cells expressing either GFP-EMTB or GFP-Ensc be-

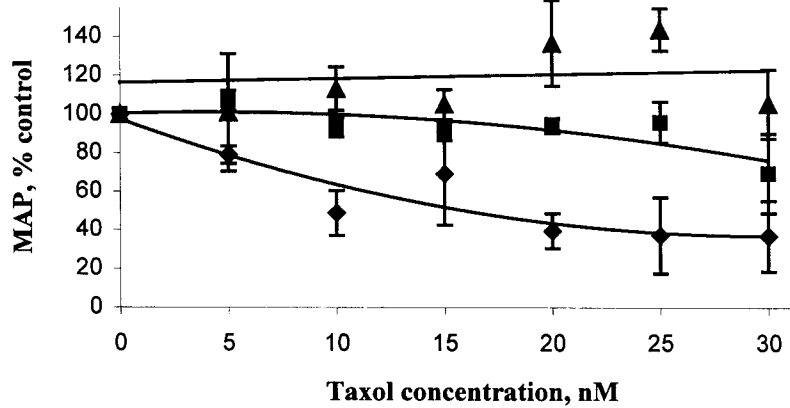
haved similarly (Fig. 4A); each showed a statistically significant decrease in transfected ensconsin chimera ( $P < 0.05$  at  $>10$  nM Taxol). The analogous behavior of both GFP-ensconsin chimeras is not surprising, since we previously showed that the MT-binding properties of both chimeras mimic each other, and endogenous ensconsin [Bulinski et al., 1999, 2001; Faire et al., 1999]. Figure 4B shows that selective killing was mediated by the MT-stabilizing drug, Taxol, but not the MT-destabilizing drug, vinblastine. Moreover, comparison of MCF-7 and HeLa cells (Fig. 4A and C) demonstrates that selective killing of highly expressing cells occurred in both lines of Taxol-sensitive human lines we tested.



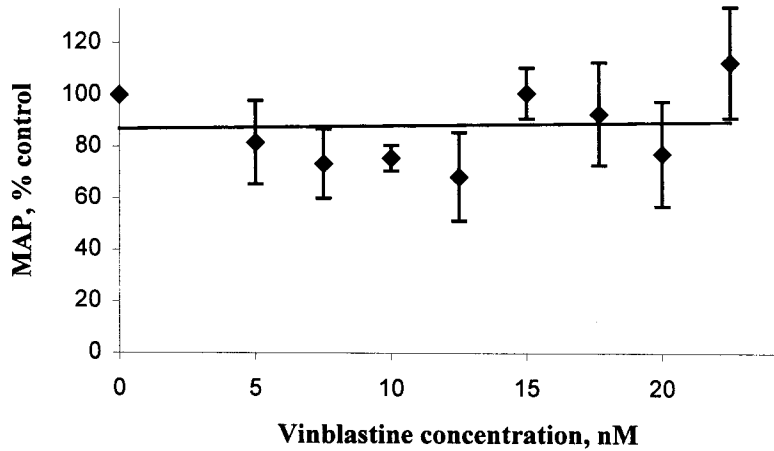
Next, we examined the Taxol responses of TC-7 cells, which are relatively insensitive to Taxol ( $LD_{50} >600$  nM; Table I). TC-7 cells stably express heterogeneous, high levels of GFP-EMTB (ranging from 1.6% of cell protein, which would saturate ensconsin's MT-binding sites, to only 0.04%) [Faire et al., 1999]. Contrary to results with MCF-7 and HeLa cells, microscopic scoring yielded no discernible difference in abundance of TC-7 cells highly expressing GFP-EMTB after treatment with Taxol concentrations close to the cells'  $LD_{50}$  (data not shown). Similarly, quantification of GFP-EMTB revealed no statistically significant change in expression at any concentration of Taxol (see Fig. 6A), or vinblastine (data not shown), suggesting that there was neither selective killing, nor survival, of cells with extra ensconsin. In addition, in TC-7 cells the level of endogenous ensconsin protein and transcript and of MAP4 protein were all unchanged by Taxol treatment (data not shown). Results in TC-7 cells differed markedly from results in HeLa or MCF-7 cells, both of which manifest 10- to 100-fold greater Taxol-sensitivity.

Fig. 3. Cells expressing higher levels of exogenous GFP-ensconsin constructs show increased susceptibility to killing by Taxol. **A:** A stably transfected MCF-7 cell line (not cloned) expressing heterogeneous levels of GFP-ensconsin (GFP-Ensc) was treated with the indicated concentrations of Taxol for 3 days, then extracts of surviving cells were immunoblotted with anti-EMTB antibody, to detect expression of GFP-Ensc and endogenous ensconsin (Ensc). GFP-Ensc expression in cells surviving Taxol treatment *decreased* as Taxol concentration *increased*, while levels of endogenous Ensc and another MAP (MAP4) remained constant. Staining with anti-actin (actin) provided a loading control. Relative mobility of each antigen was as follows: GFP-Ensc, 145 kDa; Ensc, 116 kDa; MAP4, 210 kDa; actin, 43 kDa. **B:** A stably transfected MCF-7 cell line expressing heterogeneous levels of ensconsin's microtubule-binding domain, GFP-EMTB, was treated with various concentrations of Taxol. Cells expressing higher levels of GFP-EMTB showed more pronounced killing by Taxol than did cells expressing GFP-Ensc (above). The same protocol as in A was used. The immunoblot shows the level of GFP-EMTB at each drug concentration; actin was used as a loading control. Relative mobility of GFP-EMTB was 55 kDa. **C:** An MCF-7 cell line expressing heterogeneous levels of ensconsin's microtubule-binding domain, GFP-EMTB, was treated with various concentrations of vinblastine; cells expressing higher levels of GFP-EMTB were *not* differentially killed by vinblastine treatment. Note that, even though cells were killed at the highest vinblastine concentrations assayed, the proportion of cell protein that was GFP-EMTB was constant. Protocol was the same as in A. GFP-EMTB content is shown after each treatment, along with the level of actin, a loading control. **D:** A HeLa cell line expressing heterogeneous amounts of ensconsin's microtubule-binding domain, GFP-EMTB, showed more pronounced Taxol-induced killing of cells expressing higher levels of GFP-EMTB, compared to cells expressing lower levels. The protocol followed was the same as in A. GFP-EMTB is shown at each drug concentration, along with the level of endogenous ensconsin, Ensc, which remained approximately constant in level. Level of actin was used to normalize extracts to detect protein load (data not shown).

A



B



C

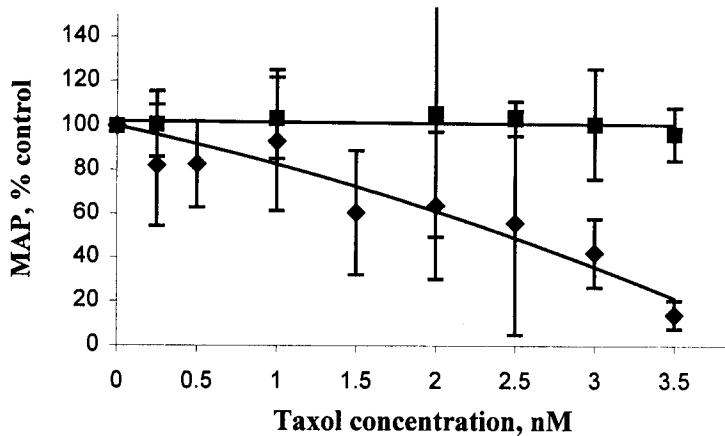


Fig. 4. Expression of GFP-ensconsin chimeras in Taxol-sensitive human cell lines sensitizes them to Taxol-killing. **A:** Quantification of GFP-MAP chimera in GFP-Ensconsin-MCF-7 (■) and GFP-EMTB-MCF-7 (◆) cells surviving 3-day treatments at the indicated Taxol concentrations. Abundance of endogenous ensconsin (▲) is also shown in GFP-Ensconsin-MCF-7 cells. Note that for cells transfected with either GFP-MAP chimera, cells with more abundant expression, i.e., cells with brightly labeled MTs at the start of the experiment, were selectively killed (see Table II). Thus, GFP-MAP chimera decreased in abundance during Taxol treatments, as shown. All three species were quantified with anti-EMTB antibody, which was normalized to the amount of anti-EMTB reactivity in untreated cells (100%). A Student's *t*-test showed that decreases in either chimera were significant for Taxol concentrations greater than 10 nM ( $P < 0.05$ ). Endogenous ensconsin showed no significant decrease at any concentration tested ( $P < 0.18$ ).  $N \geq 3$  for each treatment. **B:** Quantification of GFP-EMTB level in GFP-EMTB-MCF-7 (◆) cells surviving 3-day vinblastine treatments. GFP-EMTB showed no significant decrease in level ( $P > 0.16$ ) at any vinblastine concentration; that is, there appeared to be no differential killing of cells with higher GFP-EMTB levels. Blots were quantified as in A.  $N \geq 2$  for each treatment. **C:** Quantification of expression of GFP-EMTB chimera (◆) and endogenous ensconsin (■) in GFP-EMTB-HeLa cells surviving Taxol treatments at concentrations indicated. The most highly expressing (i.e., most brightly fluorescent cells) were killed most easily by Taxol; thus, HeLa cells showed a decreased abundance of GFP-EMTB that was statistically significant ( $P < 0.05$ ) at Taxol concentrations  $> 2$  nM. Blots were quantified as in A;  $N \geq 3$  for each treatment.

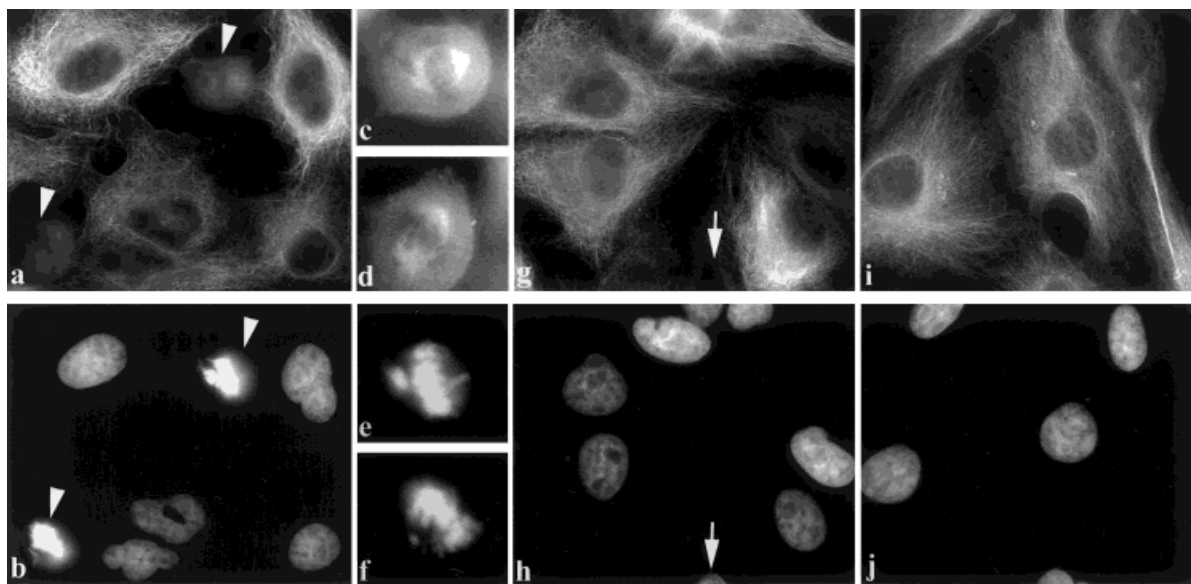


Fig. 5. Verapamil increases efficacy of Taxol-treatment in GFP-EMTB-TC-7 cells. Micrographs of TC-EMTB cells treated with (a–f) Taxol and verapamil (300 nM, 6 hr, and 10  $\mu$ M, 18 hr, respectively), (g, h) Taxol only (300 nM, 6 hr), and (i, j) verapamil only (10  $\mu$ M, 18 hr). Images of GFP-EMTB fluorescence (a, c, d, g, i) illuminate the MT network and DAPI stain (b, e, f, h, j) shows the nuclei or chromosomes. Note that c–f are images of mitotic cells that are marked by arrowheads in the field shown in a, b. The images in c–f were

acquired at a higher focal plane to capture details of the more spherical dividing cells. Many cells treated with both Taxol and verapamil (a–f) displayed abnormal nuclei or had progressed into mitosis within the treatment period, while cells treated with Taxol or verapamil alone more closely resembled untreated cells (data not shown). Arrows in g, h highlight one of the cells expressing little GFP-EMTB; these cells characteristically displayed normal nuclear morphology in the presence of Taxol.

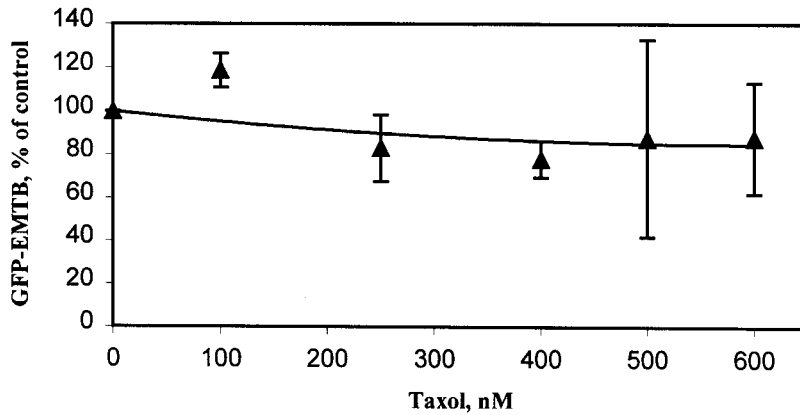
TC-7 cells may be less sensitive to Taxol than MCF-7 or HeLa cells partly because they express high levels of the drug export pump, p-glycoprotein, which can be partially inhibited by verapamil [Brouty-Boye et al., 1995]. Addition of 1–10  $\mu$ M verapamil to GFP-EMTB-TC-7 cells did not affect cell viability or MT organization (Fig. 5i,j, and data not shown). However, pre-incubation of either GFP-EMTB-TC-7 or naive TC-7 cells with 10  $\mu$ M verapamil potentiated Taxol's effects on cell morphology. Figure 5 shows images of EMTB bound to the MT network (visualized with GFP) and nuclear structure and cell cycle stage (visualized with DAPI chromatin staining) in Taxol-treated GFP-EMTB-TC-7 cells. Note that no obvious MT bundling was observed in cells treated with Taxol and verapamil (Fig. 5a,b), but many cells showed blebbing and/or fragmentation of nuclei, and mitotic arrest and/or multipolar spindles, all characteristic effects of Taxol [Long and Fairchild, 1994; Mooberry et al., 1999] (mitotic figures in Fig. 5a,b, are shown at a higher focal plane in Fig. 5c–f, to facilitate visualization of spindles). Cells treated with Taxol and verapamil for 6 hr showed an elevated mitotic index ( $9.5 \pm 2.7\%$ ;  $n=2$ ). In contrast, cells treated with Taxol alone (Fig. 5g,h) showed few abnormalities in nuclear morphology and only slightly elevated mitotic index ( $4.8 \pm 2.1\%$ ;  $n=2$ ). Cells treated with verapamil

alone (Fig. 5i,j) had normal nuclei and mitotic index ( $1.9 \pm 1.3\%$ ;  $n=2$ ), indistinguishable from that of untreated cells ( $2.9 \pm 0.9\%$ ;  $n=2$ ). These data show that verapamil pre-incubation increases Taxol sensitivity of GFP-EMTB-TC-7 cells.

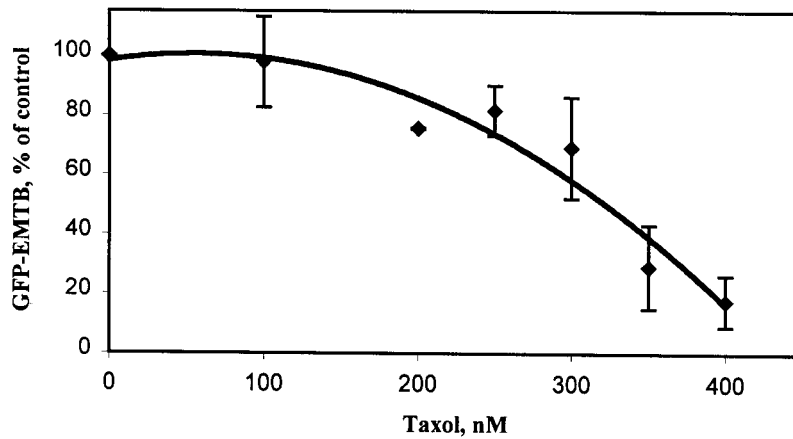
We next tested whether treatment of GFP-EMTB-TC-7 with verapamil might render their behavior more like that of cells naturally more Taxol-sensitive, such as MCF-7 and HeLa. In GFP-EMTB-TC-7 cells exposed to verapamil and various concentrations of Taxol, we noted that, although  $\sim 80\%$  of cells expressed GFP-EMTB at the start of the experiment, after drug treatment the proportion of cells brightly labeled with GFP-EMTB declined (Table IIB), as did the cells' GFP-EMTB content (Fig. 6B;  $P < 0.05$  at Taxol concentrations  $>300$  nM). Thus, GFP-EMTB expression decreased because cells expressing the highest levels of chimera were killed more effectively by Taxol. Selective killing was also measurable as a change in the  $LD_{50}$  for Taxol; in the presence of 10  $\mu$ M verapamil, naive TC-7 cells'  $LD_{50}$  for Taxol was 400 nM, while that of highly expressing GFP-EMTB-TC-7 cells was 300 nM (Table IIB).

No significant change in abundance of cells highly expressing GFP-EMTB was observed after treatment with vinblastine and verapamil (Table IIB; Fig. 6C). For both naive and GFP-EMTB-expressing TC-7 cells, the

A



B



C

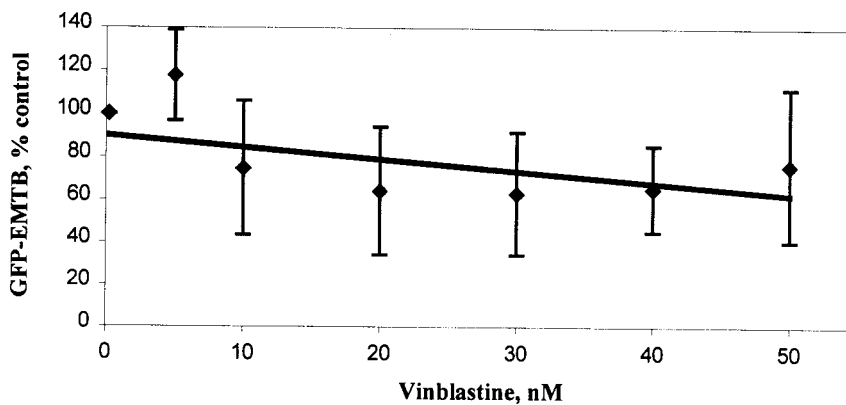


Fig. 6. Expression of GFP-EMTB chimera in Taxol-treated GFP-EMTB-TC-7 cells with or without verapamil pre-treatment. **A:** Quantification of expression of GFP-EMTB (▲) in GFP-EMTB-TC-7 cells surviving 3-day Taxol treatments at the indicated concentrations. GFP-EMTB-TC-7 cells, which are relatively insensitive to killing by Taxol, showed no statistically significant change in abundance of GFP-EMTB at any Taxol concentration administered ( $P > 0.16$ ). Quantification was performed as in Figure 4A.  $N = 2$  for each treatment. **B:** Quantification of expression of GFP-EMTB (◆) in GFP-EMTB-TC-7 cells surviving 3-day treatments with verapamil and Taxol. GFP-EMTB-TC-7 cells were pretreated for 12 hr with verapamil (10  $\mu$ M) to inhibit p-glycoprotein-mediated drug efflux [Brouty-Boye et al., 1995] and then subjected to 3-day treatments with Taxol at the indicated concentrations. In the presence of verapamil, cells expressing the highest amounts of GFP-EMTB were killed more effectively by Taxol; the cell population showed a decreased abundance of GFP-EMTB that was statistically significant ( $P < 0.05$ ) at Taxol concentrations greater than 300 nM. Blot quantification was performed as in Figure 4A.  $N \geq 2$  for each Taxol concentration. **C:** Quantification of expression of GFP-EMTB (◆) in GFP-EMTB-TC-7 cells treated with verapamil and vinblastine. GFP-EMTB-TC-7 cells were pretreated with verapamil as in B, followed by 3-day treatment with vinblastine, at each concentration indicated. Quantification, performed as in Figure 4A revealed that GFP-EMTB-TC-7 cells showed no change in abundance of GFP-EMTB that was statistically significant at any vinblastine concentration used ( $P \geq 0.10$ ).  $N = 2$  for each treatment.

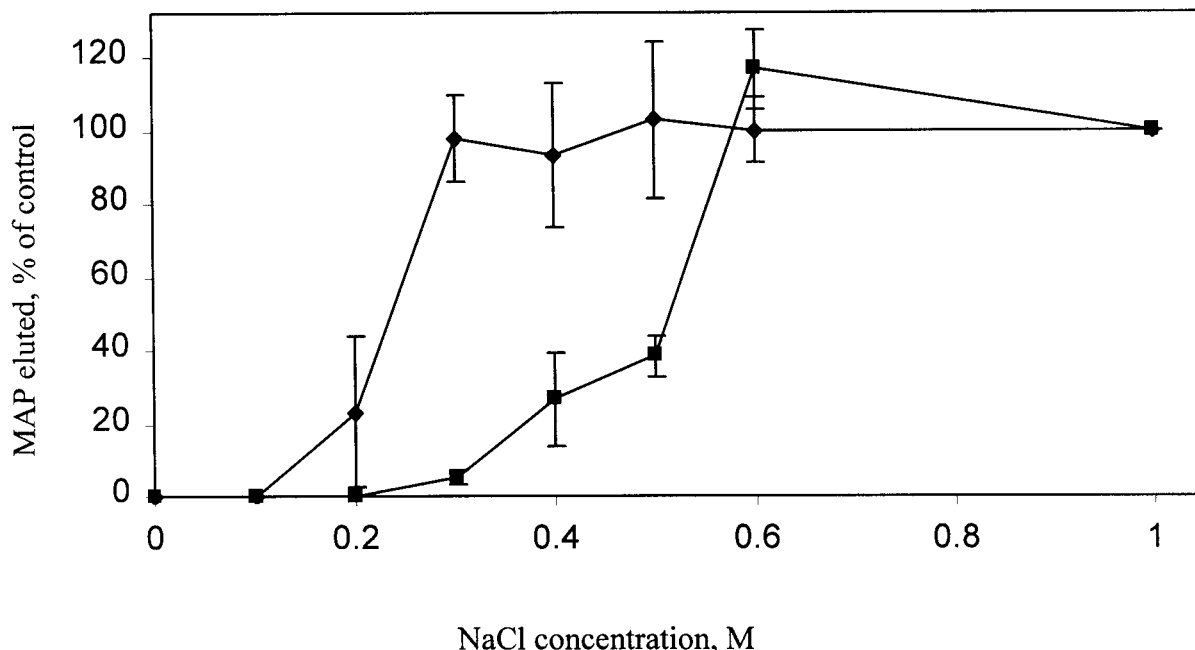


Fig. 7. Ensconsin's binding to MTs is tightened by pre-treatment with Taxol. MT-binding of ensconsin to HeLa cell MT samples was assayed by elution with increasing concentrations of NaCl in PDEM buffer (see Materials and Methods for details). Shown is quantification of Western blots with anti-EMTB of proteins eluted from MTs prepared from (■) HeLa cells pretreated with Taxol (1  $\mu$ M, 2 hr) and from (◆) HeLa cells treated with vehicle alone (0.1% DMSO). Note that elution of ensconsin from Taxol-treated cell MTs requires higher concentrations of NaCl than elution from control cell MTs.

LD<sub>50</sub> for vinblastine was 50 nM. Thus, TC-7 cells were selectively killed by Taxol only when the cells were pre-sensitized to drug treatment with verapamil. Also, selective killing was specific to Taxol; it was not triggered by vinblastine ( $P \geq 0.15$  for all vinblastine concentrations).

Taken together, results on cells that vary widely in Taxol sensitivity suggest that the presence of extra ensconsin presented a selective disadvantage for cell survival in the presence of Taxol. The fact that endogenous ensconsin protein and transcript were unaffected by Taxol treatment of naive or GFP-MAP-transfected MCF-7 or HeLa cells suggests that Taxol did not directly alter ensconsin transcription or protein synthesis. Rather, our data suggest that MT-binding of exogenous ensconsin or its domains may exert deleterious effects on cells in the presence of Taxol.

Previous work showed that introducing a moderate amount of GFP-EMTB chimera into untreated cells did not alter MT dynamics [Faire et al., 1999]; however, this study did not address whether Taxol-induced MT stabilization occurred identically on MTs containing different complements of bound MAPs. Previous *in vitro* studies showed that adding Taxol to MT-ensconsin solutions caused tightening of ensconsin:MT binding [Bulinski and Bossler, 1994]. If this also occurred *in vivo*, altered

MT dynamics and cell cycle progression might be expected to ensue.

To detect biochemically any Taxol-induced tightening of ensconsin:MT binding, we compared the MT-binding of ensconsin isolated from HeLa cells pretreated with 1  $\mu$ M Taxol, with that from cells treated with vehicle alone (DMSO, 0.1%). We chose treatment conditions based upon the knowledge that incubation of 5 $\times$ GFP-EMTB-TC-7 cells in 1  $\mu$ M Taxol for 2 hr increased the residence time ( $t_{1/2}$ ) of EMTB on the MT  $\sim$ 10-fold [Bulinski et al., 2001]. Because ensconsin and EMTB bind ionically to MTs, we used a salt-elution protocol to assay avidity of the MAP for MTs within crude preparations [Bulinski et al., 1999]. Cells pretreated with Taxol contained bundled MTs and numerous mitotic spindles. Concerns that these structures would not be disassembled in preparing cell lysates, resulting in the loss of important components from cell extracts [e.g., Bulinski et al., 1997], were precluded by modifying existing protocols for preparing extracts (see Materials and Methods).

Salt-elution data (Fig. 7) suggest that ensconsin was more avidly associated with MTs isolated from Taxol-treated cells than with those isolated from control cells; that is, a higher salt concentration ( $\sim$ 0.5 M) was required to elute 50% of the ensconsin from MTs isolated

from cells pretreated with Taxol than from cells treated with vehicle alone ( $\sim 0.25\text{M}$ ). As previously reported, altered elution of ensconsin was seen both in naive and GFP-EMTB-HeLa cells pretreated with Taxol; elution of ensconsin and GFP-EMTB from MTs occurred under the same salt-elution conditions [Bulinski et al., 1999] (data not shown). This result constitutes the first evidence that *in vivo* binding of any MAP to MTs is significantly changed when cells are treated with Taxol.

## DISCUSSION

Multiple factors influence cells' responses to the chemotherapeutic drug, Taxol. For example, cells may be altered in Taxol sensitivity because of changes in efficiency of either drug uptake or drug efflux. Alternatively, cells may be altered in apoptotic responses, or in biochemical or dynamic characteristics of their MTs [reviewed by Dumontet and Sikic, 1999]. In some cells, ability to withstand heightened Taxol exposure is accompanied by alterations in total tubulin content [Jaffrezou et al., 1995], tubulin isoform composition [Haber et al., 1995; Rangenathan et al., 1996; Kavallaris et al., 1997], or expression of mutant tubulins [Cabral and Barlow, 1989; Giannakakou et al., 1997]. Although it has proven difficult to demonstrate a causal relationship between any of these changes and the Taxol response, one can readily envision more stable, less dynamic MTs being more acutely affected by the MT-stabilizing properties of Taxol.

In addition to tubulin, MTs also contain MAPs bound along their length. One MAP ubiquitously expressed in non-neuronal cells, MAP4, elicits MT behaviors analogous to those elicited by low concentrations of Taxol. For example, it stabilizes MTs *in vivo* and increases the cellular content of total tubulin and polymeric MTs [Nguyen et al., 1997, 1999]. MAP4 is also down-regulated during p53-mediated apoptosis [Murphy et al., 1996], suggesting that it may modulate apoptosis induced by Taxol. Indeed, Zhang et al. [1998] showed that overexpression of MAP4 was sufficient to increase cells' sensitivity to Taxol, although in other cell types no increased Taxol sensitivity was conferred by MAP4 overexpression [Nguyen et al., 1997]. Since low levels of Taxol, which cause only subtle alterations in MT dynamics, are sufficient to manifest dramatic effects on cell division and cell motility [Jordan et al., 1993; Liao et al., 1995; Tanaka et al., 1995], even a slight alteration in Taxol's ability to stabilize MTs in MAP4-overexpressing cells could change cell survival or mitotic properties significantly. In the absence of drugs, though, MAP4-overexpressing cells showed decreased growth rate and organelle transport [Nguyen et al. 1997; Bulinski et al., 1997], suggesting that changes in drug sensitivity in

MAP-overexpressing cells might be unrelated to changes in MT dynamics.

In this paper, we focused on ensconsin, a MAP reported not to be MT-stabilizing [Faire et al., 1999], as a potential effector of the Taxol response. Our initial finding, that ensconsin expression correlated positively with sensitivity to the drug, suggested the hypothesis that raising cellular ensconsin level might increase Taxol sensitivity. To test this hypothesis, we used heterogeneous populations of stable transfectants to detect any impact a cell's ensconsin content might have on its survival during Taxol treatment. Our experimental design allowed us to rule out other variables effectively, including integration point mutations, gene expression changes caused by transfected cDNAs, or spontaneous alterations in Taxol sensitivity that might arise during extended culture. The fact that all stably expressing cells were exposed to the same culture conditions and drug treatments provided an internal control in our experiments. We noted that treatment of heterogeneous, uncloned stable transfectants might also simulate an *in vivo* situation relevant to cancer; in essence, our experiments measured "survival of the fittest" during a drug treatment of cells that differed *only* in level of expressed ensconsin.

In support of our hypothesis, Taxol-sensitive cells such as MCF-7 and HeLa that were engineered to overexpress ensconsin or its MT-binding domain were killed more readily by moderate Taxol treatments. However, in TC-7, a cell line  $\sim 300$ -fold less sensitive to Taxol than HeLa, Taxol's potency was not changed by overexpression of ensconsin domains unless we first treated the cells so as to accentuate their Taxol sensitivity. Our results indicate that ensconsin may, indeed, potentiate Taxol action, but the TC-7 results suggest that its effects are not potent enough to be detected in cells that tolerate high levels of Taxol.

Accumulated data provide clues about how ensconsin might affect Taxol sensitivity. First, unlike MAP4, ensconsin neither slows cell growth nor alters MT dynamics in interphase cells when it is expressed at normal or slightly overexpressed levels (4–10 times the endogenous concentration) [Faire et al., 1999]. Heightened expression of ensconsin does, however, increase mitotic index and incidence of multipolar spindles [Faire et al., 1999]. Thus, it is unlikely that Taxol is more effective in ensconsin-overexpressing cells because the cells' growth has already been deleteriously affected by ensconsin expression. Rather, the retardation of M-phase progression and decreased mitotic fidelity caused by ensconsin overexpression may instead work additively or synergistically with Taxol's similar effects on mitotic progression. It is striking that both MAP4 and ensconsin are each mimetic of different Taxol effects.

Second, in MTs prepared from cells pre-treated with Taxol, ensconsin appeared to be more tightly bound to MTs. Together with data from Masson and Kreis [1995], who found that M-phase phosphorylation of ensconsin concomitantly lessened its affinity for MTs, this result raises the possibility that Taxol treatment may alter ensconsin phosphorylation, and may increase ensconsin's MT-binding affinity, thus increasing the amount of MAP bound to the MT at any time. This hypothesis is supported by the fact that tighter binding of ensconsin (as in Taxol-treated cells) and increased level of ensconsin bound along the MT (as in ensconsin-overexpressing cells) both lead to arrest and/or retardation of mitosis. In addition, studies of ensconsin's MT-binding dynamics demonstrate that ensconsin does increase its residence time on the MT in cells treated with Taxol, protein kinase inhibitors, or media that reduce cytoplasmic ATP concentration [Bulinski et al., 2001]. These results suggest that ensconsin exists in more than one state with regard to MT binding, and that the shift to the dynamically bound state depends on phosphorylation of ensconsin or other proteins. It is even possible that ensconsin becomes an MT-stabilizing MAP when it is bound to MTs in its less dynamic state (i.e., long residence time,  $t_{1/2}$ ).

Our results suggest several potential mechanisms by which cells expressing more ensconsin could be rendered more susceptible to killing by Taxol. A plausible model is that Taxol changes ensconsin's MT binding, either its tenacity or its dynamics of binding and release, or both. MTs possessing high levels of tightly or statically bound ensconsin may be more highly stabilized by Taxol, and these hyper-stabilized MT structures may interfere with intracellular trafficking, mitosis, or motility [Hamm-Alvarez et al., 1996; Jordan et al., 1993; Liao et al. 1995; Bulinski et al., 1997]. Alternatively, ensconsin's less dynamic MT binding may harm cells because the MAP's static association with MTs results in sequestration of an ensconsin-binding partner on the MTs. Examples abound of important regulatory molecules, such as kinases and phosphatases, that are tethered to MTs through association with a MAP [Obar et al., 1989; Ookata et al., 1995; Liao et al., 1998; Lee et al., 1998]. Activity of these enzymes would be expected to change in potentially deleterious ways if the MAP to which they bound changed its dynamics of MT-binding.

We do not yet have information about the phosphorylation state of ensconsin, with or without Taxol treatment. Altered phosphorylation of ensconsin or other proteins could occur either as a direct effect of Taxol treatment or as a function of Taxol's MT stabilization, which may inhibit the normal mitotic regulation of ensconsin. Several striking changes in phosphorylation of other proteins and of kinase activity have been noted in Taxol-treated cells, including tyrosine phosphorylation

of *shc* [Wolfson et al., 1997], novel phosphorylation of another cytoskeletal protein, vimentin [Vilalta et al., 1998; Chu et al., 1998], and a block in intracellular activation of MAP kinase [Jackson et al., 1997]. In addition, reports have documented both *activation* of PKA, leading to BCL2 hyperphosphorylation and induction of apoptosis [Srivastava et al., 1998], as well as the converse, *decreased* PKA activity [Wang et al., 1999]. Decreased PKA activity has been suggested to be a widespread effect of Taxol treatment that is necessary but not sufficient to confer Taxol sensitivity [Wang et al., 1999]. Results presented here, suggesting that cells containing more ensconsin are more susceptible to downstream effects of Taxol, impel us to investigate the mechanisms by which Taxol treatment changes ensconsin's MT-binding properties and how it impacts upon mitosis and cell survival.

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