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Cross-resistance of Nocodazole-Resistant Mutants of CHO Cells Toward Other Microtubule Inhibitors: Similar Mode of Action of Benzimidazole Carbamate Derivatives and NSC 181928 and TN-16

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SUMMARY

Stable mutants which are between 1.6- and 2.2-fold resistant to the microtubule inhibitor, nocodazole (Noc^R mutants) have been isolated in Chinese hamster ovary cells after a single-step selection. The different Noc^R mutants exhibit specific changes in their cross-resistance or collateral sensitivity toward various microtubule inhibitors (viz. colchicine, podophyllotoxin, taxol, vinblastine, and maytansine), but they show no change in resistance toward unrelated compounds, indicating that the genetic lesions in these mutants are microtubule related. The set of Noc^R mutants examined and a second-step podophyllotoxin-resistant cell line, Pod^{RII}6 (which is highly resistant to nocodazole) were found to exhibit proportionately increased cross-resistance toward various benzimidazole carbamate derivatives (viz. mebendazole, fenbendazole, carbendazim, parbendazole, oxibendazole, albendazole, benomyl, and cambendazole) as well as two additional microtubule inhibitors, NSC 181928 (ethyl 5-amino-1,2-dihydro-3[*N*-methylanilino)methyl]pyrido[3,4-*b*]-pyrazin-7-ylcarbamate) and TN-16 [3-(1-anilinoethylidene)-5-benzyl-pyrrolidine-2,4-dione]. These results indicate that the mechanism of action of these latter two inhibitors is very similar to that of nocodazole. The lack of cross-resistance of the Noc^R and Pod^{RII}6 cell lines to thiabendazole provides evidence that the mechanism of action of this compound is different from that of other benzimidazole carbamate derivatives. Based on structure-activity relationship studies between various nocodazole-like compounds, a number of structural features of these compounds which appear important/essential for this type of biological activity have been identified.

The benzimidazole carbamate group of compounds exhibits a broad range of clinically and agriculturally useful biological activity. For example, a number of compounds of this group (viz. mebendazole, oxibendazole, albendazole, cambendazole, fenbendazole, parbendazole) show potent anthelmintic activity against a variety of pathogenic nematodes, cestodes, and hematodes, whereas other compounds such as benomyl and thiabendazole have proven effective as fungicides (1–3). Nocodazole, which is another compound of this group, has shown antineoplastic activity in a number of experimental systems (4). Earlier studies with benzimidazole carbamate derivatives have shown that they inhibit cell division by interfering with the mitosis step. In cells treated with these drugs, MTs are depolymerized and they also inhibit tubulin polymerization and binding of colchicine to tubulin under *in vitro* conditions (5–8).

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For the past few years, we as well as others have been employing a genetic approach to investigate the mechanism of action of different MT inhibitors in mammalian cells (9–19). Using this approach in CHO cells, mutants resistant to a number of different MT inhibitors (viz. colchicine, colcemid, griseofulvin, taxol, podophyllotoxin, vinblastine) have been isolated (9–19). Although many of these mutants are affected in α- and β-tubulins (10, 11, 13), a number of mutants resistant to podophyllotoxin and griseofulvin are specifically altered in MT-related proteins other than tubulin (14, 17, 18, 20). The detailed cross-resistance studies with some of these mutants have provided valuable insight into the possible similarity or differences in the modes of action of different MT inhibitors (19, 21).

In the present paper, we report the selection and some characteristics of CHO cell mutants resistant to the benzimidazole carbamate derivative, nocodazole (Noc^R mutants). The cross-resistance studies with the Noc^R mutants show that they

ABBREVIATIONS: MT, microtubule; CHO, Chinese hamster ovary; NSC 181928, ethyl 5-amino-1,2-dihydro-3[(*N*-methylanilino)methyl]-pyrido(3,4-*b*)-pyrazin-7-ylcarbamate; TN-16, 3-(1-anilinoethylidene)-5-benzyl-pyrrolidine-2,4-dione; WT, wild type; EMS, ethyl methanesulfonate; Noc^R, nocodazole resistant; Pod^R, podophyllotoxin resistant.

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exhibit highly specific and characteristic patterns of cross-resistance toward various benzimidazole carbamate derivatives that possess antimitotic activity. These studies indicate that two additional MT inhibitors, NSC 181928 and TN-16, possess the same type of biological activity as nocodazole and other benzimidazole carbamate derivatives and that all of these inhibitors contain common structural determinants which may be responsible for their activities.

Materials and Methods

Cell lines and culture conditions. The parental nocodazole-sensitive CHO line is referred to as WT in our work (16–19). The selection of Noc^R mutants is described in the text. $Pod^{RII}6$ is a two-step podophyllotoxin-resistant mutant of CHO cells, which has been extensively studied in our laboratory (14, 21). The cell lines were routinely grown in monolayer cultures in alpha minimal essential medium supplemented with 5% fetal bovine serum by procedures described earlier (22).

Selection of mutants. An exponentially growing semiconfluent dish of WT cells was treated with 300 μg/ml of the mutagen EMS for 20 hr at 37°. This treatment results in about 50% cell killing. The mutagen-treated cells were grown for 4 days in nonselective medium to allow time for mutation fixation and expression of the mutant phenotype (22). The selection of Noc^R mutants from control or EMS-treated cells was carried out by plating 5×10^5 cells/100-mm-diameter dish in a large number of dishes in medium containing the indicated concentration of the drug. The observed mutant frequencies have been corrected for the plating efficiency of the cells at the time of selection.

Degree of resistance of mutant cells toward various drugs. The degree of resistance of mutants toward various drugs was determined from their relative plating efficiencies in the presence of different concentrations of the drugs, as described previously (19, 21). The D_{10} value of a drug toward a cell line refers to the dose of the drug which reduces relative plating efficiency of a cell line to 10% as compared to the untreated control cells.

Drugs and chemicals. The sources of various drugs were as follows. Nocodazole was purchased from Aldrich Chemical Co. Inc., Milwaukee, WI. Mebendazole (Vermox) which was obtained from Ortho Pharmaceuticals (Canada) was kindly provided by Dr. U. G. G. Hennig of University of Alberta, Edmonton, Canada. Fenbendazole (Panacur) was provided by Dr. R. K. Muser, Hoechst-Roussel Pharmaceuticals, Inc., Sommerville, NJ; carbendazim and benomyl by Drs. C. T. Bennett and H. J. Thome of E. I. du Pont de Nemours & Co., Wilmington, DE; oxibendazole (SK & F 30310), albendazole (SK & F 62979), and parbendazole by Dr. R. C. Parish of Smith Kline Animal Health Products, West Chester, PA; and cambendazole and thiabendazole by Drs. C. A. Cutler and C. M. Fraser of Merck, Sharp and Dohme Research Laboratories, Rahway, NJ. NSC 181928 was kindly provided by Dr. G. P. Wheeler (23) of Southern Research Institute, Birmingham, AL, as well as by Dr. V. L. Narayanan of the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. TN-16 was purchased from Wako Pure Chemical Industries Ltd., Tokyo, Japan. A small sample of TN-16 was also kindly provided by Dr. T. Arai (24), University of Tsukuba, Ibaraki, Japan.

The initial stock solutions of the drugs were made in dimethyl sulfoxide which were then diluted with sterile H_2O to obtain secondary stock solutions. The final concentration of dimethyl sulfoxide in all experiments was less than 0.1%, which has no toxic or other observable effects on cells.

Other procedures. The two-dimensional gel electrophoresis of [^{35}S] methionine-labeled cellular proteins was carried out as described earlier (14, 18).

Results

Selection of nocodazole-resistant mutants of CHO cells and their cross resistance pattern toward other microtubule inhibitors. The dose-response curve of nocodazole toward WT CHO cells is shown in Fig. 1A. The drug was toxic to CHO cells in the range of 5–10 ng/ml, and at 15 ng/ml, surviving clones were obtained at a frequency of approximately 1×10^{-6}. The selection of mutants resistant to nocodazole was carried out by plating EMS-treated WT cells in medium containing 15, 20, and 25 ng/ml of nocodazole (see Materials and Methods for details). After 10–12 days of incubation at 37°, a few discrete colonies were observed in dishes containing 15 and 20 ng/ml of nocodazole (approximate frequency 1×10^{-5} and 3×10^{-6}, respectively), but no surviving colonies were observed at 25 ng/ml or higher concentrations of the drug. A few of the well formed colonies which were showing good growth under these conditions were picked and, after growth in nonselective medium, their degree of resistance toward nocodazole was determined. All of the eight clones thus examined showed increased resistance to nocodazole in comparison to WT cells. The dose response curves for nocodazole for four of these mutants (Noc^R-3, Noc^R-11, Noc^R-12, and Noc^R-14) are shown in Fig. 1A. The mutants exhibited between 1.6- and 2.2-fold resistance to nocodazole, and their level of resistance was in the order of Noc^R-3 ≥ Noc^R-14 > Noc^R-11 ≥ Noc^R-12 > WT. Similar results with these mutants have been obtained in three independent experiments. The drug-resistant phenotype of these mutants has remained stable upon subcloning and prolonged growth (>6 months) in nonselective medium. Due to the low level of resistance of the mutants, a second-step selection in the presence of higher concentrations of nocodazole was also attempted on some of the Noc^R mutants. However, the second-step selection has thus far not yielded any mutants

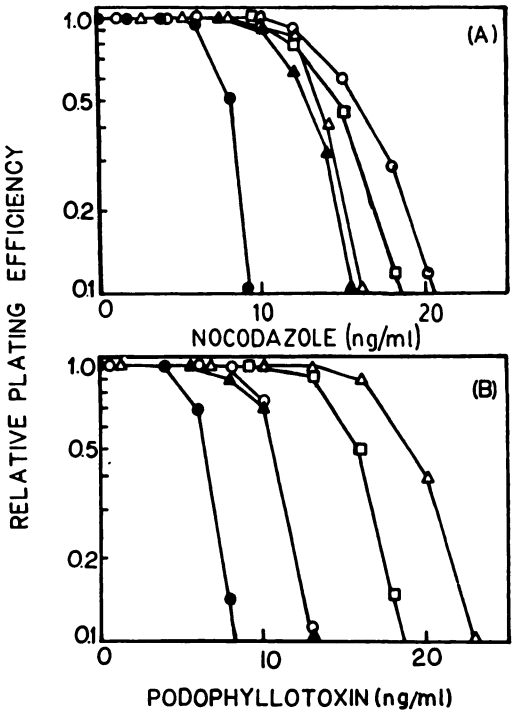

Fig. 1. Dose response curves of the parental (i.e., WT) and Noc^R mutants toward nocodazole (A) and podophyllotoxin (B). ●, WT; ○, Noc^R-3; △, Noc^R-11; ▲, Noc^R-12; □, Noc^R-14.

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which are significantly more resistant than the mutants obtained after a single-step selection (results not shown).

The cross-resistance pattern of the Noc^R mutants towards various MT inhibitors was next investigated. In earlier studies with Pod^R mutnts of CHO cells, it was observed that all Pod^R mutants exhibited at least some degree of cross-resistance to nocodazole (14, 21). In particular, most of the Pod^{RII} mutants (obtained after two single-step selections in the presence of podophyllotoxin), exhibited between 3- and 4-fold resistance to nocodazole in comparison to the parental cells (14). Therefore, it was of interest to examine whether the Noc^R mutants also exhibited cross-resistance toward podophyllotoxin. From the results of these studies, shown in Fig. 1B, it is evident that all of the Noc^R mutants exhibited increased resistance (2–3 fold) to podophyllotoxin, but the relative degree of resistance of different mutants to nocodazole was not in the same order as that observed for nocodazole (Fig. 1A). Results of cross-resistance studies for some of the other MT inhibitors are shown in Table 1. Interestingly, the various Noc^R mutants showed enhanced sensitivity toward taxol which acts by stabilizing the MT structure (25). Previously, mutants resistant to podophyllotoxin and benomyl (a benzimidazole carbamate derivative) have also been found to exhibit enhanced sensitivity toward taxol, (14, 15, 21). In contrast to the results obtained with podophyllotoxin and taxol, the various Noc^R mutants exhibited interesting differences in their cross-resistance behavior toward other MT inhibitors. For example, whereas Noc^R-12 and Noc^R-14 showed enhanced sensitivity toward colchicine, Noc^R-3 and Noc^R-11 displayed increased resistance to this drug. Similarly, for vinblastine and maytansine, whereas some mutants exhibited either no change in sensitivity or higher sensitivity in comparison to the parental cells, the others were found to have become more resistant to the drugs. The above mutants, however, showed no change in their degree of resistance toward the MT inhibitor, griseofulvin, as well as unrelated drugs such as puromycin or daunomycin (Table 1), to which the mutants affected in membrane permeability are known to exhibit cross-resistance (19, 26–28). The observed specific changes in the cross-resistance patterns of the Noc^R mutants toward various MT inhibitors and their lack of cross-resistance to the drugs to which membrane permeability mutants exhibit increased resistance provide strong suggestive evidence that the genetic lesions in these mutants are MT related.

TABLE 1

Cross-resistance pattern of the Noc^R mutants toward different MT inhibitors and other compounds

Compound	D_{10} value for WT cells[a]		Relative degree of resistance of different mutants[a]			
			Noc^R-3	Noc^R-11	Noc^R-12	Noc^R-14
	μg/ml					
Colchicine	0.08	(1)	1.9	1.2	0.6	0.6
Vinblastine	0.01	(1)	1.6	2.5	1.4	1.0
Maytansine	0.0015	(1)	1.3	0.9	0.8	0.8
Taxol	0.09	(1)	0.35	0.20	0.6	0.30
Griseofulvin	4.5	(1)	1.0	1.0	0.9	1.0
Puromycin	3.0	(1)	1.0	1.0	1.0	1.0
Daunomycin	0.02	(1)	1.0	1.0	1.0	1.0

[a] The effect of different concentrations of the drugs on plating efficiencies of cells was determined in parallel as in Fig. 1. Assuming the D_{10} value of any drug for the WT cells to be 1 (shown in parentheses), relative degree of resistance/sensitivity of other cell lines was determined. In two independent experiments that have been carried out with these drugs, the relative resistance/sensitivity of the mutant cell lines differed by < 10%.

To determine whether any of the Noc^R mutants showed any electrophoretic alteration in any of the known MT proteins, two-dimensional (2-D) gel electrophoretic analysis of total cellular proteins from the WT and the mutant cells was carried out. Results of these studies have thus far failed to reveal any apparent electrophoretic alteration in the Noc^R mutant cells (results not shown).

Cross-resistance pattern of the Noc^R mutants toward benzimidazole derivatives. The cross-resistances of the Noc^R mutants toward a number of different benzimidazole derivatives, whose structures are shown in Fig. 2, was next examined. These studies were carried out with three of the Noc^R mutants (viz. Noc^R-3, Noc^R-12, and Noc^R-14) and the Pod^{RII}6 cell line, which is about 3.2-fold resistant to nocodazole (14). Results of these studies are presented in Table 2. For all of the compounds studied except thiabendazole, the various Noc^R mutants and the Pod^{RII}6 cell line showed proportionately increased resistance in comparison to the WT cells. However, no cross-resistance was observed for thiabendazole for any of the mutant cell lines. In earlier studies with emetine-resistant and Pod^R mutants, we have observed that, for different derivatives of a compound which act in the same manner, molar concentrations of the compounds which produce equivalent cellular toxicity toward WT cells (viz. D_{10} values) provide a good measure of their relative activities (21, 29–31). Assuming the molar D_{10} value for nocodazole to be 1, the relative toxicities of various benzimidazole derivatives which show similar activity have been calculated (Table 2). Table 2 also gives the IC_{50} values (drug concentration causing 50% inhibition) for inhibition of tubulin polymerization by various benzimidazole derivatives as reported in the literature (7, 8). If cellular toxicity of various benzimidazole carbamate derivatives to which the Noc^R mutants exhibit cross-resistance was due to their effects on MTs, then the relative activities of these compounds in inhibiting tubulin polymerization should show a good correlation to their relative cellular toxic concentrations. As seen from Table 2, these two values indeed show a good correlation (correlation coefficient 0.82), which supports the view that the cellular toxicity of these compounds is due to their effect on microtubules.

Wheeler *et al.* (23, 32, 33) in recent years have reported that a number of 1,2-dihydro[3,4-*b*]pyrazines show potent antimitotic activity against mammalian cells both *in vitro* and *in vivo*. One of the most active compounds in this series is NSC 181928. Previous biochemical studies with NSC 181928 show that, like other benzimidazole carbamate derivatives, it competes with colchicine for binding to tubulin and, like nocodazole, its antimitotic effect on cells is reversible (23, 32, 34, 35). Arai (24) has described another synthetic MT inhibitor, TN-16, which also competitively and reversibly inhibits binding of colchicine to tubulin. Since NSC 181928 and TN-16 bear structural similarity to the benzimidazole carbamates (see Fig. 2), it was of much interest to examine cross-resistance of the Noc^R and Pod^{RII}6 cell lines toward these compounds. From the results of these studies, shown in Fig. 3, it is evident that, similar to nocodazole and other benzimidazole carbamate derivatives, the various Noc^R mutants and the Pod^{RII}6 cell line exhibited proportionately increased resistance to both NSC 181928 and TN-16. These results indicate that the mechanisms of action of NSC 181928 and TN-16 should be very similar to those of nocodazole and other benzimidazole carbamate derivatives.

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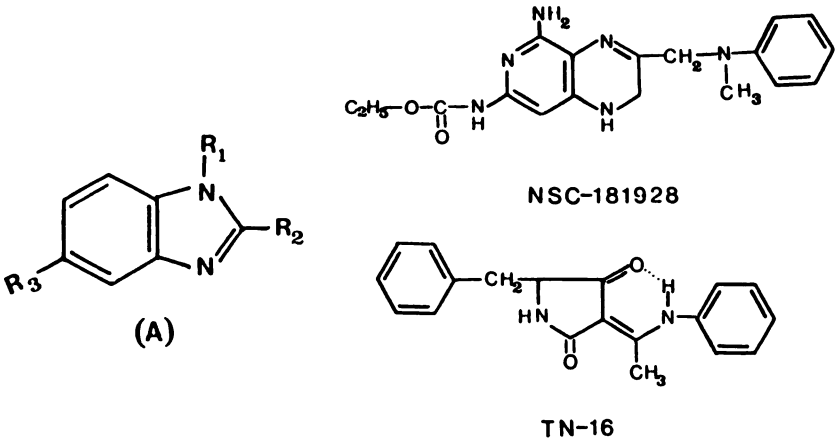

(A)	R_1	R_2	R_3
Nocodazole	-H	$-NHCO_2CH_3$	
Mebendazole	-H	$-NHCO_2CH_3$	
Fenbendazole	-H	$-NHCO_2CH_3$	
Carbendazim	-H	$-NHCO_2CH_3$	-H
Parbendazole	-H	$-NHCO_2CH_3$	$-C_4H_9$
Oxibendazole	-H	$-NHCO_2CH_3$	$-OC_3H_7$
Albendazole	-H	$-NHCO_2CH_3$	$-SC_3H_7$
Benomyl	$-CONHC_4H_9$	$-NHCO_2CH_3$	-H
Cambendazole	-H		$-NHCO_2CH(CH_3)_2$
Thiabendazole	-H		-H

Fig. 2. Chemical structures of various benzimidazole derivatives (*A*), NSC 181928, and TN-16.

Discussion

The benzimidazole carbamate derivative, nocodazole, is a specific inhibitor of MT assembly *in vivo* and *in vitro* (5–8). Results presented in this paper show that stable mutants which are between 1.6- and 2.2-fold resistant to nocodazole can be readily isolated in CHO cells after a single-step selection in the presence of the drug. The different Noc^R mutants exhibit specific changes in their cross-resistance/sensitivity toward various MT inhibitors, but their sensitivity toward unrelated compounds such as puromycin and daunomycin, to which the mutants affected in membrane permeability exhibit increased resistance, was found to be unaltered. These results indicate that the genetic lesion(s) in these mutants is MT specific and affects a cellular component(s) that is involved in the interaction of these drugs with MTs. Earlier studies with mutants of *Aspergillus nidulans* resistant to the benzimidazole carbamate derivatives, benomyl, showed that a number of these mutants contained electrophoretically altered forms of β-tubulin (36). However, 2-D gel electrophoretic analysis of the Noc^R mutants has not shown any change in the mobility of tubulin or any other proteins. Since mutations that lead to a charge alteration comprise only a small fraction ($\simeq$15%) of all mutations, our failure to observe an electrophoretic change may be due to the small number of mutants that were investigated. Nevertheless, in view of these results, other possible mechanisms for drug resistance could not be entirely excluded at present.

The cross-resistance studies with the Noc^R mutants toward various known MT inhibitors provide information regarding similarity and differences in the mode of action of different drugs. Our observation that the Noc^R mutants do not show a similar pattern of cross-resistance to colchicine, taxol, vinblastine, maytansine, and griseofulvin, as observed for nocodazole, suggests that the mechanisms of action of these inhibitors differs from that of nocodazole. The reciprocal cross-resistance of the Noc^R and Pod^R mutants to podophyllotoxin and nocodazole (21) indicates some similarity in the mode of action of these two drugs. However, the failure of these mutants to show proportionate cross-resistance to the other drug indicates that their mechanisms of action, although similar, are not identical. In contrast to the results with these inhibitors, the set of Noc^R mutants exhibited proportionately increased cross-resistance

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TABLE 2

Cross-resistance pattern of the Noc^R and Pod^{RII}6 mutants toward various benzimidazole derivatives: relative activity of different compounds

Compound	D_{10} value for WT cells[a]	Relative degree of resistance of the mutant cell lines[a]			IC_{50} values for inhibition of MT assembly *in vitro*[b]	MT-inhibitory inhibitory activity relative to nocodazole[c]	Cellular toxic concentration relative to nocodazole[d]
		Noc^R-3	Noc^R-12	Pod^{RII}6			
	M				*M*		
Nocodazole	3.3×10^{-8}	2.0	1.6	3.2	1.97×10^{-6}	1	1
Mebendazole	1.3×10^{-7}	2.1	1.5	3.4	5.20×10^{-6}	0.38	0.25
Fenbendazole	1.4×10^{-7}	1.6	1.3	3.6	6.32×10^{-6}	0.31	0.24
Carbendazim	4.2×10^{-6}	1.6	1.3	3.0	90.10×10^{-6}	0.022	0.008
Parbendazole	3.6×10^{-8}	2.0	1.5	3.6	3.63×10^{-6}	0.54	0.92
Oxibendazole	1.2×10^{-7}	1.5	1.3	3.5	2.54×10^{-6}	0.77	0.28
Albendazole	1.9×10^{-7}	2.0	1.4	3.4	20.0×10^{-6}	0.1	0.17
Benomyl	6.2×10^{-6}	1.8	1.3	3.1	58.30×10^{-6}	0.033	0.005
Cambendazole	1.0×10^{-6}	2.0	1.6	4.5	64.20×10^{-6}	0.030	0.033
Thiabendazole		1.0	1.0	1.0	>1000	*e*	*e*

[a] The D_{10} values for various cell lines toward different benzimidazole derivatives were determined from survival curves similar to those shown in Fig. 1. Assuming the D_{10} value of any drug for the WT cells to be 1, the relative degree of resistance of the mutant cell lines was calculated from the ratios of the D_{10} values for the mutant cell lines as compared to that for the WT cells. Similar results (±10%) for these lines have been obtained in three independent experiments. The Noc^R-14 cell line showed a level of resistance toward various derivatives similar to that seen here for the Noc^R-3 mutant.

[b] The IC_{50} values represent concentrations of the drugs which inhibit polymerization of bovine brain tubulin by 50% under *in vitro* conditions. Except for albendazole, the IC_{50} values shown here have been taken from the work of Friedman and Platzer (7). The IC_{50} values for albendazole are from the work of Ireland *et al.* (8).

[c] Assuming the molar IC_{50} value for nocodazole to be 1, the relative MT-inhibitory concentrations of other compounds have been calculated.

[d] Assuming the molar toxic concentration for nocodazole to be 1, the relative toxic concentrations for other derivatives have been calculated.

[e] Does not show MT-inhibitory or nocodazole-like activity.

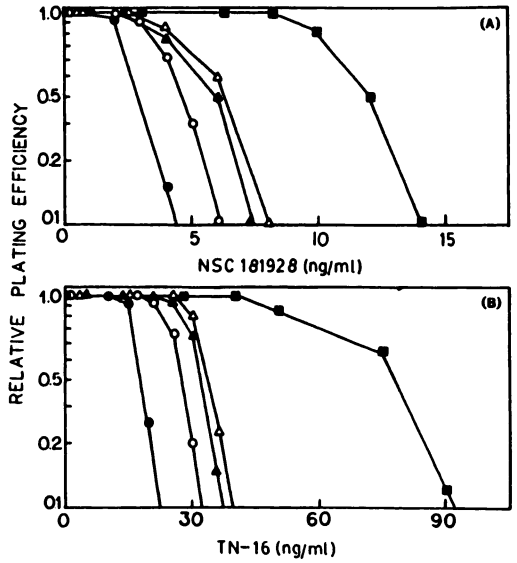

Fig. 3. Dose response curves of the parental and the mutant cell lines in the presence of increasing concentrations of NSC 181928 (A) and TN-16 (B). ●, WT cells; △, Noc^R-3; ○, Noc^R-12; ▲, Noc^R-14; ■, Pod^{RII}6.

to a large number of benzimidazole carbamate derivatives (all except thiabendazole, which lacks the carbamate group) as well as to two other MT inhibitors, NSC 181928 and TN-16. These results provide strong suggestive evidence that the mechanism of cellular action of all these inhibitors (except thiabendazole) should be similar to that of nocodazole. This inference is consistent with previous biochemical studies which show that all of these compounds, except thiabendazole, inhibit tubulin polymerization *in vitro* and, like nocodazole, they compete with colchicine for binding to tubulin (5–8, 23, 24, 34, 35). These results also indicate that cross-resistance studies with the set of Noc^R and Pod^R mutants provide a simple and specific mean for identifying other compounds which act in a similar manner.

From the chemical structures of various benzimidazole derivatives (Fig. 2) and their relative cellular toxicities or activities (Table 2), a number of inferences regarding structure-activity relationship between these compounds could be made. If one examines the chemical structures of various benzimidazole derivatives that show nocodazole-like activity in our studies (viz. mebendazole, fenbendazole, carbendazim, parbendazole, oxibendazole, albendazole, benomyl, and cambendazole), then all of these are found to contain an aminocarboalkoxy

$$\begin{matrix} & & O & \\ & & \| & \\ -NH- & C & - & R \end{matrix}$$

(—NH—C—R) group at either position R_2 or R_3. The importance of this group in the biological activity of these compounds is indicated by several observations. 1) If one compares the chemical structures of cambendazole and thiabendazole, then these differ only in one regard, i.e., cambendazole contains a

$$\begin{matrix} & & O & \\ & & \| & \\ -NH- & C & - & R \end{matrix}$$

—NH—C—R group at position R_3 in place of a —H in thiabendazole. Since the former compound behaves like nocodazole, whereas the latter does not, this provides strong evidence

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regarding the essential nature of the —NH—C—R group for nocodazole-like activity. 2) The two other MT inhibitors, NSC 181928 and TN-16 (23, 24), which show the same type of behavior in cross-resistance studies as benzimidazole carba-

$$\begin{matrix} & & O & \\ & & \| & \\ -NH- & C & - & R \end{matrix}$$

mate derivatives, both contain a —NH—C—R group in their structures. Furthermore, the structure-activity studies of 1,2-dihydropyrido-[3,4-*b*]pyrazine derivatives by Wheeler *et al.* (33) have shown that, when this group is absent (as in compound No. 35 in Ref. 33), then the antimitotic activity of these compounds is totally lost, thus again indicating the essential

$$\begin{matrix} & & O & \\ & & \| & \\ -NH- & C & - & R \end{matrix}$$

nature of the —NH—C—R group for this kind of antimitotic

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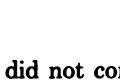
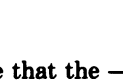
activity. 3) In an extension of the present studies, we have examined the cross-resistance of the set of Noc^R and Pod^R mutants toward a large number of other benzimidazole derivatives which contain different substitutent groups at the $—R_2$ position. Results of these studies show that 2-benzimidazolyl

urea, which contains a $—NH—C—NH_2$ group at the R_2 position, exhibits a cross-resistance pattern and a biological activity similar to those of nocodazole, whereas the other compounds



examined which did not contain the —NH—C—R group are inactive in this regard.[1] All of the above observations together



strongly indicate that the —NH—C—R group plays a critical role in the interaction of nocodazole and related drugs with tubulin.

Another structural feature that is common in all compounds which show nocodazole-like activity and may be required for such activity is the presence of two rings fused to each other in [3,4-*b*] positions. One of these should be a six-membered ring (which may be heterocyclic), but the other could be either a five (such as in benzimidazole)- or a six (as in NSC 181928)-membered ring. The compound TN-16, which also shows this type of activity, appears at first as an exception in this regard. However, this compound could also form a six-membered ring which is linked to the five-membered ring by hydrogen bond formation between the —NH group on aniline and the carbonyl

(—C—) group on the 4-position in the pyrrolidine ring (see Fig. 2).

Finally, if one compares the relative activity of various benzimidazole derivatives which show nocodazole-like activity, it is evident that those compounds which contain a large nonpolar group in the R_3 position show much higher activity in comparison to others which contain —H in this position (viz. benomyl, carbendazim). The nonpolar substituent in the R_3 position could be either an aromatic or a heterocyclic ring (as in nocodazole, mebendazole, and fenbendazole), or it could be a linear aliphatic chain (as in parbendazole, oxibendazole, and albendazole). Similar nonpolar substituents are also present in an analogous position in NSC 181928 and TN-16 (see Fig. 2). It is possible that the nonpolar group at the R_3 position, due to its hydrophobic interaction, may stabilize the binding of nocodazole to tubulin. A similar inference regarding the importance of substituent at the R_3 position on the activity of the benzimidazole carbamate derivatives has been reached by other investigators (37).

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