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IDENTIFICATION OF 2-BENZIMIDAZOLYL UREA AS A NEW ANTIMITOTIC COMPOUND BASED ON CROSS RESISTANCE STUDIES WITH NOCODAZOLE RESISTANT MUTANTS OF CHO CELLS

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The cross resistance patterns of a set of nocodazole-resistant (Noc^R) and podophyllotoxin-resistant (Pod^R) mutants of Chinese hamster ovary cells, which exhibit highly specific cross resistance towards compounds that show nocodazole-like antimitotic activity, towards a large number of benzimidazole derivatives have been examined. Of the various compounds examined, the Noc^R and the Pod^R mutants were found to exhibit increased cross resistance towards only 2-benzimidazolyl urea, indicating that this compound may possess similar biological activity as nocodazole. The nocodazole-like antimitotic activity of 2-benzimidazolyl urea has been confirmed by its ability to block cells in mitosis, and by its competition of ^{3}H-podophyllotoxin binding to microtubule proteins in cell extracts. The nocodazole-like behavior of 2-benzimidazolyl urea and lack of similar activity in other benzimidazole derivatives examined, provides valuable information regarding structural features that are required for this type of biological activity.

In an earlier paper, cross resistance of a set of nocodazole-resistant (Noc^R) mutants of Chinese hamster ovary (CHO) cells towards various microtubule inhibitors and benzimidazaole anthelimintics was examined (1). Results of these studies showed that the cross resistance pattern of the Noc^R mutants was highly specific and that these mutants exhibited proportionately increased cross resistance to various benzimidazole carbamate derivatives which showed nocodazole like antimitotic activity (viz. mebendazole, fenbendazole, carbendazim, parbendazole, oxidbendazole, albendazole, carbendazim and benomyl), but not to thiabendazole which lacked similar activity in mammalian cells. The cross resistance studies with the set of Noc^R mutants thus provided a sensitive and specific assay for identifying other compounds which possessed similar biological activity as nocodazole. Based on these studies, it was inferred that the microtubule inhibitors NSC 181928 and TN-16, which do not contain the

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benzimidazole ring system in their structures (2,3) also interacted with MTs in the same manner as nocodazole and other benzimidazole anthelimintics (1). The structure-activity relationship studies on these compounds indicated that a NH - C - R group (where R is an alkyloxy group), was an essential requirement for the nocodazole-like activity.

To further investigate the importance of - NH - C - R group in nocodazole-like activity, I have examined cross resistance of the set of Noc^R mutants and a podophyllotoxin-resistant mutant (Pod^R), which exhibit high degree of resistance to nocodazole, towards a large number of benzimidazole derivatives which contain different substituent groups in the R_2 position (see Figure 1). These studies have led to the identification that 2-benzimidazolyl urea (2-BU) possesses the same kind of biological activity as nocodazole. This inference is supported by the blockage of cells in mitosis by 2-BU and by its ability to competitively inhibit binding of [^{3}H]podophyllotoxin to microtubule proteins in vitro. These results further reinforce and extend the important role of the - NH - C - R group in the biological activity of nocodazole-like compounds.

MATERIALS AND METHODS

Cell Lines and Plating Efficiency Determination: The parental Chinese hamster ovary (CHO) cell line is referred to as WT (wild-type) in our work (4,5). The selection and characteristics of the two nocodazole-resistant mutants Noc^R-3 and Noc^R-14 have been described recently (1). $Pod^{RII}6$ is a two-step podophyllotoxin-resistant line of CHO cells which exhibits specific high degree of resistance to nocodazole and other antimitotic benzimidazole carbamate derivatives (1,6,8). The degree of resistance of the mutant cell lines as compared to the parental WT cells towards various drugs was determined from their relative cloning efficiencies in presence of different concentrations of the drugs, as described previously (1,8). The effect of treatment with drugs on mitotic index of cells was determined as described earlier (9).

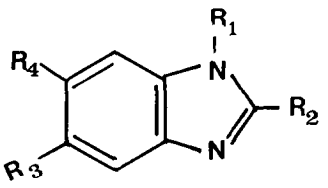
Drugs and Chemicals: 2-Guanidino benzimidazole, 2-phenyl benzimidazole, 2-(0-amino phenyl)benzimidazole, 5-chloro-2(trichloromethyl)benzimidazole, 2-hydroxy benzimidazole, 2-amino benzimidazole, 1-ethyl-2-methyl benzimidazole, 2-amino-5,6-dimethyl benzimidazole and 2-benzimidazolyl urea, were purchased from Aldrich Biochemical, Inc, Milwaukee, WI. The other chemicals viz. 5,6-dimethyl-2-guanidino benzimidazole (Cat. No. S57311-6), 5,6-dimethyl benzimidazole-1-acethyarazide (S34671-3), 2-amino methyl-5,6-dimethyl benzimidazole hydrochloride (S34118-5), 2-(5,6-dimethyl-2-benzimidazolyl amino)-6-methyl-4-pyrimidinol (S37409-1) and (5,6-dimethyl-2-benzimidazolyl)-carbamonitrile (S39107-7) were purchased from Alfred Bader Library of Rare Chemicals, Milwaukee, WI. The sources of other drugs and chemicals has been described earlier (1,8). Competition studies on the binding of [^{3}H]-podophyllotoxin to microtubular proteins in CHO cell extracts was carried out as described previously (8, 10-12).

RESULTS AND DISCUSSION

Cross resistance of the Noc^R and the Pod^{RII} 6 Mutants Towards Benzimidazole Derivatives: We have earlier shown that the present set of the Noc^R and Pod^R mutants exhibited highly specific cross resistance towards benzimidazole derivatives which show antimitotic activity and this property of the mutants could be used to screen for other compounds which behaved in a similar manner (1). With the aim of identifying additional compounds that may show this kind of activity, cross resistance of the Noc^R and the Pod^R cell lines towards a large number of benzimidazole derivatives, none of which was known to exhibit antimitotic acitivty, was investigated. The chemical structures of these derivatives are shown in Figure 1. These derivatives contain different substituent groups in the R_2-position in place of the carbamate group (NH - C(=O)-OCH_3) found in nocodazole and a number of other benzimidazole derivatives which show antimitotic activities (1). A number of these compounds also contain other substitutions in the R_1, R_3 and R_4 positions. We expected that if any of these compounds behaved in a similar manner as to nocodazole, then the Noc^R and Pod^R mutants would exhibit increased cross resistance towards it.

The cross resistance of the mutants towards these compounds was investigated by determining the relative cloning efficiences of the parental and the mutant cell lines in medium containing different concentrations of the drugs. For all of the compounds shown in Fig. 1, except No. 11 (i.e. 2-benzimidazoyl urea; 2-BU), no cross resistance was observed for the Noc^R and the Pod^R mutants i.e. their sensitivity was similar to the parental WT cells (results not shown). However, the above mutants were found to exhibit proportionately increased resistance to the compound No. 11, (i.e. 2BU) (Fig. 2), which was similar to that observed for nocodazole in earlier studies (1). Similar results with these compounds have been obtained in at least three independent experiments.

The increased resistance of the Noc^R and the Pod^R mutants to 2-BU suggested that this compound may show antimitotic activity as nocodazole. To investigate this, effect of treatment with 2-BU as well as a number of known antimitotic drugs on the mitotic index of WT cells was examined. From the results of



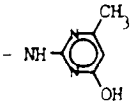
COMPOUND No.	CHEMICAL NAME	R_1	R_2	R_3	R_4
1	2-GUANIDINO BENZIMIDAZOLE	H	$-NH-C(=NH)-NH_2$	H	H
2	2-PHENYL BENZIMIDAZOLE	H		H	H
3	2-(O-AMINOPHENYL BENZIMIDAZOLE)	H		H	H
4	5-CHLORO-2(TRICHLOROMETHYL) BENZIMIDAZOLE	H	$-CCl_3$	Cl	H
5	5,6,DIMETHYL-2-GUANIDINO BENZIMIDAZOLE	H	$-NH-C(=NH)-NH_2$	CH_3	CH_3
6	2-HYDROXYBENZIMIDAZOLE	H	$-OH$	H	H
7	2-AMINOBENZIMIDAZOLE	H	$-NH_2$	H	H
8	1-ETHYL-2-METHYL BENZIMIDAZOLE	C_2H_5	$-CH_3$	H	H
9	2-AMINO-5,6-DIMETHYL BENZIMIDAZOLE	H	$-NH_2$	CH_3	CH_3
10	5,6-DIMETHYL BENZIMIDAZOLE 1-ACETHYDRAZIDE	$-CH_2-C(=O)-N(H)-NH_2$	H	CH_3	CH_3
11	2-BENZIMIDAZOLYL UREA	H	$-NH-C(=O)-NH_2$	H	H
12	2-(5,6,DIMETHYL-2-BENZIMIDAZOLYL AMINO) 6-METHYL-4-PYRIMIDINOL	H		CH_3	CH_3
13	(5,6,DIMETHYL-2-BENZIMIDAZOLYL)-CARBAMONITRILE	H	$-NH-C\equiv N$	CH_3	CH_3
14	2-AMINOMETHYL-5,6-DIMETHYL BENZIMIDAZOLE	H	$-NHCH_3$	CH_3	CH_3
15	BENZIMIDAZOLE	H	H	H	H

Figure 1. Chemical structures of different benzimidazole derivatives.

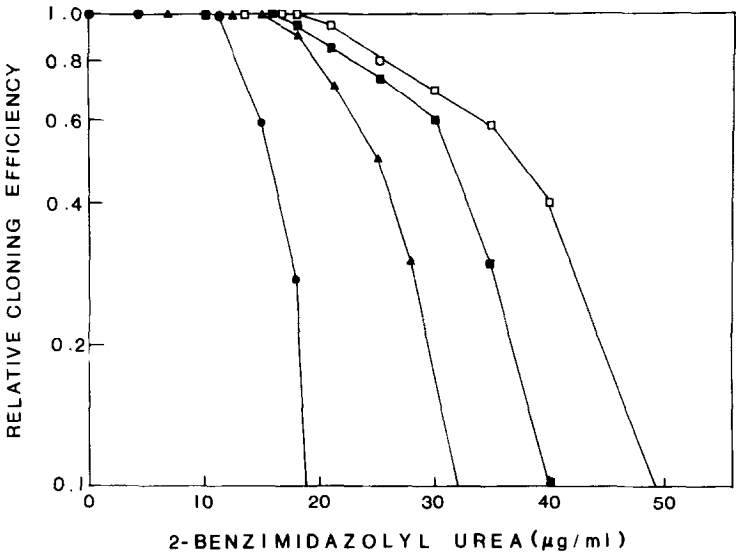


Figure 2. Survival curves of the parental and the mutant cell lines towards 2-benzimidazolyl urea. (●), wild-type CHO cells; (▲), Noc^R-3; (■), Noc^R-14 and (□), Pod^{RII}6 cell line.

these studies presented in Table 1, it is evident that similar to treatment with known antimitotic drugs (viz. nocodazole, colcemid and podophyllotoxin) 2-BU-treatment caused a large time-dependent increase in the fraction of cells which were arrested in mitosis. The other benzimidazole derivatives shown in Fig. 1 were inactive in this regard (results not shown).

Since nocodazole and related benzimidazole carbamates are competitive inhibitors of colchicine and/or podophyllotoxin binding to microtubule (MT)

Table 1

Effect of 2-Benzimidazolyl Urea and Other Inhibitors on the Mitotic Index of Cells

Compound	Concentration	% of cells in Mitosis				
		0 hr	1 hr	4 hr	8 hr	24 hr
Nocodazole	0.1 μg/ml	0.25 ± 0.10	0.72	23.4	54.5	70.0
Podophyllotoxin	0.05 μg/ml	0.25 ± 0.10	0.80	16.2	22.5	16.8
Colcemid	0.1 μg/ml	0.25 ± 0.10	0.70	14.3	23.5	21.1
2-Benzimidazolyl Urea	50 μg/ml	0.25 ± 0.10	0.50	3.8	10.7	12.5

The WT CHO cells were treated with the indicated concentration of various drugs (which are about 5 to 10 fold higher than the D_{10} values for the WT cells) for the specified times and % of cells which accumulated in mitosis was estimated as described previously (9).

Table 2

Competition of ^{3}H-Podophyllotoxin Binding to Microtubule Proteins

Competing Drug	Concentrations	^{3}H-Podophyllotoxin binding	
		Amount of drug bound to cell extracts (c.p.m.)	% Binding
None	Control	7,700	100
Nocodazole	1 µg/ml	7,250	94.1
	2 µg/ml	3,940	51.2
	5 µg/ml	1,630	21.2
Colchicine	1 µg/ml	7,580	98.4
	5 µg/ml	3,290	42.7
	10 µg/ml	1,530	19.9
2-Benzimidazolyl Urea	10 µg/ml	7,350	95.5
	20 µg/ml	6,500	84.4
	50 µg/ml	2,520	32.7
	100 µg/ml	1,250	16.2

Binding of ^{3}H-podophyllotoxin (3.4 Ci/mmol; final concentration 5 x 10^{-7} m) to MT proteins in WT cell extracts in presence of indicated concentrations of the competing drugs was determined as described in earlier studies (8, 10). Assuming the drug binding in absence of any competing drug as 100%, the % binding in presence of different drug concentrations was calculated. Results shown are average of triplicate samples which differed by <10%.

proteins (13-15) to obtain further evidence that 2-BU acted in a similar manner as nocodazole, competition studies on the binding of ^{3}H-podophyllotoxin to MT proteins were carried out. As seen from Table 2, binding of ^{3}H-podophyllotoxin to MT protein in WT cell extracts was decreased in a concentration dependent manner by the MT inhibitors nocodazole and colchicine. Very interestingly, 2-BU also competitively reduced the binding of ^{3}H-podophyllotoxin to MT proteins in a similar manner, although in comparison to nocodazole and colchicine about 10 fold higher concentration of 2-BU was required to reduce the drug binding to a similar extent. These results indicate that the affinity or 2-BU for the drug binding site is lower in comparison to nocodazole, colchicine or podophyllotoxin.

DISCUSSION

The cross resistance and biochemical studies which are presented here provide evidence that the compound 2-benzimidazolyl urea shows similar biological (i.e. antimitotic) activity as nocodazole and other benzimidazole

carbamate derivatives. Based on studies with antimitotic benzimidazole carbamate derivatives we have previously inferred that the presence of - NH - C(=O) - R group (where R is generally $-OCH_3$) at either position R_2 or R_3 in the molecule (see Fig. 1) is an essential structural requirement for this kind of activity (1). This inference is strongly supported and further extended by the result of the present studies. Of various benzimidazole derivatives containing different substituent group in the $-R_2$ position which were examined, only 2-BU which contain a - NH - C(=O) - NH_2 group showed similar activity as nocodazole in various experiments. The nocodazole-type activity of 2-BU indicates that the R-group in the - NH - C(=O) - R could be either alkyloxy (viz. OCH_3 or OC_2H_5 as in nocodazole and NSC 181928) or it could be a - NH_2 group. The lack of similar activity in the compound Nos. 1 and 5, which are 2-guanidino (i.e. NH - C(=NH) - NH_2) derivatives of benzimidazole indicate that the carbonyl group in the NH-C(=O) - R side chain plays an important role in the activity of these compounds.

The compound 2-BU represents structurally the simplest benzimidazole derivatives which shows this type of activity. Based on its relative cellular toxicity, its activity is about ≈ 1000-fold less than that of nocodazole and about 5-10 fold less than that of carbendazim and benomyl (see 1). The lower activity of 2-BU which contains no substituents in the benzene ring, in comparison to the other compounds is as may be expected since the nocodazole-like activity of benzimidazole derivatives is known to increase upon substitution of large alkyl or aromatic groups in the molecule (1,16). Based on this knowledge, it should be possible to synthesize additional 2-BU derivatives which show higher activity than the parental compound. Since benzimidazole group of antimitotic drugs exhibit a broad range of clinically and agriculturally useful biological activity (17,18), the information provided by the present study should prove useful in the synthesis and screening of new drugs with this type of biological activity.

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