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Kinetic analysis of the in vitro cell-killing action of neocarzinostatin*

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Summary. The relationship between the drug concentration and exposure time of neocarzinostatin (NCS) for a definite cell-killing effect was kinetically analyzed, taking into consideration its loss in biological activity during incubation. Its cell-killing activity was determined by a colony-forming inhibition assay, which was conducted at room temperature (25° C) for 0.5–30 min exposure and at 37° C for 5 min – 96 h exposure. Drug degradation at both temperatures was also investigated by bioassay. NCS lost its biological activity much faster at 37° C than at 25° C and the rate of loss in activity was higher at the lower initial concentration. Thus, the initial NCS concentrations necessary for 90% cell kill corresponding to each exposure time and a drug degradation constant were applied to a mathematical equation for the cell-killing effect of cell-cycle-phase-nonspecific agents. As a result, the curves for IC_{90}-exposure time relationships predicted from drug degradation constants for 37° C and 25° C were fairly well fitted to the respective experimental data. These results indicate that the cell-killing action of NCS can be expressed by this mathematical equation with scrutiny of drug degradation and is dependent on the concentration-time product (C × T).

Introduction

NCS is a unique antitumor antibiotic, which is composed of protein moiety and a nonprotein chromophore [10]. To quantitatively correlate its in vitro cytotoxicity with its in vivo antitumor activity, it is quite important to establish the relationship between the drug concentration and duration of exposure of NCS for a definite cell-killing effect.

For the in vitro analysis of cytotoxicity, Shimoyama [12] has studied the colony-forming inhibition of a wide variety of antitumor agents, showing that the cell-killing action of NCS is completely dependent on concentration and independent of exposure time when cells are exposed for 60 min or longer [12]. On the other hand, we have attempted to establish a kinetic analysis for the cell-killing effect of cell-cycle-phase-nonspecific antitumor agents [13] through in vitro colony-forming inhibition studies [11]. In the latter report, the relationship of cell-killing effects to drug concentration and exposure time was expressed by mathematical equations. We also showed that it is very important to take drug degradation during incubation into consideration.

It has been reported that the NCS chromophore is very labile and that the protein moiety plays a role in protecting the NCS chromophore from loss of activity [10]. Therefore, the cytotoxic activity of NCS should be investigated for short periods of exposure such as from one to a few minutes before most biological activity of the drug is lost as well as for longer periods of several hours. We have carried out colony-forming inhibition studies using Chinese hamster lung V79 cells with high plating efficiency. To conserve a constant temperature during drug exposure, we conducted experiments using a brief exposure (0.5–30 min) at room temperature (25° C); for relatively longer periods of exposure (5 min – 96 h), experiments were done at 37° C. In addition, analysis of the cell-killing kinetics of NCS at 25° C and 37° C was carried out, estimating the decomposition of the drug at 25° C and 37° C, respectively. By these experiments, we attempted to determine the concentration-exposure time relationship of NCS for a definite cell-killing effect.

Materials and methods

Chemicals. NCS was purchased from Yamanouchi Seiyaku Co. Ltd., Tokyo, Japan. All other chemicals were of analytical grade.

Cell culture. Chinese hamster V79 cells were grown in RPMI 1640 medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer supplemented with 10% fetal bovine serum and 100 μg/ml kanamycin at 37° C in a humidified atmosphere of 5% CO_2 and 95% air.

Estimation of the cell-killing effects of NCS. The cell-killing effects of NCS were determined by a colony-forming inhibition assay described previously [11], except that cells were inoculated in each plastic plate at a cell density of 100–12,800.

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Loss in the biological activity of NCS during incubation. Incubation of the drug was done in culture medium containing V79 cells at a density of 330 cells/ml at 25° C or 37° C for appropriate periods. At the end of each incubation, a certain amount of the culture medium was taken and filtered through 0.45 μ Disposable Syringe filters (Corning) to exclude cells. Immediately after the filtration, an appropriate volume of the filtrate was added to each assay plate containing 3.0 ml culture medium. For samples incubated at 25° C, the assay plates were left for a further 30 min at 25° C and then placed into a CO_2 incubator; samples incubated at 37° C were directly placed in a CO_2 incubator. The titration curve for the incubation at 25° C was prepared by exposing the cells to known concentrations of NCS for 30 min at 25° C, then incubating them at 37° C for 4 days. To prepare the titration curve for incubation at 37° C, the cells were incubated with various concentrations of the drug at 37° C for 4 days.

Results

Model analysis

Based on the basic pharmacodynamic model for cell-cycle-phase-nonspecific agents proposed by Jusko [4], we have expressed the relationship between drug concentration and exposure time necessary for 90% cell kill by the following equation [11]:

$$C_{m,90} \times \frac{1 - e^{-\alpha t}}{\alpha} = \frac{2.3}{kK}, \quad (1)$$

where $C_{m,90}$, α, t, k, and K represent an initial concentration of the drug necessary for 90% cell kill, a first-order degradation constant of the drug, the exposure time, a drug-induced irreversible cell death rate constant, and an equilibrium constant between intra- and extracellular concentration of the drug, respectively. Since the area under the concentration-time curve of the drug (AUC) can be written as $\int_0^t C_{m,90} \times e^{-\alpha t} dt$,

$$AUC = \int_0^t C_{m,90} \times e^{-\alpha t} dt = C_{m,90} \times \frac{1 - e^{-\alpha t}}{\alpha} = \frac{2.3}{kK} \quad (2)$$

is obtained by considering Eq. (1). This result indicates that C × T or the AUC for 90% cell kill of cell-cycle-phase-nonspecific agents is constant.

Degradation of NCS during incubation at 37° C or 25° C

Degradation of NCS in the culture medium at 37° C or 25° C was investigated. As shown in Fig. 1, the decomposition rate was considerably affected by temperature and NCS lost its biological activity much faster at 37° C than at 25° C at an initial concentration of ca. 0.4 μg/ml. Furthermore, the degradation rate was dependent on the initial concentration; NCS seemingly underwent first-order degradation at a given initial concentration. Thus, first-order degradation constants (α) and half-lives ($t_{1/2}$) for different concentrations and temperatures are summarized in Table 1, which indicates that the loss in NCS activity is faster at a low than at a high initial concentration at the same temperature.

Table 1. Degradation constants and half-lives of NCS under various conditions

Initial concentration (μg/ml)	Temperature (°C)	α[a]	$T_{1/2}$[b]
0.39	37	0.085	8.2
0.14	37	0.44	1.6
0.42	25	0.013	53.3
0.078	25	0.074	9.4

[a] A first-order degradation constant of the drug (min^{-1})

[b] Half-life (min) $\left(= \log_e \frac{2}{\alpha} \right)$

Relationship between concentration and exposure time of NCS for 90% cell-killing effect

V79 cells were exposed to NCS for various periods at 37° C or 25° C, dose-response curves were determined, and IC_{90} values (concentration for 90% kill) were derived. The experimental data are illustrated with closed circles in Fig. 2 (at 37° C) and in Fig. 3 (at 25° C). As shown in Fig. 2, IC_{90}s were almost constant for an exposure longer than 5 min, which indicates that the cell-killing action of NCS is dependent exclusively on concentration over 5 min exposure and independent of exposure time, as Shimoyama [12] reported. We showed fast decomposition (α = 0.44 min^{-1}, $t_{1/2}$ = 1.6 min) at 37° C with an initial concentration of 0.14 μg/ml. Since the IC_{90}-exposure time relationship expressed by Eq. (1) gives an asymptotic curve, the limiting value of IC_{90} ($t \to \infty$) is expressed as $\frac{2.3\,\alpha}{kK}$, a result of drug decomposition under the culture conditions. The kK value is calculated to be 17.2 ($ml \cdot \mu g^{-1} \cdot min^{-1}$) using the limiting IC_{90} of 0.059 ($\mu g \cdot ml^{-1}$) (Fig. 2) and α of 0.44 (min^{-1}). Thus, IC_{90}s for shorter periods of exposure than 5 min can be simulated, and the predicted IC_{90}-exposure time curve is shown in Fig. 2. This result implies that

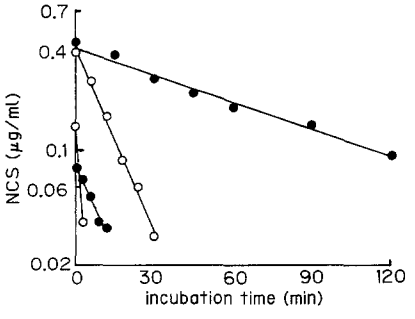

Fig. 1. Degradation kinetics of NCS with various initial concentrations at 25° C (– ● –) or 37° C (– ○ –). The *ordinate* is on a log scale

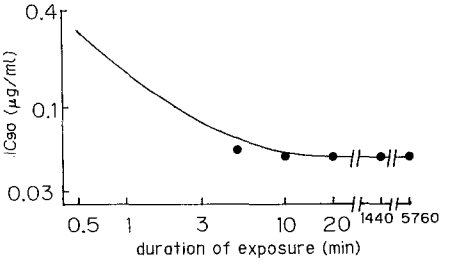

Fig. 2. Log-log relationship between IC_{90} and exposure time (0.5–30 min) for NCS acting at 37° C. IC_{90} values were plotted against exposure times on a log scale (●). The kK value was calculated from the limiting IC_{90} of 0.059 (μg/ml) and α of 0.44 (min^{-1}), and the curve was generated from Eq. (1)

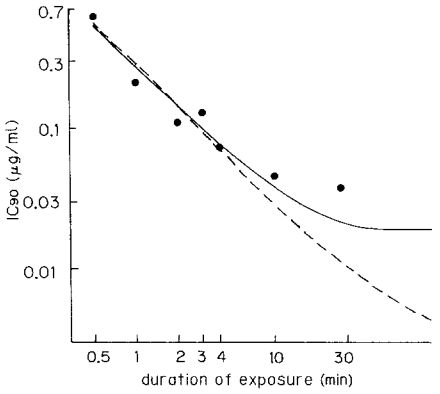

Fig. 3. Log-log relationship between IC_{90} and exposure time (0.5–30 min) for NCS acting at 25° C. IC_{90} values were plotted against exposure times (●) on a log scale. Eq. (1) was fitted to the experimental data, and the best-fitted curve was generated with α of 0.074 (min^{-1}) (*solid line*) and α of 0.013 (min^{-1}) (*dotted line*) by nonlinear regression analysis

IC_{90}s estimated by drug exposure at 37° C reach the lower limit within 5 min due to the drug's extremely fast decomposition.

Because of technical complications in experiments using very short drug exposure, drug treatment was done at room temperature (25° C). As shown in Fig. 3, a higher concentration was necessary for 90% cell kill when the cells were exposed to NCS for <4 min. The decay of NCS was also determined at 25° C (Table 1). Two α values were obtained with initial concentrations of 0.42 and 0.078 μg/ml. Equation (1) with the above α values was fitted to the data of IC_{90}s for various periods of exposure by nonlinear regression analysis [16]. As a result, respective kK values were calculated, and the curves were illustrated by a solid line for α of 0.074 (min^{-1}) and by a dotted line for α of 0.013 (min^{-1}) in Fig. 3. For exposure times shorter than 4 min, both curves were fitted considerably well to the experimental data, but the curve simulated with α of 0.013 (min^{-1}) (*dotted line*) was not fitted to the data for long exposure time in comparison with that with α of 0.074 (min^{-1}) (*solid line*). In contrast, Eq. (1) with α of 0.074 (min^{-1}) was better fitted to the data for exposure times longer than 3 min.

As described above, the degradation constant of NCS was markedly dependent on its initial concentration. It is noteworthy that the initial NCS concentration was

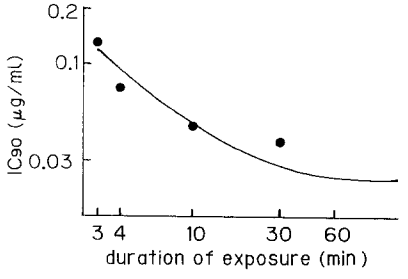

Fig. 4. Log-log relationship between IC_{90} and exposure time (3–30 min) for NCS acting at 25° C. IC_{90} values were plotted against exposure time longer than 3 min (●) on a log scale. The kK value was calculated from the IC_{90} for incubation times longer than 3 min and α of 0.074 (min^{-1}), and the curve was generated from Eq. (1)

0.078 μg/ml when α of 0.074 (min^{-1}) was observed. This concentration was similar to IC_{90} values for exposure times longer than 3 min but significantly different from those for exposure times of 0.5 and 1 min (Fig. 3). Therefore, the kK value was calculated to be 7.2 ($ml \cdot \mu g^{-1} \cdot min^{-1}$) from IC_{90} values for only these relatively long exposure times and the above α value. By the use of this kK value, a log-log relationship was simulated and illustrated as a curve in Fig. 4, indicating more improved fitting to the corresponding experimental data. This implies that to establish the exact relationship between IC_{90} and exposure time, α should be related with the initial concentration of the drug.

Using Eq. (1), the C × T value (i.e., the integration of concentration upon time or AUC) turned out to be constant for 90% cell kill [Eq. (2)]. These results indicate that since the IC_{90}-exposure time relationship of NCS can fairly well be expressed by Eq. (1) using the accurate value of degradation, the cell-killing action of NCS is dependent on C × T.

Discussion

NCS is an antitumor protein antibiotic possessing low-molecular-weight nonprotein chromophore [1, 2] at a molar ratio of 1:1 [8]. The binding of NCS chromophore to apo-NCS (the protein moiety of NCS) may be due to not only ionic interaction between the acidic side chain of apo-NCS and the basic center of an aminosugar moiety of NCS chromophore but also hydrophobic interaction between the hydrophobic amino acids of apo-NCS and hydrophobic moieties of NCS chromophore [3]. The chromophore was found to be released from NCS above pH 7.

The NCS chromophore is responsible for both DNA strand scission and growth inhibition of tumor cells [7, 9, 14, 15]. Shimoyama [12] has classified cell-cycle-phase-nonspecific antitumor agents [13] as concentration-dependent drugs according to their mode of cytotoxic action. The cell-killing action of NCS has also been shown to be completely dependent on concentration at exposure times of >60 min [12].

We have analyzed the cell-killing effect of mitomycin C, nitrogen mustard, and ACNU as examples of cell-cycle-phase-nonspecific agents on a kinetic basis, demonstrating that the cell-killing action of this class of agents is dependent on C × T when drug decay is taken into consideration [11]. If DNA strand breaks caused by NCS are lethal to cells, it can be speculated that NCS is expected to be a cell-cycle-phase-nonspecific agent because a number of DNA-damaging agents have been thus classified by Skipper et al. [13].

NCS chromophore is very labile to heating and exposure to UV light [5]. Therefore, we attempted to analyze the cell-killing action of NCS for short periods of exposure, such as from 0.5 min to several minutes, considering drug degradation during incubation. The results show that NCS reveals C × T-dependent cell-killing activity. The C × T dependence of NCS cytotoxicity implies that the amount of cellular damage resulting in cell death is dependent on the C × T value.

Our present analysis of NCS cytotoxicity includes the first-order drug degradation constant. However, the drug decomposition rate was influenced by the initial concentration of the drug. Therefore, in Eq. (1) of the relationship between IC_{90} and exposure time, the relationship of α to

$C_{m,90}$ (initial concentration) should be established for more accurate analysis. The reason for the concentration dependence of the drug degradation is unclear. Kohno et al. [6] have analyzed the degradation of NCS in detail and reported that under either direct or indirect sunlight, NCS loses its biological activity but that this cannot be attributed to either a first-order or zero-order reaction. These authors also showed that when residual activity after exposure to sunlight at various initial concentrations was studied, the highest ratio of loss in activity was observed at the lowest initial concentration of NCS [6]. This is in complete accordance with our results (Fig. 1).

Between 25° C and 37° C, there was a difference in kK values: kK at 37° C was greater than that at 25° C, indicating that cellular sensitivity at 37° C is higher than that at 25° C. This may be due to a higher level of drug uptake and a higher rate of cellular damage at 37° C in comparison with those at 25° C. On the other hand, active species of NCS disappeared much more rapidly at 37° C than at 25° C. As a result of these inverse effects, no great difference in IC_{90} values was observed between 25° C and 37° C.

In conclusion, we analyzed the cell-killing action of NCS on a kinetic basis and revealed that it is C × T- or AUC-dependent, as are other cell-cycle-phase-nonspecific agents. Although the cell-killing action of NCS appears to be concentration-dependent due to its extremely rapid decomposition during incubation, its kinetic characteristics were clearly elucidated by the introduction of precise data on its loss of biological activity.

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