Low-Dose Cisplatin and 5-Fluorouracil in Combination Can Repress Increased Gene Expression of Cellular Resistance Determinants to Themselves

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ABSTRACT

The synergistic mechanism of cisplatin (CDDP) and 5-fluorouracil (5-FU) in combination remains unclear, despite its substantial antitumor activity, which has been demonstrated clinically. To clarify the mechanism(s), we determined the sensitivity or resistance factors to either drug in seven gastrointestinal cancer cell lines and then analyzed the altered gene expression after different exposures to CDDP and 5-FU. At the basal gene expression level, glutathione S-transferase (GST) expression correlated with the observed resistance to CDDP, whereas dihydropyrimidine dehydrogenase (DPD) and multidrug resistance-associated protein (MRP) expression was related to 5-FU resistance. GST, DPD, and MRP expression increased in response to the respective drug, but they also increased in response to the other drug as well. Additionally, 5-FU revealed a drastically increased thymidylate synthase (TS) gene expression in 5-FU-resistant cells. However, the increasing actions of CDDP and 5-FU on GST, DPD, and TS expression varied according to the exposure time, concentration, and schedule. A low concentration of CDDP (1 μg/ml, 30 min) followed by 5-FU (0.5 μg/ml, 72 h) was found to cause a less increased expression of GST, MRP, GST, and TS than either drug alone, thus resulting in synergistic cytotoxicity in 5-FU-resistant COLO201 and CDDP-resistant HCC-48 cells. The sequential combination of CDDP and 5-FU inhibited the growth of human normal renal proximal tubule cells by less than 20%. Low concentrations of CDDP followed by continuous exposure to 5-FU can repress increased gene expression related to both drug resistances, thereby being synergistically cytotoxic in human gastrointestinal cancer cells.

INTRODUCTION

Numerous experimental and clinical studies have revealed a pronounced antitumor activity of a combination of CDDP and 5-FU in various types of human tumors (1, 2). The combination is active even in chemoresistant gastrointestinal cancers, but the response and toxicity vary considerably according to the schedule and dose used (3, 4). The optimal treatment protocol for the combination has not yet been found, despite extensive clinical efforts. The synergistic mechanism has been studied intensively in the laboratory to establish the rational treatment protocol, but the mechanism remains unclear.

The synergistic mechanism has been attributed to the fact that CDDP (3) can increase the availability of the reduced folate necessary for tight binding of FdUMP, a 5-FU metabolite, to TS, thus enhancing the cytotoxicity of the CDDP and 5-FU combination (2). However, the increasing effect of CDDP on intracellular pools of 5,10-methylenetetrahydrofolate and tetrahydrofolate is not always the main mechanism of the synergism (5). Johnston et al. (6) have suggested that the interaction of 5-FU and CDDP is associated with a greater degree of fragmentation of both nascent and parent DNA, but not with an increase in intracellular reduced folate pool.

A variety of studies on drug resistance have given us useful information about other possible factors conferring the synergistic activity of a 5-FU/CDDP combination such as the initial rate-limiting enzyme in the 5-FU catabolism, DPD, and a GSH-related detoxification system for CDDP (7–9). Some reports have suggested that several TS enzyme-related changes exist in an acquired multidrug-resistant cell associated with GP-170 or MRP (10, 11). Those findings led us to hypothesize that some combination of TS, DPD, GP-170, and MRP may

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3 The abbreviations used are: CDDP, cisplatin; 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-UMP; TS, thymidylate synthase; DPD, dihydropyrimidine dehydrogenase; F-β-Ala, 2-fluoro-β-alanine; FUPA, 2-fluoro-3-ureidopropionic acid; NQO, NADPH/quinone oxidoreductase; P450R, NADPH/cytochrome P450 reductase; MSH, glutathione S-transferase; GSH, glutathione; GP-170, 170-kDa P-glycoprotein; MRP, multidrug resistance-associated protein; γ-GCS, γ-glutamylcysteine synthetase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
confers the synergistic activity of a 5-FU/CDDP combination in addition to its increasing effect on intracellular folate pool.

To clarify the mechanism of CDDP/5-FU synergy, in the present experiments we determined which properties correlate with the observed sensitivity or resistance to CDDP and 5-FU and then studied the detailed action of 5-FU and CDDP, alone or in combination, on critical determinant(s) through gene expression analysis. We used seven unselected human gastrointestinal cancer cell lines and normal human renal cells as a control to obtain more insight into the cellular characteristics that account for the differential sensitivity or resistance to CDDP, 5-FU, and a combination of the drugs.

MATERIALS AND METHODS

Chemicals. CDDP was generously provided by Bristol-Myers-Squibb (New York, NY). 5-FU was obtained from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). F-β-Ala and [6-3H]-5-FU were purchased from Tokyo Chemical Industries Co., Ltd. (Tokyo, Japan) and Japan Radioisotope Association (Tokyo, Japan), respectively; and FdUMP was obtained from Sigma (St. Louis, MO). All other chemicals were of analytical grade and were purchased from Wako Pure Chemicals (Osaka, Japan) and Sigma.

Cells. Human colon adenocarcinoma lines COLO201 and COLO320DM and a human gastric carcinoma cell line, MKN45, were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). HSC-42 (human gastric carcinoma), HEC-46 (human esophageal cancer), and HCC-48 and HCC-50 (human colon carcinomas) cell lines were kindly provided by Dr. Kazuyoshi Yanagihara (National Cancer Center, Tokyo, Japan). Normal human renal proximal tubule cells (CC-2553) were purchased from Clonetics (San Diego, CA). Human cancer cell lines were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% heat-inactivated fetal bovine serum (BioWhittaker, Verviers, Belgium). A REGM BulletKit (BioWhittaker) was used for the culture of CC-2553 cells. Cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ and maintained in continuous exponential growth by passing every 3 days.

Evaluation of 5-FU Efficacy. The efficacy of 5-FU was evaluated after a 30-min or 72-h exposure of cells to the drug. For the 30-min exposure, exponentially growing cultured cells were concentrated to 5 × 10⁶ cells/ml, exposed to the indicated drug concentrations for 30 min, and washed twice; cells were then seeded in 24-well plates at a density of 2.5 × 10⁴ cells/ml in RPMI 1640 with 10% fetal bovine serum. For the 72-h continuous exposure, exponentially growing cells (2.5 × 10⁶ cells/ml) were exposed to the indicated drug concentrations for 72 h. After this 72-h incubation, surviving cells were counted with a Coulter counter (Hialeah, FL) and the trypan blue exclusion test.

Biochemical Quantitative Assay. Activities of NQO, P450R, GST, and the content of GSH were measured as described previously (12). NQO activity was measured as the Dicoumarol-sensitive reduction of 2,6-dichlorophenol indophenol. P450R activity was measured as the NADPH-dependent reduction of cytochrome c. GST activity was measured with 1-chloro-2,4-dinitrobenzene as a substrate by monitoring the enzyme-dependent change in absorbance at 340 nm. Total cellular GSH content was measured by an enzyme recycling procedure in which GSH was sequentially oxidized by 5,5′-dithiobis-(2-nitrobenzoic acid) and reduced by NADPH in the presence of GSH reductase.

The DPD activity was measured by a 5-FU degradation assay according to the method of Ikenaka et al. (13). After the enzymatic reaction in the presence of [6-3H]-5-FU, the radioactivity of FUPA, F-β-Ala, and the 5-FU fraction collected via silica gel thin-layer chromatography was counted. DPD activity was calculated according to the following equation: 5-FU degradation (%) = [(F-β-Ala + FUPA)/(F-β-Ala + FUPA + 5-FU) (cpm)] × 100. Specific activity (nmol/mg protein/h) was calculated as 2.5 nmol × 5-FU degradation (%) × [1/(mg protein × (1/dilution fold))].

The total concentration of TS was determined by a binding assay according to the method of Spears (14). The enzyme solution (0.2 ml) collected from the cells was preincubated for 3 h at 25°C with 0.1 ml of buffer A [600 mM NH₄HCO₃, 100 mM NaF, 15 mM 5′-CMP, and 100 mM β-mercaptoethanol (pH 8.0)] to dissociate free FdUMP. The solution was placed in an ice-cold bath and incubated at 30°C for 20 min with 0.2 ml of [6-3H]FdUMP and 0.1 ml of cofactor solution B [50 mM potassium phosphate buffer, 2 mM tetrahydrofolate, 16 mM ascorbic acid, 9 mM formaldehyde, 150 mM 5′-CMP, 20 mM β-mercaptoethanol, 100 mM NaF, and 2% BSA (pH 7.4)]. The total TS concentration was calculated from the radioactivity according to the following equation: TS (pmol) = FdUMP (dpm) × 2.45 × 10⁻⁵.

Protein content was measured with the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

RNA Preparation and Northern Analysis. Total RNA isolation and Northern analysis were performed as described previously (12). The probes used for hybridization were as follows: (a) a 0.5-kb EcoRI cDNA fragment of the human DPD gene kindly provided by Dr. Masaaki Motoyama (Fujii Memorial Research Institute, Ohsu, Japan; Ref. 15); (b) a 0.775-kb EcoRI cDNA fragment of the human TS gene (16); (c) a 0.7-kb fragment of human GP-170 cDNA (MDR1) derived from K562 cells (17); (d) a 0.9-kb EcoRI cDNA fragment of the human MRP gene (18); (e) a 0.5-kb canalicul multispécific organic anion transporter (cMOAT) cDNA from K562 cells (19); (f) a 0.75-kb EcoRI cDNA fragment of the human GSTπ clone pGPl2 (20); (g) a 1.1-kb fragment of the human NQO1 cDNA derived from K562 cells (21); (h) a 0.8-kb fragment of human P450R cDNA derived from HCC-48 cells (22); (i) a 0.88-kb fragment of the heavy subunit of human γ-GCS cDNA from KB cells (23); and (j) a 0.85-kb fragment containing the exons 4–7 of the human GAPDH gene (24).

Data Analysis. The Student t test and linear regression analysis were used as appropriate. The statistical calculations and tests were performed using StatView 4.11 software (Abacus Co., Berkeley, CA) on a Macintosh computer.
Low-Dose CDDP and Genes Related to 5-FU Resistance

RESULTS

Determinants of Cellular Resistance to CDDP and 5-FU. When normalized relative to GAPDH, the basal level of GST\(\pi\) gene expression was correlated with the IC\(_{50}\) value for CDDP in seven gastrointestinal cancer cell lines (Fig. 1), whereas the expression of DPD and MRP mRNA correlated with the IC\(_{50}\) value for 5-FU. Also, GST and DPD activity was correlated with the cellular CDDP and 5-FU resistance, respectively, except in the 72-h CDDP treatment setting (Fig. 2). GST (for CDDP) and DPD and MRP (for 5-FU) appear to be important resistance factors in the seven cancer cell lines. In contrast, the basal level of TS mRNA and protein expression was not related to 5-FU resistance. The levels of MDR1, cMOAT, NQO1, P450R, and \(\gamma\)-GCS gene expression did not correlate with the IC\(_{50}\) values for either CDDP or 5-FU (data not shown). The amount of enzymatic activity of GST and NQO and GSH content were also not correlated with the cellular resistance to either CDDP or 5-FU (data not shown).

Modulative Effect of CDDP and 5-FU on Gene Expression Related to Respective Drug Resistance. GST\(\pi\), DPD, MRP, and TS expression was analyzed in HCC-48 and COLO201 cells after a 30-min exposure to CDDP or to 5-FU (Fig. 3). HCC-48 cells were sensitive to 5-FU (IC\(_{50}\), 43.0 \(\mu\)g/ml for the 30-min exposure and 1133 ng/ml for the 72-h exposure), but resistant to CDDP (IC\(_{50}\), 9.6 \(\mu\)g/ml for the 30-min exposure and 125 ng/ml for the 72-h exposure). Conversely, COLO201 cells were less sensitive to 5-FU (IC\(_{50}\), 174.3 \(\mu\)g/ml for the 30-min exposure and 2093 ng/ml for the 72-h exposure) but were sensitive to CDDP (IC\(_{50}\), 4.2 \(\mu\)g/ml for the 30-min exposure and 47.7 ng/ml for the 72-h exposure). Treatment with 3.5 \(\mu\)g/ml CDDP for 30 min caused a greater increase in GST\(\pi\) expression in CDDP-resistant HCC-48 cells than in CDDP-sensitive COLO201 cells. Treatment with 40 \(\mu\)g/ml 5-FU for 30 min caused a greater increase in DPD and MRP expression in COLO201 cells, which are not as sensitive to 5-FU, than in 5-FU-sensitive HCC-48 cells. GST plays an important role in CDDP resistance, and DPD and MRP may participate in 5-FU resistance not only because of its increased level in the steady state, but also because of the response to the respective drug causing an increase of the gene expression. The 30-min 5-FU treatment revealed the greatest increase of TS mRNA, but the increase occurred both in COLO201 and HCC-48 cells (Fig. 3). CDDP and 5-FU each also caused an increase in gene expression related to the resistance to the other drug. Continuous treatment with 0.1 \(\mu\)g/ml CDDP revealed an increased expression of both GST\(\pi\) and DPD (Fig. 4). Continuous treatment with 0.5 and 1 \(\mu\)g/ml 5-FU-enhanced DPD and TS expression but also enhanced GST\(\pi\) expression as well. Although increased expression of DPD and TS by 5-FU was observed only in COLO201 cells, which are less sensitive to 5-FU, 5-FU caused an increase in GST\(\pi\) expression related to CDDP resistance in both cell lines. The continuous exposure of cells to 5-FU increased TS expression the most (294-fold after a 12-h continuous exposure to 1 \(\mu\)g/ml 5-FU) in the less sensitive COLO201 cells. TS appears to have a role in 5-FU resistance through a drastic increase in the gene expression in response to continuous 5-FU treatment. However, there appear to be different modes of the action of CDDP and 5-FU. In contrast to the continuous exposure, the short-period treatment (30 min) with either CDDP or 5-FU enhanced the MRP expression more but caused a smaller increase in the expression of DPD, TS, and GST\(\pi\) (Fig. 5). At the maximum dosage, CDDP (3.5 \(\mu\)g/ml for 30 min) caused a 3.2-fold increase in MRP expression in HCC-48 cells, and 5-FU (40 \(\mu\)g/ml for 30 min) produced a 2.5-fold increase in COLO201 cells. In contrast, none of the other 30-min treatments caused more than a 15-fold increase in TS expression; in fact, I...
mg/ml CDDP decreased the level of DPD and MRP expression in COLO201 cells, which are less sensitive to 5-FU.

The CDDP/5-FU Combination and Gene Expression Related to the Resistance to CDDP and 5-FU. When cells were treated with 1 or 3.5 mg/ml CDDP for 30 min followed by 0.5 mg/ml 5-FU, GSTπ, DPD, MRP, and TS expression in COLO201 and HCC-48 cells remained within a 4-fold increase relative to the basal level (Fig. 6). As compared to treatment with 0.5 mg/ml 5-FU alone, the maximum level of gene expression after these combinations was less than 50% for DPD, 2%
for TS, and 40% for GSTπ in COLO201 cells. A less than 2-fold increase in MRP expression was observed in HCC-48 cells after treatment with 3.5 μg/ml CDDP for 30 min followed by 0.5 μg/ml 5-FU, although treatment with CDDP alone revealed a 3.2-fold increase. Treatment with CDDP followed by continuous exposure to 5-FU repressed an increase in gene expression related to the cellular resistance to both 5-FU and CDDP. The repressive action was most remarkable on TS gene expression.

**DISCUSSION**

A clear understanding of the critical determinant(s) of each drug action is of key importance when investigating drug interactions. Among 10 possible factors, we demonstrated here that GST (for CDDP) and DPD, MRP, and TS (for 5-FU) were closely related to resistance in seven human gastrointestinal cancer cell lines. Although both CDDP and 5-FU caused increased gene expression of these sensitivity determinants when given alone, a low concentration of CDDP (a less than 18% inhibitory concentration; 1 μg/ml) followed by 5-FU (a less than 18% inhibitory concentration; 0.5 μg/ml) was found to repress the increase in DPD, MRP, and TS gene expression while retaining the basal level of GSTπ expression, thereby revealing a synergistic cytotoxicity. The low concentration of CDDP appeared to act mainly as a modulator to enhance 5-FU activity. The sequential exposure to CDDP and 5-FU reduced cell growth.
to approximately 50% of the control in both cells that were less sensitive to 5-FU and CDDP-resistant cancer cells, whereas in human normal renal cells, the reduction was 80% below that of the control.

The pronounced clinical activity of a CDDP/5-FU combination has been demonstrated in several treatment protocols (2, 4). Among these studies, the low-dose CDDP/5-FU combination has attracted much attention due to its fairly high response rate (about 50%) for advanced and recurrent gastric cancer and its low toxicity (4). Our results in this study can explain why low-dose CDDP enhances 5-FU antitumor activity in gastric cancer patients without increasing the renal toxicity and may prove the sequential combination to be a rational treatment protocol using CDDP and 5-FU.

In the laboratory, the potent activity of such a sequential combination was first demonstrated by Scanlon et al. (1), although their suggested mechanism is different than ours. They showed that low concentrations of CDDP did not alter TS activity but increased the reduced folate in A2780, a human ovarian carcinoma cell line. A combination of CDDP with 5-FU; however, does not always cause an increase of intracellular reduced folate pools in cells. In human colon carcinoma NCI H548, no notable differences can be detected in the intracellular folate pools, FdUMP, or 5-fluoro-UTP pools or 5-FU incorporation into RNA or DNA with the addition of CDDP to 5-FU (6). In contrast, numerous studies have shown that TS-related changes and altered DPD action are crucial in 5-FU-induced cytotoxicity (7, 8). An increase in TS mRNA in 5-FU-resistant cells has been observed (25). More than 85% of administered 5-FU is degraded through the catabolic pathways in humans (26), and intracellular DPD is demonstrated to play an important role in 5-FU-induced anticancer activity. We have shown previously that inhibition of DPD activity by 5-ethyluracil resulted in increased 5-FU cytotoxicity in the seven cell lines used in this study (27). The enhancing effect of 5-ethyluracil on 5-FU activity was found to vary according to the DPD activity of the cells, although the difference in the activity was less than 2-fold. MRP is involved in resistance to various types of anticancer agents, and its action as a GSH-conjugate transporter and its coordinated gene expression with several xenobiotic detoxifying genes such as γ-GCS gene are intriguing (28, 29). Repressive action of CDDP on TS, DPD, and MRP expression enhanced by 5-FU may be a crucial event in CDDP/5-FU combination-induced synergistic activity.

However, CDDP and 5-FU showed different modes of action, depending on the concentrations used and the contact times with cells. The critical mechanism of interaction and the antitumor activity in the CDDP/5-FU combination might vary greatly with changes in the treatment protocols. In the continuous exposure treatment, CDDP (0.1 µg/ml) caused an increase in GSTπ and DPD expression, and 5-FU (0.5 and 1 µg/ml) enhanced DPD and, most notably, TS expression. Alternatively, after the short-period treatment (30 min), an increase in MRP expression was more striking; short-period exposure to 5-FU-

![Fig. 5 Altered expression of GSTπ, DPD, MRP, and TS mRNA after short-period exposure (30 min) of HCC-48 and COLO201 cells to CDDP or 5-FU. Exponentially growing cells (5×10⁶ cells/ml) were incubated for 30 min in the absence (−−−) or presence of 1 µg/ml (○), 3.5 µg/ml CDDP (●), or 40 µg/ml 5-FU (▼). mRNA was prepared for Northern blot analysis after incubation for 1, 6, 24, 48, and 72 h in drug-free medium following the initial treatment. mRNA levels for each gene are expressed relative to the mean values of the basal levels. Each data point represents the mean ± SD for three individual experiments.](https://clincancerres.aacrjournals.org)
and CDDP caused less of an increase in \( \text{GST}_\pi \), \( \text{DPD} \), \( \text{MRP} \), and \( \text{TS} \) expression than the continuous exposure treatment. These different modes of 5-FU action are shown in a human colon cancer cell variant, HT-29/5-FU/S, which is developed by repeated 1-h exposure to 5-FU. The cells are resistant to short-term 5-FU exposure, but they are more sensitive to long-term exposure than the parent cell line (30). Sobrero et al. (31) have also shown that the HCT-8 human colon cancer cell variant resistant to short-term 5-FU exposure retains sensitivity to continuous exposure, and they suggested that bolus 5-FU cytotoxicity is predominantly a RNA mechanism and that continuous exposure is a TS mechanism. Despite the limited data, our data have suggested that alternative bolus administration of CDDP and 5-FU could be another potent protocol due to its reduced induction of the

\[ \begin{align*}
\text{Table 1} & \quad \text{Inhibition of cell growth by CDDP and 5-FU} \\
\hline
\text{Concentration (\( \mu \text{g/ml} \))} & \text{CDDP (30-min exposure)} & \text{5-FU (72-h exposure)} & \text{HCC-48} & \text{COLO201} & \text{CC-2553} \\
\hline
1.0 & 98.7 \pm 2.3^b & 82.3 \pm 8.3 & 87.0 \pm 6.6 \\
3.5 & 73.5 \pm 5.8 & 53.2 \pm 5.3 & 76.3 \pm 5.0 \\
10.0 & 42.5 \pm 5.8 & 28.7 \pm 4.9 & 67.9 \pm 5.6 \\
 & 82.2 \pm 3.0 & 84.5 \pm 3.8 & 88.8 \pm 4.0 \\
 & 39.8 \pm 7.3 & 51.6 \pm 2.3 & 62.3 \pm 3.3 \\
 & 50.7 \pm 3.3 & 51.7 \pm 2.8 & 80.9 \pm 6.6 \\
1.0 & 49.7 \pm 7.5 & 50.4 \pm 2.3 & 66.5 \pm 6.9 \\
3.5 & 32.2 \pm 4.3 & 21.0 \pm 3.5 & 49.3 \pm 2.9 \\
10.0 & 45.7 \pm 4.9 & 39.1 \pm 2.6 & 48.7 \pm 7.6 \\
1.0 & 31.5 \pm 1.5 & 21.8 \pm 3.0 & 40.2 \pm 2.2 \\
3.5 & 20.2 \pm 1.3 & 18.7 \pm 2.9 & 36.6 \pm 4.9 \\
10.0 & & & & \\
\hline
\end{align*} \]

\(^a\) % Control growth represents the percentage of control in cell growth, which was determined after a 30-min exposure of cells (5 \( \times 10^6 \) cells/ml) to CDDP or a 72-h continuous exposure of cells (2.5 \( \times 10^6 \) cells/ml) to 5-FU. In the combination, cells (5 \( \times 10^6 \) cells/ml) were exposed to CDDP for 30 min and then incubated for 72 h at a concentration of 2.5 \( \times 10^6 \) cells/ml in medium with 5-FU. Surviving cells were counted using a Coulter counter and the trypan blue dye exclusion test, and the percentage control growth was determined.

\(^b\) Data represent the mean \( \pm \) SD of three separate experiments. The mean of percentage control growth for each individual experiment was determined through analysis of the mean cell count in three samples.
resistant factors, such as DPD, TS, and GST. In this combination treatment, MRP would be a key factor. Because MRP is understood not to play an important role in 5-FU resistance in general, the detailed function of MRP on 5-FU is the subject of an ongoing study (27).

We demonstrated here that TS gene expression increased remarkably in response to 5-FU in 5-FU-resistant cells more than in 5-FU-sensitive cells, although the basal level of TS did not relate as closely to 5-FU activity as did DPD and MRP. In addition, the increase in DPD, MRP, TS, and MRP expression in response to 5-FU and CDDP exposure was transient and returned almost to the basal level within 72 h. Kigawa et al. (32) recently used gene expression analysis to reveal an important role of γ-GCS and MRP in clinical chemoresistance in surgical ovarian tumor specimens before and after chemotherapy, although the basal expression level of γ-GCS and MRP did not relate to the clinical resistance. Because exposure to CDDP and 5-FU results in numerous alterations both intracellularly and at the membrane, depending on the treatment concentrations and schedules (24, 33), a poor understanding of the mechanism of each drug will inhibit any clear understanding of the synergistic mechanism. Gene expression analysis after exposure to drug(s) in several treatment settings will be indispensable for determining a critical event or factor responsible for the cytotoxic activity.

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