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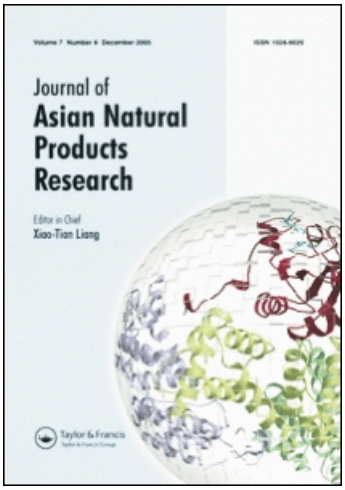

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
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To cite this Article Xu, Cheng-Xiong and He, Jian-Hua(1999) 'Anticancer Effect of Howiinol A and its Mechanism of Action', Journal of Asian Natural Products Research, 2: 1, 1 — 19
To link to this Article: DOI: 10.1080/10286029908039885
URL: http://dx.doi.org/10.1080/10286029908039885

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JANPR, Vol. 2, pp. 1–19
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ANTICANCER EFFECT OF HOWIINOL A AND ITS MECHANISM OF ACTION

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(*Received 3 March 1999; Revised 30 March 1999; In final form 29 April 1999*)

Howiinol A (GHM-10) is a kind of phenylethylene pyrone compounds isolated from *Goniothalamus howii*. By using the techniques of cell growth curve determination, MTT test, soft agar colony assay and experimental therapy of transplantable tumors in mice, it is found that GHM-10 exerts potent inhibitory effect on cancer cells but its influence on normal cells is relatively slight; the sensitivity of a drug-resistant cell line, KB/VCR 2000, to GHM-10 is similar to its parent cell line KB. Remarkable therapeutic effect can be seen in mice bearing H22 hepatoma and Lewis lung cancer and in mice with ascetic sarcoma 180 when GHM-10 is orally or intraperitoneally administered.

The IC_{50}s of L1210 cells treated with GHM-10 for 1 and 24 h are 6.85 and 3.32 $\mu g \cdot ml^{-1}$ respectively. The ratio of IC_{50} 1 h and IC_{50} 24 h is only 2.06, indicating that the action of GHM-10 is conformed to a cell cycle non-specific cytotoxic agent. By using trypan blue exclusive test and morphological examination, it is demonstrated that the main effect of GHM-10 is to inhibit the cell proliferation. Flow cytometery technique is used to analyze the cell cycle of L1210 cells. The results show that to some extent, GHM-10 blocks the cell cycle transition from G1 phase to S phase. By using [^{3}H] labeled precursor incorporation technique, it is shown that GHM-10 significantly suppresses the biosynthesis of DNA, RNA and protein in L1210 cells, and the DNA synthesis is mostly affected. At 1 h after the cells were treated with GHM-10, these inhibitory effects have already been irreversible, suggesting that GHM-10 may cause structural damage on DNA molecules. However, GHM-10 is unable to intercalate into DNA molecules or to destroy its structure directly. By using single cell gel electrophoresis and alkaline elution technology, it is confirmed that GHM-10 causes DNA molecule damage and single strand breakage in L1210 cells. Further studies show that GHM-10 markedly inhibits DNA dehelix induced by DNA topoisomerase II both inside and outside the cells, indicating that GHM-10 is acting as an inhibitor of DNA topoisomerase II.

Keywords: Anticancer agent; *Goniothalamus howii*; DNA damage; Cytotoxicity; DNA topoisomerase II

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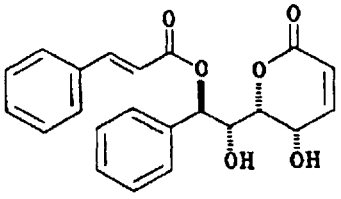
INTRODUCTION

Plants of the Annonaceae family are growing in the tropic and subtropic areas. In recent years, they attracted great attention of the scientists as a series of new chemical components – annonaceous acetogenins, were isolated from this family, and some of them showed potent anticancer activity [1]. There are 24 genera, 103 species and 6 subspecies of plants belonging to the Annonaceae family growing in China [2]. They are mainly located in Guangxi, Yunnan and Hainan provinces. Howiinol A (GHM-10) is a kind of phenylethylene pyrone compounds, isolated from *Goniothalamus howii* by Professor De-Quan Yu of the Institute of Materia Medica. Chinese Academy of Medical Sciences [3]. GHM-10 is a new compound not found in literature. Its chemical structure is relatively simpler than those of acetogenins, and chemical synthesis has been accomplished. GHM-10 has shown strong anticancer effect both *in vitro* and *in vivo*. In the present paper, the results of its anti-tumor effect and its mechanism of action were reported.



The chemical structure of GHM-10

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RESULTS AND DISCUSSION

1. Effect of GHM-10 on the Growth of L1210 Cells

After treatment with various concentrations of GHM-10, the growth of L1210 cells was inhibited and the inhibition was both concentration and time dependent. It could be seen in Fig. 1 that the inhibitory rate of cell growth reached approximately 66.7% when the L1210 cells were treated with GHM-10 at a concentration of $2\,\mu g \cdot ml^{-1}$ (for the method of calculation, see Ref. [4]).

2. Effect of GHM-10 on Cancer Cells and Normal Cells *In Vitro*

MTT test was used for observing the effect of GHM-10 on human ovary cancer cells A2780, human hepatoma cells Bel 7402, human oral cavity

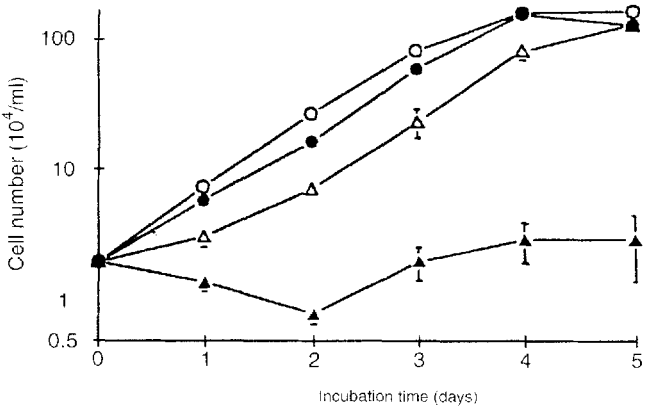

FIGURE 1 Inhibition of L1210 cell growth by different concentration of GHM-10 ($n = 3$, mean ± SD). ○—○ Control; ●—● GHM-10 1 μg · ml^{-1}; △—△ GHM-10 2 μg · ml^{-1}; ▲—▲ GHM-10 4 μg · ml^{-1}.

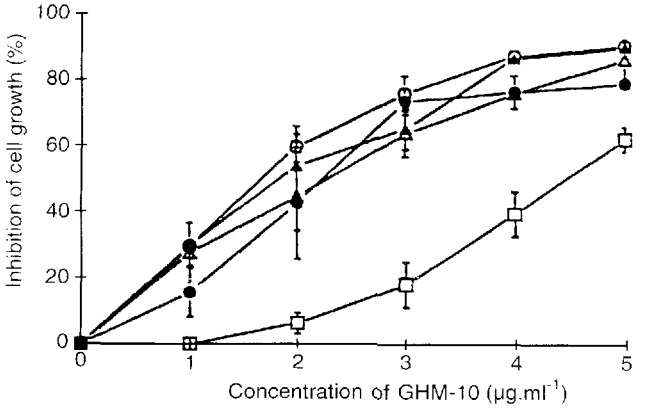

FIGURE 2 Cytotoxic effect of GHM-10 on cancer cell lines and a normal cell line ($n = 3$, mean ± SD). ○—○ Human ovary cancer cell line A2780; ●—● human colon cancer cell line HCT-8; △—△ human oral cavity cancer cell line KB; ▲—▲ human hepatoma cell line Bel 7402; □—□ Chinese hamster lung cell line CHL.

cancer cells KB and human colon cancer cells HCT-8, as well as a normal cell line of Chinese hamster lung cells CHL *in vitro*. As shown in Fig. 2, cancer cells were more sensitive to GHM-10 than normal cells.

3. Effect of GHM-10 on Drug-resistant Cells

Multiple drug resistance is a serious problem in cancer chemotherapy. Using the technique of MTT test, we found that GHM-10 exerted similar

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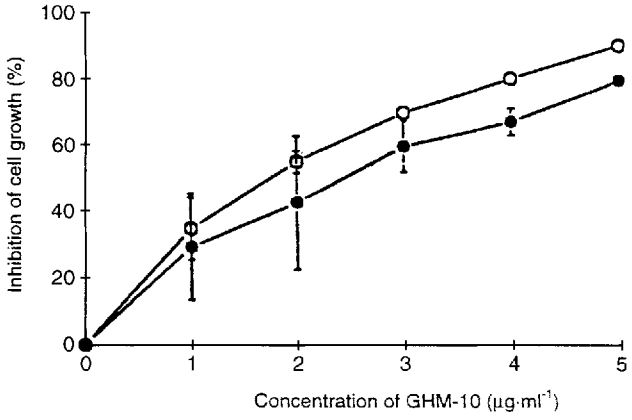

FIGURE 3 Cytotoxic effect of GHM-10 on a multiple drug resistant cell line ($n=3$, mean ± SD). ○—○ KB cells (parent cell line); ●—● KB/VCR200 (resistant cell line).

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cytotoxic effect on KB/VCR200 cells and their parent KB cells (Fig. 3). It is suggested that there is no cross resistance between GHM-10 and vincristine. Since resistance to vincristine belongs to multiple drug resistance, KB/VCR200 is a multiple drug resistant cell line. That is to say, the multiple drug resistant cell lines are still sensitive to GHM-10.

4. Effect of GHM-10 on the Colony Forming Capacity of Various Cells

Double-layer soft agar assay was used for observing the effect of GHM-10 continuous exposure on the colony forming efficiency in several cell lines. Colony forming inhibitory rate of cells after treatment with drugs in various concentrations was calculated by taking the inhibitory rate of the control group as 0. It could be seen in Fig. 4 that HL-60 were the most sensitive cells to GHM-10, L1210 cells were the next and the sensitivity of normal bone marrow granulocyte–macrophage colony forming cells (GM-CFC) was lower than that of cancer cells. The 50% lethal concentrations (IC_{50}) to GHM-10 for HL-60, L1210 and GM-CFC were 1.24, 1.57 and 5.34 $\mu g \cdot ml^{-1}$ respectively.

5. The Therapeutic Effect of GHM-10 on Mouse H22 Liver Cancer and Mouse Lewis Lung Cancer

We can see from Table I that GHM-10 administered either orally or intraperitoneally can inhibit the growth of H22 liver cancer and Lewis lung cancer markedly.

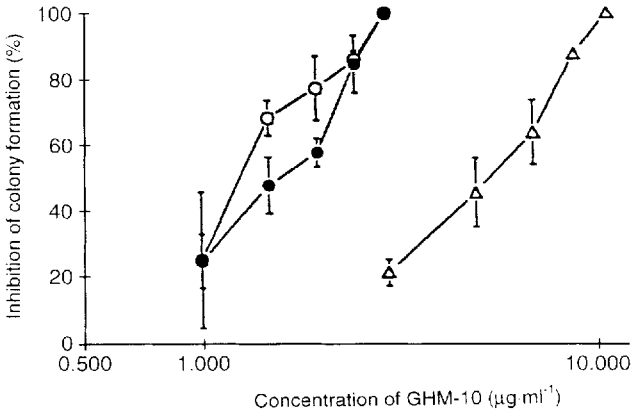

FIGURE 4 Effect of GHM-10 on clonogenicity of cancer cells and murine bone marrow cells (GM-CFC) in soft agar ($n = 3$, mean ± SD). ○ ○ Human promyelocytic leukemia cell line HL-60; ●–– ● mouse lymphocytic leukemia cell line L1210; △––△ mouse GM-CFC.

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6. The Therapeutic Effect of GHM-10 on S-180 Bearing Mice

The results in Table II showed that GHM-10 injected intraperitoneally can prolong the life span of mice bearing S-180 ascites.

7. The Colony-forming Capacity of L1210 Cells after Treated with GHM-10 for Different Time

L1210 cells were treated with GHM-10 in different concentrations for 1, 24 h and 7 days and the inhibitory rates of colony formation were shown in Fig. 5. When cells were continuously exposed to GHM-10 for 7 days, GHM-10 3 $\mu g \cdot ml^{-1}$ were able to suppress the colony formation totally. Most cells lost their colony forming capacity after treatment with GHM-10 4 $\mu g \cdot ml^{-1}$ for 24 h. The ratio of IC_{50}-24 h (3.32 $\mu g \cdot ml^{-1}$) to IC_{50}-7 days (1.59 $\mu g \cdot ml^{-1}$) is 2.09; the ratio of IC_{50}-1 h (6.85 $\mu g \cdot ml^{-1}$) to IC_{50}-7 days (1.59 $\mu g \cdot ml^{-1}$) is 4.31. It is generally held that the cytocidal effect of a cytotoxic agent *in vitro* is dependent on the drug concentration (C) and the time of exposure (T). Usually for producing the same level effect, the higher the concentration provided, the shorter the time needed. That is to say, $C \times T$ is a constant. When the L1210 cells were treated with GHM-10 for 1 h, the IC_{50} was 6.85 $\mu g \cdot ml^{-1}$, $C \times T = 6.85$. When they were treated for 24 h, the IC_{50} was 3.32 $\mu g \cdot ml^{-1}$, $C' \times T' = 79.68$. If $C \times T = C' \times T'$, IC_{50}-24 h would be $6.58/24 = 0.27\ \mu g \cdot ml^{-1}$, it is far less than the IC_{50}-24 h detected (3.32 $\mu g \cdot ml^{-1}$), indicating that the efficiency of large dose GHM-10 exposure for a short time is higher than small dose exposure for a long time.

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TABLE I Inhibition of GHM-10 on transplantable hepatoma H22 and Lewis lung cancer in mice

Tumor	*Group*	*Dose* ($mg \cdot kg^{-1} \cdot days$)	*Animal no.* (*initial/final*)	*Body wt.* (g) (*initial/final*)	*Tumor wt.* (g) (*mean* ± *SD*)	*Inhibition* (%)
H22	Control		10/10	20.4 ± 1.03/30.4 ± 3.53	3.60 ± 0.43	
	GHM-10	20 × 10 p.o.	10/10	21.4 ± 1.35/28.1 ± 5.95	2.10 ± 0.76**	41.6
		60 × 10 p.o.	10/8	21.4 ± 2.13/29.9 ± 5.90	1.65 ± 0.83**	54.2
		10 × 10 i.p.	10/10	21.2 ± 1.75/28.2 ± 4.39	1.45 ± 0.88**	59.7
		40 × 10 i.p.	10/10	21.4 ± 1.50/24.8 ± 2.65	1.28 ● 0.46**	64.4
Lewis	Control		10/9	21.7 ± 1.16/18.7 ± 2.63	2.36 ± 0.41	
	GHM-10	20 × 10 p.o.	10/10	20.4 ± 0.97/20.2 ± 1.16	0.96 ± 0.36**	59.4
		60 × 10 p.o.	10/10	20.8 ± 0.91/19.5 ± 1.19	0.49 ± 0.22**	79.2

**$P < 0.01$, vs control.

TABLE II Effect of GHM-10 on the survival time of mice with S-180 ascites tumor

Group	*Dose* (mg · kg^{-1} · days)	*Animal no. (initial/final* 30 days)	*Survival time* (days)	*IST** (%)	*P*
Control GHM-10		12/0	18.1 ± 1.2		
	10 × 7 i.p.	10/3	26.7 ± 5.3	47.5	< 0.01
	20 × 7 i.p.	10/9	29.2 ± 3.2	61.3	< 0.01
	40 × 7 i.p.	10/10	30.0 ± 0.0	65.7	< 0.01

*Increased survival time, mice which live longer than 30 days are calculated as 30 days.

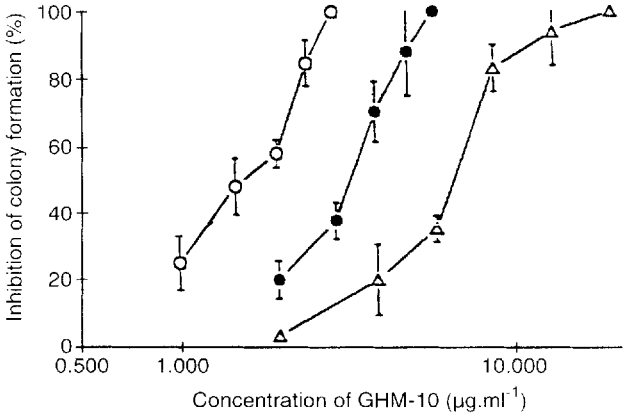

FIGURE 5 The dose–response curves of L1210 cells treated with GHM-10 for different time ($n = 3$, mean ± SD). ○—○ Treated for 7 days; ●—● treated for 24 h; △—△ treated for 1 h.

8. Effect of GHM-10 on the Cell Cycle Transition of L1210 Cells

Flow cytometer was used for analyzing the cell cycle and the effect of GHM-10 (4–12 μg · ml^{-1}, exposure for 6–18 h) on L1210 cells was observed. When the cells were treated with GHM-10 in concentrations of 4–12 μg · ml^{-1} for 6 h, no effect on cell cycle distribution could be detected. The percentage of cells in G_1, S and G_2 + M remained in the normal range. Since the concentrations of GHM-10 used in this experiment were enough to suppress the growth of L1210 cells (see Fig. 5, after treated with GHM-10 4–12 μg · ml^{-1} for 1 h, the colony formation of L1210 cells suppressed 20–90%), the results indicated that GHM-10 may block cell cycle transition in cells of all phases. Along with the increase of GHM-10 concentration or exposure time, there was a slight increase in the percentage of G_1 cells, and a slight decrease in the percentage of S cells. It is suggested that GHM-10 may block the cell transition from G_1 to S (Fig. 6 and Table III).

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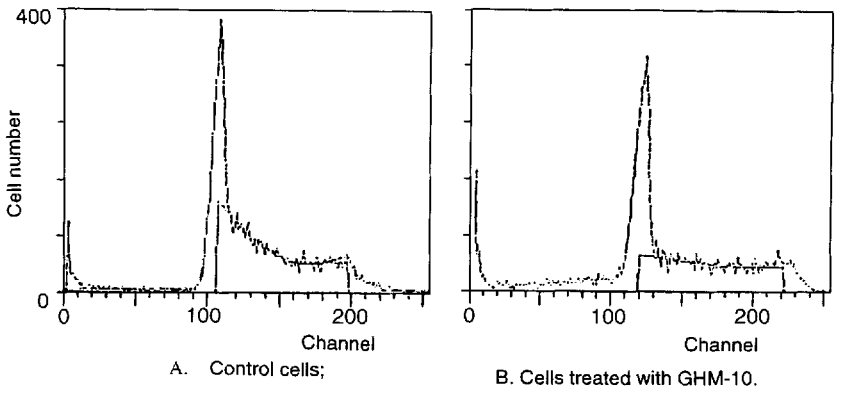

FIGURE 6 Flow cytometric DNA histogram of L1210 cells treated with GHM-10 ($4\,\mu g \cdot ml^{-1}$) for 12 h.

TABLE III Effect of GHM-10 on cell cycle kinetics in L1210 cells

Incubation time (h)	*GHM*-10 ($\mu g \cdot ml^{-1}$)	*% of cell population*		
		G_1	S	G_2
	0 (Control)	31	64	5
6	4	32	64	4
6	6	33	61	6
6	12	38	58	4
6	24	45	46	9
	0 (Control)	22	69	9
12	4	41	50	9
18	4	40	52	8

9. Effect of GHM-10 on DNA, RNA and Protein Biosynthesis in L1210 Cells

Thymidine (TdR), uridine (UR) and leucine (Leu) are the precursors for DNA, RNA and protein synthesis respectively. Therefore, the incorporation rate of [^{3}H]TdR, [^{3}H]UR and [^{3}H]Leu reflexes the metabolism and proliferation of the cells. As shown in Fig. 7, inhibition of DNA, RNA and protein synthesis to different extent was induced by GHM-10 in concentrations of $4-12\,\mu g \cdot ml^{-1}$. This effect was dose dependent and the inhibition of DNA synthesis was the most obvious one, followed by RNA, and the protein synthesis was less affected. It could be seen in Fig. 8 that the inhibition of DNA and RNA synthesis was time dependent when $4\,\mu g \cdot ml^{-1}$ of GHM-10 were used.

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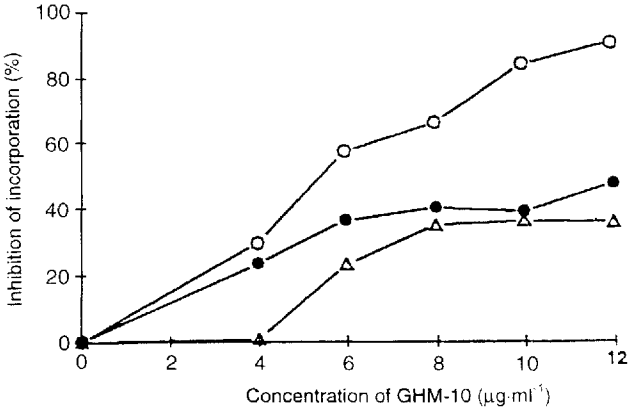

FIGURE 7 Effect of GHM-10 at various concentrations on the incorporation of [^{3}H] labeled precursors into macromolecules of L1210 cells. L1210 cells were treated with GHM-10 for 6 h. ○—○ [^{3}H] TdR; ●—● [^{3}H] UR; △—△ [^{3}H] Leu.

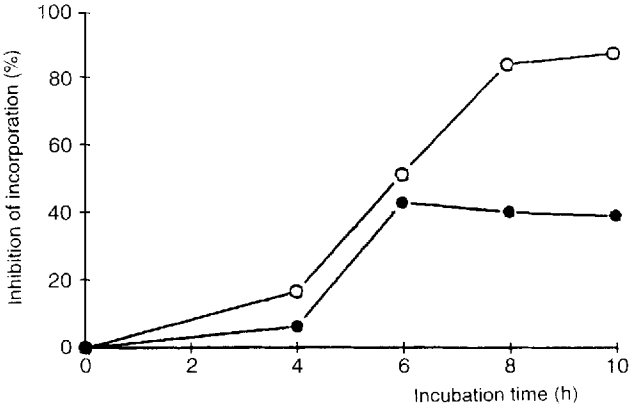

FIGURE 8 The time–effect curve of GHM-10 4 μg · ml^{-1} on the inhibition of [^{3}H] labeled precursors incorporation into macromolecules of L1210 cells. ○—○ [^{3}H] TdR; ●—● [^{3}H] UR.

10. Analyzing the Mode of Action for DNA Synthesis Inhibition Induced by GHM-10

The inhibition of DNA synthesis may be caused by the damage of DNA molecules or caused by metabolic interference. From Fig. 9 we can see that after L1210 cells were treated with 6–8 μg · ml^{-1} for 1 h, the [^{3}H]TdR incorporation curve was decreased progressively, indicating GHM-10 may induce damage in DNA molecules.

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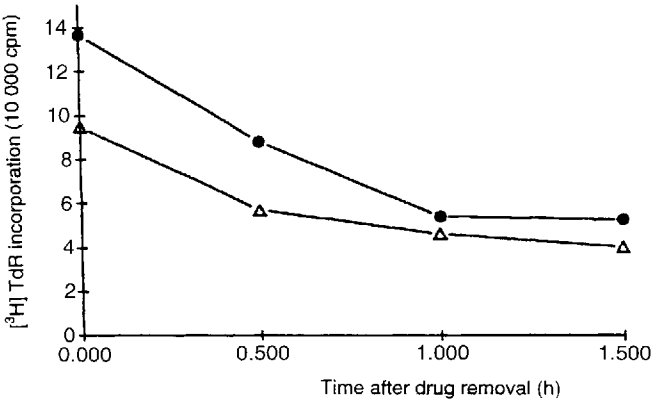

FIGURE 9 Post-exposure effect of GHM-10 on DNA synthesis. L1210 cells were exposed to GHM-10 for 1 h; 0.5–1.5 h after GHM-10 was removed, the cells were incubated with [^{3}H] TdR for 4 h and the incorporation rate of [^{3}H] TdR was detected. ●—● GHM-10 6 μg · ml^{-1}; △ △ GHM-10 8 μg · ml^{-1}.

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11. The Effect of GHM-10 on UV Spectra of Calf Thymus DNA

Some drugs like ethidium bromide (EB) can be combined with DNA directly to form a drug–DNA complex. The DNA absorption spectra would be changed by such combination (shift occurred in the peak of absorption curve). There was no change in the absorption spectra after calf thymus DNA was treated with GHM-10. It was indicated that GHM-10 was not able to intercalate into DNA molecules directly (Fig. 10).

12. DNA Damage in L1210 Cells Detected by Single Cell Gel Electrophoresis

Under the fluorescence microscope, undamaged cells were seen as bright fluorescent round spots. In the cells with damaged DNA, however, comet like tails could be seen towards the positive electrode. The brightness of the tail depended on the degree of DNA damage. As shown in Fig. 11, when cells were treated with 4 μg · ml^{-1} GHM-10, comet like tails appeared; when the concentration of GHM-10 increased to 10 μg · ml^{-1}, the brightness of tails was also increased.

13. DNA Single Strand Break Induced by GHM-10

The principle of alkaline elution technique for estimating DNA single strand break is that in an alkaline solution, the double strands of DNA will be

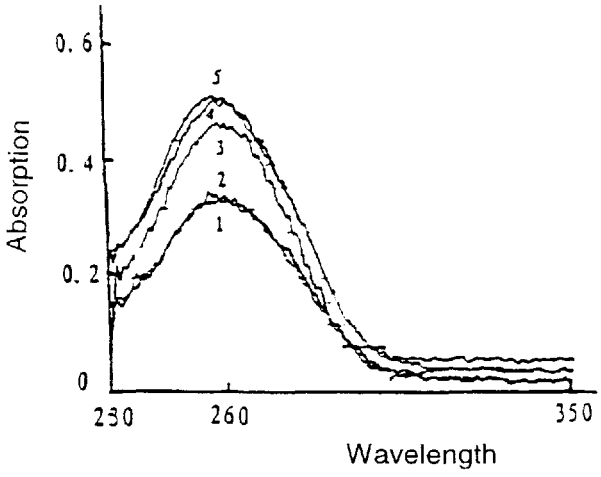

FIGURE 10 The effect of GHM-10 on UV spectra of calf thymus DNA. Calf thymus DNA were treated with GHM-10 10 μg · ml $^{-1}$ for 24 h at 22°C. UV absorption spectra were scanned in a spectrophotometer. (1) Control; (2) DNA 10 μg · ml $^{-1}$; (3) DNA 20 μg · ml $^{-1}$; (4) DNA 25 μg · ml $^{-1}$; (5) DNA 30 μg · ml $^{-1}$.

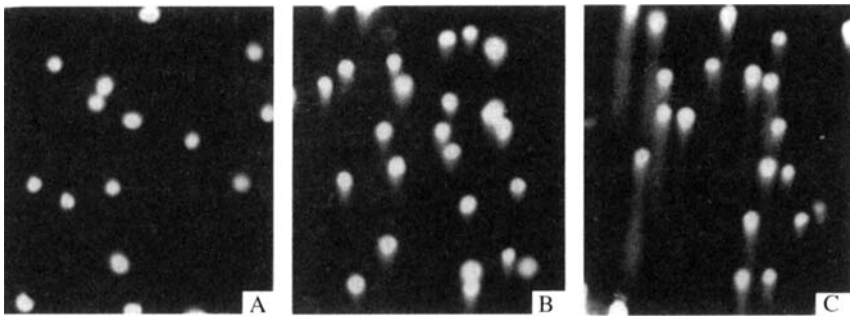

FIGURE 11 Comet like tails were formed in L1210 cells treated with different concentrations of GHM-10. Photographs of cells under fluorescence microscope after single cell gel electrophoresis. (A) Untreated cells; (B) GHM-10 treated cells, 4 μg · ml^{-1}; (C) GHM-10 treated cells, 10 μg · ml $^{-1}$.

totally separated into two single strands. If the filter membrane does not adsorb DNA molecules, the speed of DNA single strand passing through the membrane will be dependent on its length. Therefore, the ratio of DNA retained on the membrane will reflect the level of DNA single strand breakage. Figure 12 showed that when L1210 cells were treated with GHM-10 (4 and 25 μg · ml^{-1}) for 5 h, the ratio of DNA retained on the membrane decreased significantly, indicating that GHM-10 is capable of inducing

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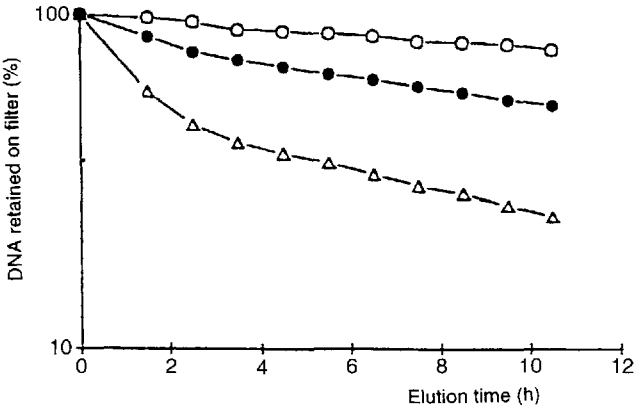

FIGURE 12 DNA single strand break induced by GHM-10. Alkaline elution technique was used for estimation. DNA single strand break was measured by the percentage of DNA retained on the filter. ○—○ Control; ●—● GHM-10 4 μg · ml^{-1} △ △ GHM-10 25 μg · ml^{-1}.

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DNA single strand break in L1210 cells. Furthermore, as the concentration of the drug increased, the ratio of DNA retained on the membrane decreased progressively. Obviously there is a dose–effect relationship.

14. Effect of GHM-10 on the Activity of DNA Topoisomerase II in L1210 Cells

DNA topoisomerase II is an important nuclear enzyme. It can modify the topo-structure of DNA molecules and induce the break and rejoin of DNA single and double strands. The capability of dehelix on super helix pUC18 DNA was used to measure the activity of DNA topoisomerase II. The results showed that compared with the control group, the activity of DNA topoisomerase II was decreased in L1210 cells treated with GHM-10 4 μg · ml^{-1} for 12 h (Fig. 13). When the DNA topoisomerase II extracted from control L1210 cells was directly exposed to GHM-10, the activity of enzyme was also decreased if the concentration of GHM-10 reached 100–500 μg · ml^{-1} (Fig. 14).

There are more than 120 genera, 2100 species of plants belonging to the Annonaceae family in the world [5]. Uvaricin was the first acetogenin compound isolated from *Uvaria acuminata* of the Annonaceae family [6]. Up to now, more than 100 acetogenins have been isolated from Annona, Rollina, Asimina, Goniothalamus plants. Among them, Bullatacin, Bullatacinone [7]

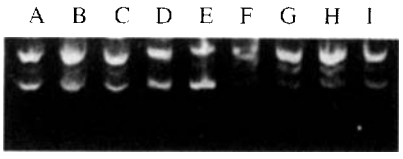

FIGURE 13 DNA topoisomerase II activity in nuclear extracts from untreated or GHM-10 treated L1210 cells. The enzyme activity was determined by uncoiling pUC18 DNA, which was located in the bottom zones of the photograph. Lane E: pUC18 DNA only; Lanes F to I: pUC18 DNA plus protein extracted from untreated L1210 cells (0.96, 0.48, 0.24 and 0.12 μg respectively); Lanes A to D: pUC18 DNA plus protein extracted from L1210 cells treated with GHM-10 4 $\mu g \cdot ml^{-1}$ for 12 h (0.96, 0.48, 0.24 and 0.12 respectively).

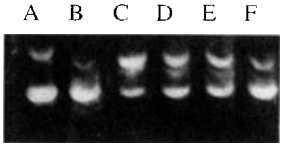

FIGURE 14 Effect of GHM-10 on the activity of DNA topoisomerase II in a cell free system. Reaction mixtures (20 μl) containing pUC18 DNA 0.42 μg, DNA topoisomerase II 0.12 μg and GHM-10 100–500 $\mu g \cdot ml^{-1}$. Lane A: pUC18 DNA only; Lane B: pUC 18 DNA plus GHM-10 500 $\mu g \cdot ml^{-1}$; Lane C: pUC18 DNA plus DNA topoisomerase II; Lanes D to F: pUC: 18 DNA plus DNA topoisomerase II and GHM-10 100, 200 and 500 $\mu g \cdot ml^{-1}$ respectively.

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and Asimicin [8] showed potent anticancer activity. Howiinol A (GHM-10) is a phenylethylene pyrone compound. Although its anticancer activity was not as strong as the above mentioned acetogenins, the content of GHM-10 in the plant is rich and has already been chemically synthesized [3]. GHM-10 exerted anticancer effect both *in vitro* and *in vivo*; it was effective in treatment of transplantable tumors in mice even when it was administered orally; the cancer cells were more sensitive to GHM-10 than the normal hematopoietic cells; the multiple drug resistant cell strain KB/VCR 2000 has similar sensitivity as its parent KB cells; in the dose of efficacy, no significant toxicity was seen [8]. All these characteristics make GHM-10 a new molecular entity with prospects for drug development. It is worth doing further studies.

Preliminary studies on mechanism of action showed that GHM-10 had no effect on inducing differentiation to cancer cells [9]. When cells were treated with GHM-10 for 24 h, the growth rate and mitotic index were decreased and the morphology of cell nuclei changed, but the cell viability remained at

normal level, indicating that GHM-10 mainly inhibited the cell proliferation [10]. The major action was to induce DNA single strand breakage, and to suppress the biosynthesis of DNA and other biological macromolecules [11]. Since GHM-10 did not bind or intercalate to DNA molecules, the damage of DNA may be caused by its action on the activity of DNA topoisomerase II and/or other mechanisms [12].

EXPERIMENTAL SECTION

Materials

Cells

Mouse lymphocytic leukemia L1210 cells and human acute promyelocytic leukemia HL-60 cells were kindly given by Dr. K. Scanlon of the Mount Sinai Medical college, USA. Human oral cavity cancer KB cells, Chinese hamster lung CHL cells were maintained by our laboratory. The vincristine resistant strain of KB cells (KB/VCR 2000) were kindly given by Prof. Xiu-Juan Ji of our Institute.

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Animals and Tumor Lines

Kunming mice and C57BL/6 mice, 18–22 g of body weight, both sexes, were supplied by the breeding center of the Institute of Experimental Animals, Chinese Academy of Medical Sciences. Transplantable animal tumors including mouse sarcoma 180, hepatoma H22 and Lewis lung cancer were maintained by our laboratory.

Drugs

GHM-10, mw 380, was supplied by the Department of Chemistry of Natural Products of our Institute. It was dissolved in DMSO, stored at 4°C, and diluted with culture medium to required concentration before use.

Methods

Determination of Growth Curve of L1210 Cells

L1210 cells in exponential growth phase were suspended in RPMI1640 medium with 10% new born calf serum in concentrations of 2×10^4 cells $\cdot$ ml^{-1}. The method for determination of growth curve was according to Ref. [4].

MTT (Thiazolyl Blue Tetrazolum Bromide) Test for Measuring the Inhibitory Rate of GHM-10 on Cancer Cells

Cells of A2780, HCT-8, KB, Bel 7402 and CHL in exponential growth phase were inoculated in 96 well plates at a concentration of 10^4 cells · ml $^{-1}$ and 200 µl per well. MTT test was used for measuring the sensitivity of different cell lines to GHM-10 [4].

Determination of the Efficiency of Soft Agar Colony Formation

Double-layer soft agar medium in 35 mm petri dishes was used for colony formation [4]. The assay for mouse bone marrow granulocyte–macrophage progenitor cells (GM-CFC) was conducted according to the literature [13].

Treatment of Mouse Lewis Lung Cancer and H22 Hepatoma of Solid Type

Tissue of solid tumor was isolated from well developed tumor-bearing mice using routine aseptic technique. Suspensions of cancer cells prepared in normal saline were inoculated into subaxillary region of Kunming mice (H22) or C57BL/6 mice (Lewis) subcutaneously. Twenty four hours after inoculation, the mice were divided into several groups randomly and drugs were administered by gastrogavage once daily for 10 days. The animals were killed on the 11th day, the body weight and tumor weight were recorded, and the inhibitory rate of tumor growth was calculated [14].

Treatment of Mouse Sarcoma 180 Ascites

Ascites was drawn from well developed tumor-bearing mice aseptically. After 1 : 1 dilution with normal saline, 0.2 ml ascites per mouse was inoculated intraperitoneally into Kunming mice. Twenty four hours after inoculation, the mice were divided randomly into several groups. Drugs were intraperitoneally administered once daily for 7 days. The life span of each mouse was recorded and the mean life span and 30 day survival rate were calculated.

Effect of GHM-10 on Colony Formation of L1210 Cells

Suspensions of L1210 cells were treated with GHM-10 in various concentrations. After incubation at 37°C and 5% CO_2 for 1 or 24 h, the suspensions were centrifuged at 1000 rpm for 5 min. The cells were washed twice then cultured in soft agar for colony assay. When the effect of continuous drug exposure on L1210 cells was tested, GHM-10 was added into the base-layer of agar directly.

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Flow Cytometer Assay

L1210 cells were exposed to GHM-10 for different time, fixed with 70% alcohol and re-suspended with 0.4 ml PBS; RNase A was added to the final concentration of $0.5\,\mu g \cdot ml^{-1}$. After incubation at 37°C for 1 h, propidium iodide (PI, Sigma product) stain solution was added to the suspension to make the final concentration at $0.5\,\mu g \cdot ml^{-1}$. The DNA content in cells and the percentage of cells in different phases of cell cycle were measured with a FACS 420 flow cytometer.

[^{3}H] Labeled Precursor Incorporation Tests

L1210 cell suspensions 4×10^5 cells $\cdot$ ml^{-1} were inoculated into 96 well plates and GHM-10 was added at the same time. The plates were placed in a 37°C, 5% CO_2 incubator for 6 h; at 4 h before the GHM 10 treatment was ended, [^{3}H] labeled precursors 1 μCi/well was added. An automatic cell collector was used to collect cells from the well and the cells were put on the glass fiber filter. Distilled water was used to wash the filter for at least 20 times. The filter was dried up in a 80°C lab oven for 15 min and put in a vial with 4 ml scintillater (0.4% PPO, 0.01% POPOP in xylene). After being stayed in a dark place for 15 min, the cpm value was counted with a Beckman LS-9800 scintillometer.

Determination of [^{3}H]TdR Incorporation Curve after the Cells Released from Drug Exposure

Suspensions of L1210 cells in exponential growth phase, 4×10^5 cells $\cdot$ ml^{-1}, were inoculated into tubes and GHM-10 was added at the same time. One hour after incubation at 37°C, the tubes were put in an ice bath to stop the reaction. The suspensions were centrifuged at 1000 rpm for 5 min, the supernatants were discarded, and the cells were washed with cold PBS twice. Fresh RPMI 1640 medium was used to suspend the cells and the suspensions were kept in a 37°C incubator for 0, 0.5, 1 and 1.5 h. Each tube was added [^{3}H]TdR 1 μCi and incubated at 37°C for 4 h. The method for measuring the incorporation of [^{3}H]TdR into cells was the same as mentioned above.

Shift of Absorption Spectrum

GHM-10 $10\,\mu g \cdot ml^{-1}$ and natural calf thymus DNA $10–30\,\mu g \cdot ml^{-1}$ were put in tubes using pH 7.25 NaH_2PO_4–Na_2HPO_4 buffer solution as reactive medium. The tubes were maintained at 22°C for 24 h and shaken

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periodically. The ultraviolet absorption spectra were scanned in a Shimadzu MPS-2000 multifunctional ultraviolet spectrophotometer.

Single Cell Gel Electrophoresis for Measuring DNA Damage in L1210 Cells

L1210 cells in exponential growth phase at a concentration of 1×10^6 cells $\cdot$ ml^{-1} were added GHM-10 4 and 10 μg $\cdot$ ml^{-1}. After incubation at 37°C, 5% CO_2 for 4.5 h, the suspensions were put in an ice bath to stop the reaction. The cells were washed with cold PBS twice and suspended again in 0.5 ml cold PBS.

Two hundred ml of 1% agar solution were evenly spread on a microscopic slide and the slide was put on an ice bath to gel the agar. The cell suspensions were kept at 37°C and mixed with 1% low melt point agarose (Sigma product) in the ratio of 1 : 3. This mixture was spread on the surface of the agar coated slide. The agarose was gelled on the ice bath again and the slide was dipped in a cytolytic solution (NaOH 0.03 mol $\cdot$ l^{-1}, NaCl 1 mol $\cdot$ l^{-1}) for 40 min. The slide was rinsed by distilled water, dried and dipped in a dehelix solution (NaOH 0.3 mol $\cdot$ l^{-1}, EDTA 1 μmol $\cdot$ l^{-1}) for 8 min. After being rinsed with double distilled water, the slide was placed in an electrophoresis bath (DF-C voltage and current stabilized electrophoresis apparatus) horizontally. The electrophoresis was performed at the voltage of 45 V for 13 min, and the slide was stained with EB for 10 min. The whole process should be protected from light and the morphology of cells was observed and photographed under a fluorescence microscope.

Alkaline Elution Technique for Estimating DNA Single Strand Break

L1210 cells in exponential growth phase, 3×10^5 cell $\cdot$ ml^{-1}, were inoculated into 33 mm dishes. Each dish contained 2 ml of suspension and [^{3}H]TdR 5 μCi. Twenty four hours after incubation at 37°C and 5% CO_2, GHM-10 were added to the final concentration of 4 and 25 μg $\cdot$ ml^{-1}. Following 5 h incubation, the cells were transferred to a millipore filtering film (Φ 0.2 μm), washed with cold PBS for three times, and 10 ml cytolytic solution (Sodium dodecyl sulfonate 0.2%, EDTA 0.02 mol $\cdot$ l^{-1}) was added. After the cells were treated for 3 min, the cytolytic solution was dropped naturally. The millipore film was further washed with 20 ml cold PBS and treated with 40 ml of alkaline elution solution (EDTA 0.02 mol $\cdot$ l^{-1}, pH was adjusted to 12.0 by NaOH) for 5 min to let the DNA become denatured. The elution solutions were collected fractionally; each fraction consisted of approximately 1.5 ml by a collecting time of 50 min. The elution process was kept

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away from light and the millipore film and elution fraction were put into vials containing 4 ml scintillater (toluene 240 ml, absolute alcohol 110 ml, triton-X-100 130 ml, PPO 1 g, POPOP 0.025 g). The radioactivity was counted and the residual rate of DNA on the millipore film ($R\%$) was calculated. The elution curve was plotted by using the $R\%$ as the function of time.

$$\text{Residual rate of DNA on film } (R\%) = (M/M + E) \times 100\%$$

M: Cpm for DNA on the film; E: Sum of cpm for DNA in the fraction of alkaline elution solutions.

DNA Topoisomerase II

When the effect of GHM-10 on DNA topoisomerase II in L1210 cells was tested, L1210 cells were treated with GHM-10 for 12 h and washed with cold PBS twice, then the enzyme was isolated and the activity measured. When the direct effect of GHM-10 on the activity of DNA topoisomerase II was tested, the enzyme was extracted from control L1210 cells first, then treated with GHM-10 and the activity measured. The methods for enzyme isolation and activity determination were according to the literature [15].

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References

[1] X.P. Fang, M.J. Rieser, Z.M. Gu, G.X. Zhao and J.L. McLaughlin, Annonaceous acetogenins: an updated review. *Phytochemical Analysis* 1993, **4**: 27.

[2] Editorial Committee for Phytography, Chinese Academy of Sciences. *Botanical record of China*, Vol. 30B, Publishing House of Science, Beijing, 1979, pp. 10–175.

[3] D.Q. Yu, R.Y. Chen, C.X. Xu, W.Z. Song, F.Z. Xie, X.P. Pan, S.Y. Sun, Z. Jiang, Y. Chen, Y.M. Ye, J.H. He, L. Ma and F. Wu, Study on the effective anti-cancer components in Annonaceous family. *Proceeding of the Annual Scientific Conference of Chinese Academy of Medical Sciences and Peking Union Medical College (1996)*. Edited by the Department of Scientific Research, CAMS & PUMC. Joint Publishing House of Beijing Medical University and Peking Union Medical College, Beijing, 1996, p. 260.

[4] C.X. Xu, *In vitro* experimental methods on cancer cells. In *Research and Development of Anticancer Drugs and Experimental Techniques*, R. Han, Ed. Joint Publishing House of Beijing Medical University and Peking Union Medical College. Beijing, 1997, p. 282.

[5] J.K. Rupprecht, Y.H. Hui and J.L. McLaughlin, Annonaceous acetogenins: a review. *J. Nat. Products* 1990, **53**: 237.

[6] S.D. Jolad, J.J. Hoffmann, K.H. Schram and J.R. Cole Jr., Uvaricin, a new antitumor agent from *Uvaria accuminata* (Annonaceae). *J. Org. Chem.* 1982, **47**: 3151.

[7] Y.H. Hui, J.K. Rupprecht, Y.M. Liu, J.E. Anderson, D.L. Smith, C.J. Chang and J.L. McLaughlin, Bullatacin and bullatacinone: two highly potent bioactive acetogenins from *Annona bullata*. *J. Nat. Products* 1989, **52**: 463.

[8] J.K. Rupprecht, C.J. Chang, J.M. Cassady, J.L. McLaughlin, K.L. Mikolajczak and D. Weisleder, Asimicin, a new cytotoxic and pesticidal acetogenin from the pawpaw, *Asimina triloba* (Annonaceae). *Heterocycles* 1986, **24**: 1197.

[9] J.H. He, Y.M. Ye and C.X. Xu, Studies on the anticancer effect of Howiinol A, a new compound isolated from *Goniothalamus howii*. *Acta Pharm. Sinica* 1998, **33**: 493.
[10] J.H. He, Y.M. Ye and C.X. Xu, Antitumor activity of Howiinol A (GHM-10) on L1210 cells *in vitro*. *Acta Pharm. Sinica* 1998, **33**: 566.
[11] J.H. He and C.X. Xu, Inhibitory effect of Howiinol A (GHM-10) on the synthesis of biological macromolecules in L1210 cells. *Acta Pharm. Sinica* 1998, **33**: 886.
[12] J.H. He and C.X. Xu, Effect of Howiinol A (GHM-10) on the structure of DNA molecules and activity of DNA topoisomerase II in L1210 cells. *Acta Pharm. Sinica* 1999, **34**: 5.
[13] N.G. Testa, J.H. Hendry and L.G. Lajtha, The response of mouse haemopoietic colony-forming units to repeated whole body X-irradiation. *Biomedicine* 1974, **21**: 431.
[14] R. Han, Experimental methods on animal transplantable tumors. In *Research and Development of Anticancer Drugs and Experimental Techniques*, Han, R. Ed. Joint Publishing House of Beijing Medical University and Peking Union Medical College. Beijing, 1997, p. 298.
[15] L.G. Wang, X.M. Liu and X.J. Ji, Determination of DNA topoisomerase II activity from L1210 cells – a target for screening antitumor agents. *Acta Pharm. Sinica* 1991, **12**: 108.

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