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Effects of Mitosene Analogues on Growth Inhibition of Human Cervical Cancer Cell Lines

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Abstract: To develop a promising alkylating agents for anti-cervical cancer chemotherapy, five mitosene analogues were synthesized. Despite the potentiality of better cytotoxicity on solid tumor cells as opposed to that on rapidly-doubled leukemic cells, there have been no reports on the inhibition of the cervical cancer cell line by mitosene analogues. The present experiment was designed to investigate whether mitosene analogues can effectively inhibit the cellular proliferation of cervical cancer cells by using an *in vitro* chemosensitivty system. The mitosene analogues displayed a potent cytotoxic effect on the tested cervical cancer cell lines. Among the analogues, (22) compound gave the best inhibitory effect on SiHa tumor colonies formation. These data indicate that mitosene analogues can effectively inhibit the growth of cervical cancer cells *in vitro*.

Key Words: Mitosene analogues, MTT, Clonogenic assay, Cervical cancer cell line

INTRODUCTION

Cervical cancer is the most common malignancy in Korean women and it's incidence tends to be higher in a rapid manner[15]. Invasive cervical cancer is regarded as a systemic disease rather than localized one[5]. Thus, surgery or radiation therapy alone little contribute to overcome advanced cervical cancer[1]. Also, many patients with cervical cancer have a little response to chemotherapy even if chemotherapy have been approved as one of the effective treatments[4]. The most appropriate adjuvant therapy has not been as yet set up and no single mode of adjuvant therapy appears to be superior to others[13]. In the clinical field, some alkylating agents such as mitomycin C and cyclophosphamide have been also introduced to treat patients with various stages of cervical cancer in a combined regimen[17]. However, the combined chemotherapy using alkylating agents has been limited in their usefulness owing to their high toxicity[18]. Therefore, the development of an effective chemotherapeutic agent is needed for the treatment of cervical cancer. Recently, some clinical studies using mitosene analogues, designed as new DNA cross-linkers mimicking mitomycin C, were reported to show potent cytostatic ac-

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tivity[6,7,8]. To reduce the toxicity of alkylating quinones and optimize their activity, mitosene was proposed as promising candidate[14]. This compound had an action of reductive alkylating quinone[16]. Alkylating quinone methides which are formed upon reduction of the quinones and subsequent elimination of the leaving groups are highly reactive and produce cytotoxic effects by reacting with the DNA (nucleophile) of tumor cells[19]. Because there is a high reduction potential in tumor cells, the reductive alkylating quinones, such as mitosene analogues, are hypothesized to be hypoxic selective agents[9,10]. Therefore it is important to judge whether the mitosene analogues can be applied in the chemotherapy of cervical cancer. However, there are few reports on the inhibition of cervical cancer cell lines by the mitosene analogues. Thus, the present study was directed to evaluate the *in vitro* cytotoxicity of the mitosene analogues (10) and (22)-(25) against human cervical cancer cell lines by using the methyltetrazoilum bromide (MTT) assay and the clonogenic assay.

MATERIALS AND METHODS

1) Chemicals

Mitosene analogues were synthesized at our insititute as previously reported[2]. Their structures are shown in Fig. 1. The reagents (4-chloro-3-nitrotoluene, pyrrolidine, morphine, and pyrroline) for synthesis of the mitosene analogues and other cytotoxic agents (mitomycin, cisplatin, adriamycin, 5-fluorouracil, and etoposide) for comparative cytotoxicity were obtained from Sigma Co., Ltd (St. Louis, USA) and Adrich Co., Ltd (Milwaukee, USA). Also, MTT agent was purchased from Sigma Co., Ltd (St. Louis, USA).

2) Cancer cell lines

The cancer cell lines for the cytotoxicity test were as follows: CaSki (human uterine cervical cancer), HeLa (human uterine cervical cancer), SiHa (human uterine cervical cancer), C33A (human uterine cervical cancer). Each cell line was maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and incubated in a highly humidified 5% CO_2 chamber at 37℃.

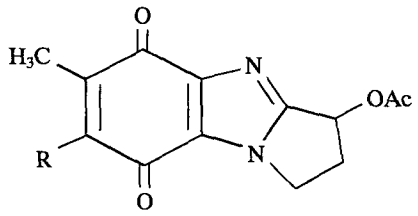
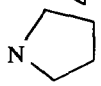
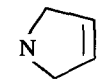
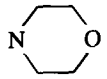


Fig. 1. Structures of mitosene analogues

Compounds	R
10	H
22	
23	
24	
25	

3) Measurement of cytotoxicity (MTT, clonogenic assay)

To evaluate cytotoxicity, the MTT method was performed essentially as described previously[11,12]. Briefly, monocellular suspension (log phase, 95% viable) was seeded at 10^4 cells with 100μl of medium per well in 96 well plates. To compare cytotoxicity between the mitosene analogues and cytotoxic agents (5-fluorouracil, cisplatin, mitomycin C, adriamycin, and etoposide) such drugs were added at varying concentrations of 50, 12.5, 3.125, 0.78, 0.195, 0.0488, 0.0122, 0.003μg/ml and cultures were continuously incubated for 48-72 hours in an incubator maintaining a highly humidified atmosphere, 5% CO_2 and 95% air without medium change. Fifty μl of the medi-

um containing MTT (5mg/ml) was added to each well. After 4 hours of exposure, the medium was partly decanted and the wells were washed with PBS, and then 150μl of DMSO were added to each well to solubilize the precipitates. The plates were transferred to an ELISA reader to measure the absorbance at 570nm with a reference wave length of 630nm. The IC_{50} value, 50% inhibition of cell growth, was calculated by regression analysis (plotting the viablity versus the concentration of the test compound) using Graphpad Prism 2.0 (GraphPad Software, Inc.). All experiments were repeated at least 3 times, with 3 wells for each concentration of test agents. As an another method to measure *in vitro* efficacy of cytotoxic drugs, twenty four well-clonogenic assay was done by modifying the 96 well based clonogenic assay[19,20]. Layers of 0.5ml, 0.5% noble agar in supplemented RPMI 1640 medium were prepared in a 24 well culture plate. SiHa cells were overlayered on the basal agar in 0.5ml of 0.3% agar containing 20% fetal calf serum (FCS). The final cell density in each culture was 5 X 10^3 per well. All experiments were done at least 3 times, with 4 wells for each concentration of the test agents. Colony formation was calculated as follows: C.F.E (colony forming efficiency) = [formed colony number / seeded colony number] X 100%.

Table 1. 50% Inhibitory concentrations of mitosene analogues on cervical cancer cell lines

Compound	IC_{50} (μg/ml)[a] of Tumor cell lines			
	SiHa	C33A	HeLa	CasKi
10	0.011	0.019	0.93	0.45
22	0.009	0.002	0.11	0.24
23	0.02	0.003	0.41	0.12
24	0.001	0.014	0.22	0.73
25	0.004	0.009	0.31	0.14
MMC	0.004	0.22	0.06	0.53
DDP	0.002	0.05	0.014	0.06
ADM	0.09	0.026	0.02	0.04
5-FU	0.04	0.01	0.02	0.25
ETP	0.004	0.005	N.D[b]	N.D

[a]: Measured by MTT assay, IC_{50} value which is defined as the concentration that caused 50% inhibition of cell growth

[b]: *Not determined*

Note, mitomycin C, MMC; cisplatin, DDP; adriamycin, ADM; 5-fluorouracil, 5-FU; etoposide; ETP

RESULTS

As shown in Table 1 and Table 2, mitosene analogues (10,22,23,24,25) showed potent cytotoxicity against the tested cell lines. In addition, all mitosene analogues showed dose-dependent cytotoxicities against all tested cell lines. All mitosene analogues showed a comparable cytotoxicity compared to cytotoxic drugs against all tested cell lines. However, each mitosene analogue revealed different cytotoxicity profiles depending on the tested cell line. In colony formation assay using SiHa cells, 50% colony forming abilities of mitosene analogues (10,22,23,24,25) were less than 0.78μg/ml (Table 2). Among mitosene analogues (22) gave the highest inhibitory effect on colony formation of SiHa cells. Of control drugs, mitomycin C exhibited the best inhibitory effect on SiHa colony formation.

Table 2. Percent colony formation of SiHa cervical cancer cell line after treatment with mitosene analogues

Compound	% C.F.E[a]				
	50	12.5	3.125	0.78	0.19 (μg/ml)
10	0	7.5	19.5	26.4	35.6
22	0	0	0	0	2.5
23	0	10.1	18.3	24.5	48.9
24	0	11.2	34.6	45.8	78.9
25	0	23.5	32.1	46.1	75.3
MMC	0	0	0	0	4.8
DDP	0	0	0	23.4	45.7
ADM	0	0	0	5.5	23.2
5-FU	0	0	0	8.2	34.3

[a]: % of Colony Forming Efficiency when CFE of control culture was 100%

Note, mitomycin C, MMC; cisplatin, DDP; adriamycin, ADM; 5-fluorouracil, 5-FU

DISCUSSION

In a previous experiment, we reported the synthesis of the same mitosene analogues (azamitosene)[2]. These mitosene analogues were designed to be highly cytotoxic on hypoxic tumor cells through reductive alkylation[19]. Mitosene analogues, containing a pyrrolobenzimidazole-based ring as a new model compound of reductive alkylating agents, were recently reported to be less toxic on normal cells compared to mitomycin C[14,22]. Therefore, mitosene analogues may have therapeutic advantages as a reductive alkylating agent for chemotherapy of cervical cancer. To test our hypothesis, we did *in vitro* chemosensitivity tests (MTT, clonogenic assay). From the chemosensitivity results, mitosene analogues (10,22,23,24,25) can be judged to have significant growth inhibition against cervical cancer cells *in vitro*. Among synthesized analogues, the hydrogen, aziridinyl, pyrrolidinyl, pyrrolinyl, and morpholinyl substituted azamitosenes revealed excellent cytotoxicity on the tested cell lines. From these results, it seems that some structures such as (10,22,23,24,25) of azamitosene may be able to enhance cytotoxicity. Recently, we reported that some of the mitosene analogues were cytotoxic to ovarian cancer cells *in vitro*[3]. Also, In the first trial of nude mouse-xenografted assay, azamitosene (22) reduced gastric tumor volume and weight (Unpublished data). Therefore, mitosene analogues including (22) may show similar activity on human cervical tumor-bearing mice. Considering these results, the mitosene analogues may have potent cytotoxicity on human cervical cancer cells. Presently, further studies are in progress to analyze the cytotoxic mechanism of mitosenes and to improve their unique cytotoxicities.

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=국문초록=

Mitosene유사체의 자궁암세포주 성장억제 효과

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실험적 자궁암 치료제로서 본 연구소에서 합성한 마이토센유사체를 인체 자궁암세포주인 SiHa, C33A, HeLa, CaSki에 처리하여, 체외 항암활성 측정시 합성 마이토센유사체들은 이 들 세포주에 대하여 대조 항암제들에 비하여 의의있는 세포독성을 보였으며, SiHa 세포주를 이용한 종양클론형성 억제검사에서는 22번 화합물만이 종양성장 억제활성이 가장 우수하였고 다른 화합물들은 대조항암제들에 비하여 활성이 낮았다. 이 마이토센유사체들에 대한 체외 항암감수성결과는 향후 전임상적인 자궁경부암 화합요법제의 기초자료로 유용할것으로 생각된다.

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