Preventive impact of alkaloids with anti-cancer effect extracted from natural herb and the derivatives

**Corresponding Author:**
Mr. Norimasa Miura,
Pharmacotherapeutics, Tottori University, 86 Nishicho, 683-8503 - Japan

**Submitting Author:**
Mr. Norimasa Miura,
Pharmacotherapeutics, Tottori University, 86 Nishicho, 683-8503 - Japan

**Article ID:** WMC00519
**Article Type:** Research articles
**Submitted on:** 11-Sep-2010, 07:55:00 AM GMT  **Published on:** 12-Sep-2010, 12:19:24 AM GMT
**Article URL:** http://www.webmedcentral.com/article_view/519
**Subject Categories:** PREVENTIVE MEDICINE
**Keywords:** Natural Herb, Alkaloids, Antitumor, Anti-hTERT


**Source(s) of Funding:**
This work was supported by grants from Japan Kampo Medical Research Institute and Japan Kampo Medicine Manufacturers Association (JKMA).

**Competing Interests:**
HK and NM, designed, performed and analyzed the research and drafted the manuscript after HM and ST gave a useful suggestion to NM. MS, WS, TT, RS, XW, ZW, ST, TH, and AM conceived of the study and helped this in vitro study as necessary. NM supervised the transfection experiments and helped to draft the manuscript. TI provided us with synthesis of apomorphine. NM and JH conducted the study as the principal investigators and contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

**Additional Files:**
FACS analysis showed induction of more than half of Topoisomerase ? activity in HLF cells is shown
hTERT mRNA downregulation by alkaloids was different
The inhibition of caspase 3 activity by alkaloids
(a) The involvement of p53 transcript in senescence
Preventive impact of alkaloids with anti-cancer effect extracted from natural herb and the derivatives


Abstract

Background

Alkaloids with pharmacological effects are often used as medications and recreational drugs, and are a group, which is one of the major elements of herbal medicine. The theme of this study is to identify novel alkaloid elements with an anti-hTERT effect present in herbs and the derivatives.

Methods

After narrowing down to 5 herbs that have strong tumor suppressive effects against a hepatocellular carcinoma cell line (HLF) by preliminary sensitivity test using extracts from kampo preparations mainly containing alkaloids, we attempted to identify a common ingredient in them. We performed sensitivity tests on each alkaloid and its derivates using respective optimal concentrations for 2 months, followed by examinations of 7 other lines including SUSM1 cells with alkaloids of interest. We examined anti-proliferative activity, hTERT mRNA downregulation, topoisomerase activity, apoptosis, cell cycle, and topoisomerase in cancer cells in the same condition used for the sensitivity test.

Results

These alkaloids have regulated cell proliferation and induced the senescence in cancer cell lines. Dicentrine, one of the alkaloids, remarkably showed anti-cancer effects. The alkaloid with the highest efficacy differed for each cancer cell line, however, dicentrine commonly showed strong anti-cancer effects for all the cell lines. In addition, anti-proliferation activity was not shown in SUSM1 cells immortalized without hTERT expression.

Conclusions

We found that as an herbal medicine element, alkaloid, with anti-hTERT effect had few adverse effects and the effect of the alkaloids is cancer-specific on the basis of a lack of the anti-proliferative effect on cells without telomerase activity. In traditional Japanese medicine, these elements have been historically administrated as a part of conventional powder, suggesting that they may have a preventive effect on cancer onset. After performing the experiment in vivo, we aim to develop derivatives that can be applied clinically as novel anti-cancer drugs.

Introduction

Recently, some fields of medical practice are focusing on products (mainly herbal extract formulations) used in traditional Japanese medicine, as options for treating chronic diseases, cancer, and pain control. However, herbs used in traditional herbal therapies are compounds of ingredients with levels that are inadequate to analyze pharmacologically. Ingredients in herbs are roughly divided into alkaloids and glycosides [1]. Alkaloids are a generic term of basic organic compounds containing nitrogen. Many alkaloids with strong biological activities are known as phytotoxins. They are chief ingredients of medicinal herb and are known as inhibitors of topoisomerase I (Irinotecan, Topotecan) or II (Etoposide, Teniposide) in clinical practice [2]. Although reports on drugs used in traditional Japanese medicine have appeared now and then, the clinical application of herbs in various fields of medicine has only been reported recently [3,4]. Fundamental studies on ingredients contained in herbs or their active form in the human body are few [5]. Herb-derived ingredients are present in a considerable percentage of anti-cancer medications. Thus, the isolation of new ingredients is essential. The enzyme telomerase has attracted attention as a chief candidate for the cancer specific antigen since about 1990. Telomerase extends telomeres located in both ends of chromosomes thereby stabilizing them [6]. Telomerase is a molecule that deviates cell aging and instructs malignant transformation [7]. The enzyme telomerase is a molecule that stabilizes chromosomal dynamics by elongating telomeres and is overexpressed in cancer cells. In addition, its activity is mainly controlled by the expression of the human telomerase reverse transcriptase (hTERT) gene and the existence of the RNA component (hTR) [8]. Most anti-cancer drugs generally affect cell proliferation of cancer cells and even normal cells, resulting in the induction of adverse effects in normal cells first. Since,
even if telomerase activity is inhibited, its effect on cancer cells is much stronger than that on normal cells. Thus, the development or discovery of effective telomerase inhibitors that can be applied to cancer cells is a potential pharmacological and translational strategy [9]. We presume that an anti-hTERT drug would show anti-cancer effects through a mild mechanism of action and that such an ingredient should be included in herbs. Here, we present novel evidence of anti-hTERT effects of aporphine alkaloids.

Methods

Herbal medicines
Active ingredients in traditional Japanese medicine are contained in some medicinal herb extracts. We screened 30 herbs containing at least one alkaloid and with tumor suppressive effect after testing their sensitivity. The selected herbs were Phellodendri Cortex, Magnoliae Flos, Magnolia Bark, Sinomenium Stem, and Coptidis Rhizoma. For this study, we were gratuitously provided all available alkaloids purified for research from Tsumura Corp. (Tsukuba, Ibaraki, Japan). We searched for an alkaloid component that is common among them from the herb database of the company.

Alkaloids
Since all five herbs contained T05827-Magnoflorine, we performed the sensitivity test using HLF cells [10]. Coptidis Rhizoma and Phellodendri Cortex were used as control for inducing tumor-suppressive phenotype as they also contain not only Magnoflorine but also Berberine, which has anti-proliferative effect against tumors [11-16]. We focused on the aporphine alkaloids with common molecular structure and performed sensitivity tests using 10 aporphine alkaloids ((+)-isocorydine.HCl, isocorydine, (+)-boldine, michepressine, dicentrine, glaucine, bulbocapnine.HCl, dehydroxy dihydrotaxilamine, apomorphine, and magnoflorine) [17,18]. Five alkaloids with anti-proliferative effect, namely, T05827-Magnoflorine, T30626-Dicentrine, T00858-(+)-Glaucine, L29150-Michepressine (Tsumura, Tokyo, Japan), and Apomorphine (Department of Environmental Engineering, Faculty of Agriculture, Tottori University, Japan) were used in a further study (Figure 1a) [19-21]. All reagents were prepared according to the manufacturer’s instructions. To reconfirm the result, we performed another cell proliferation assay using Quick Cell Proliferation Assay Kit (BioVision, Inc., Exton, PA, USA).

Real time quantitative RT–PCR
We examined the level of mRNA expression of genes, including hTERT. Real-time PCR with Light Cycler (Roche Diagnostics, Bagel, Switzerland) with OneStep RT-PCR kit (Qiagen, Tokyo, Japan) was performed using SYBR Green I (Roche Diagnostics, Bagel, Switzerland) to examine hTERT mRNA expression. The primer sets that were used for RT-PCR is sense primer [5′-CGG AAG AGT TGG AGC AA-3′] and anti-sense primer [5′-GGA TGA AGC GGA GTC TGG -3′] [22,23]. We estimated the relative expressive ratio three times, and compared it with β-actin mRNA expression.

Telomerase Assay
Telomerase activity per 2.5× 104 cells was assayed by the stretch PCR method with TeloChaser (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. Briefly, the cells were washed with PBS, pelleted by centrifugation, lysed, and centrifuged at 15,000 rpm for 20 min. The supernatants were subjected to telomerase reactions at 30° for 30 min. The products were denatured at 95° for 2 min 30 s, amplified by PCR (26 cycles of 95° 30 s, 68° 30 s, and 72° 45 s) using rTaq polymerase and anti-Taq high (Toyobo), electrophoresed in 10% polyacrylamide gels.
in TBE buffer for 60 min at 100 V, stained with 0.5 I g/ml ethidium bromide for 10 min, and quantified with an image analyzing system. An extract was considered to be telomerase positive if a DNA ladder of 6-bp periodicity was present and no corresponding signal was present in the heat-treated control except for a band of internal controls. The assay was performed in duplicate. The signal intensity and area were measured by a densitometer software (ATTO, Tokyo, Japan).

**Telomere length**

We measured the telomere length using TeloTAGGG Telomere Length Assay kit (Roche Diagnostics K.K, Basel, Switzerland), according to the manufacturer’s instruction. Mean terminal restriction fragment (TRF) length has been defined according to the following formula: mean TRF length = \( \frac{\sum(ODi)}{\sum(ODi/Li)} \). ODi is the chemiluminescent signal and Li is the length of the TRF at position i [24]. The calculation takes into account the higher signal intensity from larger TRFs due to multiple hybridizations of the telomere-specific hybridization probe.

**β-galactosidase staining**

We cultured alkaloid-treated cells on the chamber slide (Nalge Nunc International, Naperville, IL, USA), performed β-gal stain after fixing with 2% of paraformaldehyde and 0.2% of glutaraldehyde in PBS, in 4~5 weeks after chemical treatments, and examined the induction of alkaloid-induced senescence in them using a Senescence Detection kit (BioVision, CA, USA).

**FACS analysis**

We examined HLF cells to check whether Annexin V as an indicator in the early phase of apoptosis was upregulated using Guava EasyCyte Mini, according to the manufacturer’s instructions (GE Healthcare UK Ltd., Buckinghamshire, UK). FACS analysis of the HLF cells was performed using the same apparatus according to the manufacturer’s instructions.

**Caspase 3 activity assay**

Caspase 3 is a positive marker of apoptosis and one of the caspase enzymes with a role to attract a trigger of much protein cutting that leads to the orderly collapse of cells. The activity of caspase3/7 was measured using the Caspase-Glo3/7-assay (Promega, Madison, WI, USA). About 5000 cells were placed in each well of a 96-well plate and preincubated at 37°C for 24 h. The assay provides a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD, in a reagent optimized for caspase activity, luciferase activity and cell lysis. Addition of a single Caspase-Glo® 3/7 Reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate and generation of a “glow-type luminescent signal that is produced by luciferase. Signals of light emission were detected using a leader microplate and a wavelength of 485EX/527EM. Ac-Asp-Glu-Val-Asp-MCA [Ac-DEVD-MCA] (peptide Institute, Osaka, Japan) which is a caspase repressor was used as control.

**DNA Fragmentation Analysis**

DNA fragmentation in cells treated with each alkaloid was detected by polyacrylamide gel electrophoresis using Quick Apoptotic DNA Ladder Detection Kit (50 tests) (BioVision, Inc., Exton, PA, USA) according to the recommended procedure. In brief, 1 × 106 cells were lysed with TE lysis buffer and DNA was extracted. Ten micrograms of DNA were loaded on 15% polyacrylamide gel, run at 150 mA for 2 h in 1× TBE buffer (100 mM Tris/90 mM boric acid/1 mM EDTA, pH 8.4) with 0.5 μg/ml ethidium bromide, and photographed.

**Topoisomerase activity assay**

To study presence or absence of other actions than anti-hTERT effect, topoisomerase I and II activity in alkaloid-treated cancer cells were examined by a Topoisomerase I assay kit and Topoisomerase assay II kit (TopoGEN, Inc., Port Orange, FL, USA), according to the manufacturer’s instructions.

**Reverse transcriptase inhibitory assay**

Furthermore, to examine the inhibitory effect of alkaloids on reverse transcriptase activity, Lenti RT Activity kit (Cavidi Corp., Uppsala, Sweden) was used according to the manufacturer’s instructions.

**Statistical analysis**

Caspase 3 activity, percent of senescent cells, and the quantitative ratio in each group treated with alkaloid were analyzed in comparison with untreated cells, using Student's t-test. Probability value of P

**Results**

**Herbal medicines, alkaloids, and the derivatives**

We identified magnoflorine as an alkaloid ingredient commonly present in five herb extracts that induced growth suppression and telomerase repression in HLF cells. Because five of ten derivatives as aporphine alkaloids suppressed proliferation, we focused on their effects in telomerase repression, senescence induction, apoptosis induction, topoisomerase and reverse transcriptase inhibition, and cell cycle analysis in other cancer cells.

**Cell growth curve and MTT assay**

Generally anti-tumor effect emerges within a short term period in drug sensitivity testing and rapidly induces cell death in a cell cycle phase-specific or
non-specific manner. While any anti-telomerase effect takes a longer time such as one to three months than general anti-tumor drug to induce apoptotic phenotypes by way of proliferative suppression, senescence induction, and cell death. MTT assay and another cell proliferation assay showed no evidence of suppression of growth at any concentration of the alkaloids of interest. However, we observed that five alkaloids tended to suppress cell growth mildly at concentrations of 10 to 100 nM (data not shown), indicating the presence of other anti-tumor components in them. More than a month later of the treatment with alkaloids (magnoflorine, glaucine, and dicentrine) and 2 months later with michepressine, we observed growth inhibition of HLF cells (Figure 1b). From MTT analyses, we found that alkaloids could accumulate in HLF cells at an intracellular concentration of ~10 nM when used at an extracellular concentration of 10 nM. Accordingly, we used 10 nM as the treatment concentration in the long-term cultivation experiments that followed.

Real time quantitative RT–PCR
We examined hTERT mRNA expression in cells subjected to alkaloid-treatment and four alkaloids (magnoflorine, glaucine, michepressine, and dicentrine) and found that the expression was significantly suppressed throughout the treatment, compared with the untreated cells. Of the four alkaloids, dicentrine showed the strongest suppressive effect on HLF cells (Figure 4d) [17].

Telomerase Assay
As hTERT mRNA was suppressed, we attempted to confirm translational inhibition. Telomerase activity showed inhibition by 4 alkaloids in a couple of months. In addition, michepressine, glaucine, and dicentrine could not produce the 6-base ladder products at all. Magnoflorine-induced inhibition of expression corresponded to 1000 positive control cancer cells (HMcl-L7) (Figure 2b). In SUSM1, we observed the extremely faint 6-base ladder and the chemical treatment had no inhibitory effects on the signal strength.

Telomere (TRF) length
We measured mean TRF using cells that showed suppressive phenotype in growth. Compared with TRF of SUSM1, other cell lines showed a shorter TRF. TRFs in alkaloid-treated cancer cells were not necessarily shorter than those of untreated-cells. It depends on the combination of kind of chemicals and cells. For example, in HLF cells, hTERT downregulation of dicentrine did not clearly influence on telomere shortening (Figure 2c). In HLF, pk-45p, and EJ-1, TRF of cells was slightly shortened by the treatment by some chemical.

β-galactosidase staining
By SA-β-gal staining a month later than chemical treatment, approximately 60% of cells treated with dicentrine were senesced significantly. Other alkaloid-treated cells were induced to 20-30% of senescence, compared with untreated cells (P).

Annexin V and Cell cycle analysis
To elucidate the beginning of apoptotic induction, we checked the presence of Annexin V in HLF cells treated over a month with alkaloids, by flowcytometer. In particular, dicentrine induced the binding of annexin V to phosphatidylserine on the cell membrane. They showed mild induction in harvested cells (data not shown). FACS analysis showed the induction of more than half of the transfected populations into the pre-G1 phase of the cell cycle. As shown below, the increased apoptosis in cells treated with glaucine is shown as red circled areas. Analysis of DNA content in the lower areas revealed that cells produced early showed typical cell proliferation, cells produced later showed reduced cell proliferation as well as early signs of cell death (sub-G1) (Supplementary Figure 1).

Caspase 3 activity assay
Caspase 3 activity was significantly elevated in four-alkaloid treated HLF cells (michepressine, magnoflorine, glaucine, and dicentrine), compared with cells treated without chemicals or with Z-VAD (P).

DNA Fragmentation Analysis
Compared with untreated cells, DNA fragmentation was observed in some of the four alkaloids-treated cells. However, dicentrine and magnoflorine showed prominent smearing signals in 2 months rather than 1 month (Figure 4b). Necrosis dominantly influenced on this process and we could not fully observe the definite effect in the late phase of apoptosis.

Topoisomerase activity assay
In alkaloids-treated cells, glaucine showed topoisomerase I inhibition activity through the inhibition of DNA uncoiling. However, the inhibitory effect found within 2 weeks disappeared within a month of treatment (Figure 4c). Alkaloids did not induce inhibition of topoisomerase II in HLF cells (Supplementary Figure 2).

Reverse transcriptase inhibitory assay
Inhibitory effects of alkaloids on HIV-derived reverse transcriptase were examined at 30 minutes, 2 hrs, and 24 hours. Magnoflorine showed only potent action in a time-dependent manner rather than in a dose-dependent manner (Figure 4d) [17].

Examinations in other cancer cell lines
Sensitivity, hTERT mRNA expression, telomerase activity, caspase 3 activity, and DNA fragmentation of alkaloids differed to some extent in other cell lines and the result is summarized in Figure 5. In brief, the
anti-hTERT effect was significantly prominent in EJ-1, A172, SW480, pk-45p, and NIH-OVCAR3 (P

Discussion

It has been known that many herbs used in traditional Japanese medicine would contain an alkaloid. Unless the routine use in the medicine, alkaloids are often potent or induce or adverse reactions in the human body, indicating that extreme caution is required regarding the dose and the method of administration to avoid risks in long-term administration. Otherwise, we hypothesize that, due to the extremely wide selection that means ruling out medicine with strong toxicity to human body, anti-telomerase components that are thought to have a mild effect on normal cells should have dominated traditional Japanese medicine for a long time. Thus, we focused on selected components, presuming that some of them might reveal some specific anti-tumor effect, including unforeseen activities. Unlike glycosides, direct effects of alkaloids can be proved by drug sensitivity tests. However, heterogenous cell populations against drug sensitivity may emerge and this renders it difficult to estimate anti-tumor action. As such, we attempted to confirm sequences such as hTERT suppression – telomerase repression – growth change – senescence [25,26] – apoptosis [27-30]”. Vinorelbine ditartrate, Vincristine, Vindesine sulfate, Vinblastine sulfate, (VLB/VBL), Docetaxel, Paclitaxel, Etoposide (VP-16), and Irinotecan HCl (CPT-11) are clinically known as anti-cancer agents derived from plants such as natural herbs. Several telomerase inhibitors have been developed for clinical use in several facilities. Here, we report other evidences of new herbal ingredients that function as hTERT transcript inhibitors. These have potent and uniform suppressive effects on hTERT transcripts and, in general, induce to apoptosis in treated-cells without destroying the hemostasis of the telomere length [31]. In this study, SUSM1, which is immortalized without depending on hTERT expression by alternative lengthening of the telomere (ALT) mechanism [32], was never sensitive to these chemicals. ALT is utilized by approximately 10% of human tumors and a higher proportion of some types of sarcomas. Although it is well-known that ALT+ cell lines and many tumors show heterogeneous telomere length, SUSM1 that we used maintained a homogenous length. By using SUSM1, these chemicals made it possible to examine a specific effect on the hTERT molecule and they may be the appropriate materials to elucidate the ALT mechanism. Thus, we can conclude that sensitivity against alkaloids depends on the cell types and their anti-proliferative effect against ALT–cells is strong [33]. Some alkaloids have other anti-tumor effects in addition to their anti-hTERT effect [34]. Based on the cell cycle analysis, (+)-glaucine showed suppressive involvement in the growth, indicating the presence of the inhibitory effect towards topoisomerase I activity like irinotecan, supports our observation [35]. Since topoisomerase I or II inhibitors that has S/G2 phase- or G2/M phase-specific action has been already used for chemotherapy, they may be applicable clinically [36]. Only one magnoflorine showed mild anti-reverse transcriptase effect, suggesting that it may become a potent and preventive anti-virus ingredient. Recently, it has been tested for anti-inflammatory, antiradical, antioxidant, antinociceptive, antifeedant, and anti-hepatitis B virus activities [37-39]. Thus, alkaloids potentially have diverse actions but the main physiological and pharmacological role of the aporphine alkaloids that we examined may be basically their anti-hTERT effect [40]. In patients who have been treated with traditional Japanese medicine or natural herb-based medicine, we can not deny that unknown ingredients also act on the bodies. Both magnoflorine and dencitrine are present in certain plants and the former are adapted as a content of medicine in traditional Japanese medicine. We have not investigated longevity or the cause of death yet, regarding patients that have been on long-term treatment with Phellodendri Cortex, Magnoliae Flos, Magnolia Bark, Sinomenium Stem, and Coptidis Rhizoma. However, it is possible to say that patients may have unknowingly received preventive anti-tumor therapy at the effective plasma concentration of certain components when they administer 7.5g/day orally as a blended prescription for routine use. For example, Magnolia Bark, which contains more magnoflorine than other herb extracts and is used as a general anti-stress and anti-anxiety agent, contains 0.06±0.03%, corresponding to 321±161µg in 2.5g of Hangekoubokutou [41]. Effective plasma concentration is estimated as approximately at 10-100ng/ml, which corresponds to 3-30 fold of the concentration used for sensitivity testing. On the other hand, IC50 is more than 50µg/ml, resulting that other anti-tumor effects, except for anti-hTERT effect, mildly acts on the human body when traditional Japanese medicine is administrated [42]. The medicine may unconsciously have made the most of magnoflorine for therapeutic purposes as well as for preventive purposes. On the basis that dencitrine acted broadly on many types of tumor cells by suppressing their growth, it is the most effective chemical material [43].
Not only is it known to be a strong topoisomerase II inhibitor, but also to have anti-hTERT effect that is masked by other anti-tumor actions, because the IC50 is 27µM, which is equivalent to over 500 fold the concentration used in our study. Generally, it has been known that the inhibition of hTERT expression causes senescence due to shortened telomere lengths in p53-transcriptionally active cells. As shown representatively in Supplementary Figure 5, p53 transcript significantly upregulated in magnoflorine-treated HLF cells, indicating that the chemicals may function as a telomerase inhibitor. On the other hands, dicentrine and apomorphine significantly induced the downregulation of p53 mRNA. Although we can not clarify how they these cells are senescing because dicentrine treatment produces 60% senescent cells, we indicate the presence of p53-independent pathway to senescence. From the standpoint of the upregulation of p53 transcripts, magnoflorine contained in herbs would be the nearest candidate of anti-hTERT inhibitor. Further studies are needed to analyze the mechanism.

Conclusions

We found no evidences that some alkaloids, as an herbal medicine element, had adverse effects on telomerase negative cells. These alkaloids have commonly anti-hTERT effect although alkaloids are famous for the diverse actions including anti-tumor effect. We may recognize them as lead compounds to develop further effective anti-tumor drugs. As mentioned above, these chemicals are contained at the effective concentration for anti-hTERT action and are prescribed for the daily clinic as herbal medicine in East Asia. After performing the experiment in vivo, we proceed to develop derivatives that can be applied clinically as more effective novel anti-cancer drugs. Further persistent investigations into natural herbs, ingredients of herbs, and the derivatives are necessary to make a progress in this field.

Acknowledgements

This work was supported by grants from Japan Kampo Medical Research Institute and Japan Kampo Medicine Manufacturers Association (JKMA).

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

HK and NM, designed, performed and analyzed the research and drafted the manuscript after HM and ST gave a useful suggestion to NM. MS, WS, TT, RS, XW, ZW, ST, TH, and AM conceived of the study and helped this in vitro study as necessary. NM supervised the transfection experiments and helped to draft the manuscript. TI provided us with synthesis of apomorphine. NM and JH conducted the study as the principal investigators and contributed to the preparation of the manuscript. All authors read and approved the final manuscript.

References


Senescence induction (β-galactosidase staining) in SUSM1 cells is shown. Senescence cells (left) and proliferative cells (right) are shown. *means P

**Figure 4. Caspase 3 activity, apoptosis induction, and reverse transcriptase inhibition in alkaloids-treated cells.**

a. Caspase 3 activity assay in SUSM1 (right) and HLF cells (left) was shown as relative activity (RLU) to value in untreated cells. The dotted line is 300 RLU, which corresponds to caspase 3 activity detected in untreated cells. Z-VAD: pancaspase inhibitor. *= P

b. Necrosis and apoptosis induction in HLF cells is shown. 1M: a month later, after continuous treatment with alkaloids, 2M: two months later, after continuous treatment with alkaloids. The treatment with dicentrine, apomorphine, and magnoflorine for 2 months, caused the mixture of cell necrosis and DNA fragmentation significantly (marked by ?). Although SUSM1 might have slight necrosis due to the treatment in the best condition, chemicals did not have any effect on some or more necrosis or DNA fragmentation in treated cells.

c. Topoisomerase I activity in HLF cells is shown. In cells treated with glaucine for 11 days and dicentrine for 2 months, inhibition of DNA uncoiling was observed.

d. Reverse transcriptase inhibition in HLF cells is shown. Magnoflorine showed only time-dependent inhibition for the enzyme till 24 hours later after the beginning of the inhibitory reaction instructed in manufacture’s manual. 1-5, and 6 at the bottom of this figure represent Log substance concentration (1, 5, 25, 125, 625, and 3125 pg RT/ml). One pg corresponds to 4µU RT activity.

**Figure 5. Summary of immortalized cell lines treated with alkaloids.**

Summary of immortalized cell lines (HLF, EJ-1, A172, SW480, pk-45p, NIH-OVCAR3, PC-9, and SUSM1) treated with alkaloids is demonstrated. These broad actions are characteristics of alkaloids but they seem to have anti-hTERT effect in common against examined cell lines. Growth suppressive effect, caspase 3 activity, DNA fragmentation, and hTERTmRNA downregulation were representatively estimated. ++: P

**Additional files**

**Supplementary Figure 1.**

FACS analysis showed induction of more than half of the transfected populations into the preG1 phase of the cell cycle. DNA content analysis revealed in the lower parts, that cells produced early showed typical cell proliferation profile, cells produced later showed reduced cell proliferation, and early signs of cell death (sub-G1), in glaucine-treated cells. In the bottom part, the histogram with gating and the threshold were set appropriately for correctly stained cells in a clean machine. The large pink peak represents the green light signal from cells. The line segment flanking the peak is the gating band for cells. Annexin V tends to bind to phosphatidyl serine on the dicentrine-treated cell surface more than the untreated cell surface. Regarding glaucine, this figure demonstrates the possibility of apoptotic induction by other anti-tumor effect.

**Supplementary Figure 2.**

Topoisomerase I activity in HLF cells is shown. Inhibition of significant decatenated TOPO II product by dicentrine-treatment was very faintly observed. kDNA represents kinetoplast DNA (catenated) and dDNA corresponds to denatured DNA.

**Supplementary Figure 3.**

hTERT mRNA downregulation by alkaloids was different in other cell lines. Although these analyses were summarized in Figure 5, Compared with control cell line without chemical treatment, cell lines except for SUSM1 were basically induced significant suppression of hTERT transcript by chemical treatments. However, EJ-1, A172, SW480, and pk-45p were strongly suppressed in comparison with NIH-OVCAR3 and PC-9. *= P

**Supplementary Figure 4.**

The inhibition of caspase 3 activity by alkaloids was different in other cell lines. Although these analyses were summarized in Figure 5, Compared with control cell line without chemical treatment, cell lines except for SUSM1 were induced significant activation of Caspase 3 by more than an alkaloid. *= P

**Supplementary Figure 5.**

(a) The involvement of p53 transcript in senescence was representatively shown in HLF cells (N=3). Magnoflorine seems to induce senescence in p53-dependent manner because p53 mRNA was significantly upregulated compared with untreated cells. Both dicentrine and apomorphine significantly downregulated p53mRNA, suggesting that they may act in other telomerase-dependent pathway rather than in p53-dependent manner. *= P
Illustrations

Illustration 1

Molecular structure of 5 alkaloids and growth curves observed in alkaloids-treated cancer cell lines

Illustration 2

Gene expression, telomerase activity, and telomere length in alkaloids-treated cells
Illustration 3

Senescence induction, FACS analysis, and Topoisomerase I activity in alkaloids-treated cells.

Illustration 4

Caspase 3 activity, apoptosis induction, and reverse transcriptase inhibition in alkaloids-treated cells.
Illustration 5

Summary of immortalized cell lines treated with alkaloids.

<table>
<thead>
<tr>
<th></th>
<th>HLF</th>
<th>B-H</th>
<th>A172</th>
<th>SW480</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>M</td>
<td>D</td>
<td>G</td>
</tr>
<tr>
<td>Growth suppression</td>
<td>-</td>
<td>+</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Caspase 3 activity</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>UTP75pDNA overexpression</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

|    | C   | M   | D    | G    | A     | C   | M   | D    | G    | A     |
|----|-----|-----|------|------|-------|-----|-----|------|------|-------|-----|-----|------|------|-------|
|    | HLF | B-H | A172 | SW480 |       | HLF | B-H | A172 | SW480 |       | HLF | B-H | A172 | SW480 |       |
Disclaimer

This article has been downloaded from WebmedCentral. With our unique author driven post publication peer review, contents posted on this web portal do not undergo any prepublication peer or editorial review. It is completely the responsibility of the authors to ensure not only scientific and ethical standards of the manuscript but also its grammatical accuracy. Authors must ensure that they obtain all the necessary permissions before submitting any information that requires obtaining a consent or approval from a third party. Authors should also ensure not to submit any information which they do not have the copyright of or of which they have transferred the copyrights to a third party.

Contents on WebmedCentral are purely for biomedical researchers and scientists. They are not meant to cater to the needs of an individual patient. The web portal or any content(s) therein is neither designed to support, nor replace, the relationship that exists between a patient/site visitor and his/her physician. Your use of the WebmedCentral site and its contents is entirely at your own risk. We do not take any responsibility for any harm that you may suffer or inflict on a third person by following the contents of this website.