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The contribution of plukenetione A to the anti-tumoral activity of Cuban propolis

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ABSTRACT

Increasing efforts are directed toward finding applications for natural products and their derivatives in the treatment of human diseases. Among such products, propolis, a resinous substance produced by honey bees from various plant sources, has been found to be a promising source of potential therapeutics. In the present work, we aimed at studying the perspective of Cuban propolis as a source of possible anti-cancer agents. We found an anti-metastatic effect in mice and considerable cytotoxicity without cross-resistance in both wild-type and chemoresistant human tumor cell lines. Plukenetione A—identified for the first time in Cuban propolis—induced G0/G1 arrest and DNA fragmentation in colon carcinoma cells. Furthermore, the activities of both topoisomerase I and DNA polymerase were inhibited, while the expression of topoisomerase II-beta, EGF receptor, and multidrug resistance-related protein genes was found repressed. We assume that plukenetione A contributes to the anti-tumoral effect of Cuban propolis mainly by targeting topoisomerase I as well as DNA polymerase.

1. Introduction

From ancient times to our days, propolis has been widely used in folk medicine for the treatment of various illnesses. For this reason, increasing interest in this natural product as a source of pharmacologically active substances has been emerging in many scientific disciplines.[1,2] Propolis' chemical spectrum is very fluctuant and complex owing to its diverse geographical and ecological origins. This offers the opportunity to discover compounds as yet unknown which might prove valuable in pharmacognosy and later on in pharmacology.[3–5]

The behavior of the honeybees to search for plant exudations in order to elaborate propolis, which they use to protect their hives against macro- and micro-adversaries, is an extraordinary biologic characteristic that we should not pass over carelessly: the bees scan a particular floral ecosystem and collect resins which comprise compounds that are most efficient for this purpose.[6] Consequently, propolis species of various origins have been shown to be potent providers of substances with anti-oxidant, hepatoprotective, immunomodulatory, anti-microbial, anti-viral, anti-parasitic, and anti-tumoral activities.[7–15]

Numerous toxicological studies carried out in animal models revealed that propolis is generally well tolerated when applied via different administrations routes.[16] The activity of a particular propolis specimen should first be assayed in both animal models and cancer cell lines in order to determine its prospective use as source of new anti-cancer drugs.[17–19] The chemical fractionation of propolis is an obligatory task to detect possible novel anti-tumoral compounds present in these resins: given that propolis is a very complex mixture of substances, its anti-tumoral activity might be attributable either to a single substance, to synergistic effects of several compounds, or to potential metabolites.[18–23] On the other hand, poor activity might be attributable to antagonistic effects of its components or the absence or low concentration of active substances. Due to the fact that several propolis specimens have been described to exhibit immunomodulatory effects, it is also important to elucidate whether the anti-cancer activity is exerted through a direct (effect on tumor biology) or indirect mechanism, that is, stimulatory effects on the immunity of the tumor recipients.[24–27] Many works aimed at identifying anti-tumoral properties of compounds isolated from propolis were published in recent years.[28] So far, we have assayed the anti-cancer activity of more than 30 propolis species collected from different geographical regions. Therein, we were able to identify several compounds that show varying degrees of cytotoxicity in a panel of cancer cell lines.

Chemical studies and activity of Cuban propolis have been published recently.[29,30] Cuban propolis collected during spring from the western part of the island, specifically from the Havana province has shown anti-tumoral activity in vivo and in vitro. To date, we have identified three different compounds with cytotoxic prop-

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erties in this propolis, two of which—mucronulatol and nemorosone—were recently characterized by our group.[31,32] Thus, we have focused this work on the third compound, plukenetione A. It is an unusual polycyclic polyprenylated acylphloroglucinol firstly isolated from *Clusia plukenetii*[33], of which no pharmacologic evaluation has been published so far. Therefore, we are reporting for the first time both the discovery of plukenetione A in Cuban propolis and the anti-tumoral activity of this compound, which exerts its anti-cancer properties inhibiting both topoisomerase I and DNA polymerase.

2. Results and discussion

2.1. Cuban propolis displays cytotoxic and anti-metastatic effects

The first evidence of biological activity of Cuban propolis was its cytotoxicity in a well-characterized panel of human tumor cell lines comprising colon, ovarian, and prostate carcinomas as well as neuroblastoma. Sub-lines resistant to doxorubicin, etoposide, 5-FU and cisplatin generated in our laboratory were included. Additionally, we examined murine Ehrlich's tumor cells which were furthermore used in our anti-metastatic mouse model (Table 1). Propolis cytotoxicity was found to be in the range of 5–23 μg/ml, depending on the cells assayed, without any detectable cross-resistance (Table 2). This effect might be caused by some components in this propolis species that are able to inhibit MDR phenotypes directly, are no substrates for these resistance mechanisms [e.g., nemorosone[32]], or a combination of theses two effects.

In regard to propolis' cytotoxicity or its isolated bioactive constituents, it was observed that some components of propolis were more or less active in the isolated form compared with the whole substance, evidencing a multiple interaction (e.g., synergism, antagonism, or additive effect, a well-known phenomenon concerning drug interaction) between the wide propolis constituents.

Previous sub-chronic toxicological studies had confirmed that Cuban propolis is well tolerated in NMRI immunocompetent mice when administered per oral or intraperitoneally at concentrations of up to 300 mg/kg. Some mildly adverse—however reversible—side effects, such as diarrhea and orchitis in a male NMRI strain, were observed at the highest applied dose (data not shown). As depicted in Figure 1, the capability of propolis (100 mg/kg) of inhibiting metastatic colony formation in the lungs of an allograft model bearing Ehrlich's ascites tumor (EAT) was comparable to 5-FU (20 mg/kg, considered a curative dose in this tumor entity) (33.25% vs. 23.80%—with relation to the respective vehicles).

For some regional propolis specimens, immunomodulatory effects have been described. Such effects might help a cancer-stricken organism to fight tumor development to some degree.[27,40] However, since we have studied cytotoxicity in immunity-free cell cultures, we conclude that Cuban propolis does exert its anti-tumoral effect primarily via direct cytotoxic action rather than immunomodulation, the latter remaining to be elucidated in future studies.

An important finding related to the anti-tumoral and cytotoxic activity of Cuban propolis is the presence of mucronulatol as recently reported by our group.[31] This flavonoid has been identified in various plant extracts which displayed anti-tumoral activity in animal models.[41,42] Other anti-tumoral compounds present in this propolis species have also been described, among which the polyisoprenylated benzophenone nemorosone might represent the most prominent.[32,43] Since the HPLC chromatogram of Cuban prop-

Table 1
Cell lines and culture

Cell Lines	Histopathology	RF	Culture medium
HT29 WT	Colon adenocarcinoma wild-type	n/a	L-15
HT29 24R	5-FU-resistant	4	
HT29 SN38	SN38-resistant	4	
HCT8 WT	Ileocecal carcinoma wild-type	n/a	L-15
HCT8 SN38	SN38-resistant	7	
HCT8 ICID	Raltitrexed-resistant	3	
MCF-7 WT	Breast adenocarcinoma wild-type	n/a	L-15
MCF-7 AD	Doxorubicin-resistant (MDR1$^+$)	22	
MCF-7 24R	5-FU-resistant	7	
A2780 WT	Ovarian adenocarcinoma wild-type	n/a	RPMI
A2780 DX5	Doxorubicin-resistant (MDR1$^+$)	15	
A2780 CP2	Cisplatin-resistant	15	
M 51 WT	Stomach carcinoma wild-type	n/a	L-15
M51 DDP	Cisplatin-resistant	8	
LAN-1 WT	Neuroblastoma wild-type	n/a	McCoy's
LAN-1 ETO	Etoposide-resistant (MDR1$^+$)	128	
LAN-1 CP	Cisplatin-resistant	32	
LAN-1 5-FU	5-FU-resistant	64	
PC-3 WT	Prostate adenocarcinoma wild-type	n/a	DMEM
LNCap WT LNCap ETO	Prostate adenocarcinoma wild-type Etoposide-resistant MDR1$^+$	n/a 20	DMEM
Jurkat WT	Acute Lymphoblastic Leukemia (ALL)	n/a	DMEM
K-562 WT	Chronic myeloid leukemia (CML)	n/a	DMEM
H460 WT	Lung large cell carcinoma wild-type	n/a	L-15
HEK293WT	Human embryonic kidney cells	n/a	L-15
MCR-5	Embryonic lung diploid fibroblasts	n/a	L-15
3T3	Swiss Mouse fibroblasts	n/a	RPMI
Ehrlich's ascites tumor (EAT)	Mouse mamma adenocarcinoma	n/a	DMEM

Designation, histology, resistance factors (RF: IC_{50} resistant/ IC_{50} wild-type), and culture media of the cell lines employed in this work. n/a, not applicable.

Table 2
Cytotoxicity of active compounds present in Cuban propolis

Cell Lines	Propolis IC_{50} (μg/ml)	Mucronulatol IC_{50} (μg/ml)	Nemorosone IC_{50} (μg/ml)	Plukenetione A IC_{50} (μg/ml)
HT29 WT	5.8 ± 0.22	—	5.25 ± 0.43	12.03 ± 0.04
HT29 24R	7.0 ± 0.21	—	5.18 ± 0.76	14.23 ± 1.01
HT29 SN38	—	—	3.50 ± 1.40	13.33 ± 0.66
HCT8 WT	8.1 ± 0.21	2.6 ± 0.11	4.23 ± 0.19	12.90 ± 1.21
HCT8 SN38	—	—	4.07 ± 0.08	11.67 ± 0.17
HCT8 ICID	—	—	4.44 ± 0.13	12.10 ± 0.16
MCF-7 WT	—	2.52 ± 0.14	4.37 ± 0.12	16.27 ± 1.47
MCF-7 AD (MDR1$^+$)	—	>50	4.28 ± 0.08	14.97 ± 0.12
MCF-7 24R	—	—	3.30 ± 0.51	10.00 ± 0.08
A2780 WT	24.4 ± 0.95	—	9.20 ± 0.82	13.33 ± 1.33
A2780 DX5 (MDR1$^+$)	15.0 ± 0.12	—	6.75 ± 1.55	14.47 ± 1.23
A2780 CP2	23.2 ± 0.40	—	6.23 ± 0.12	15.90 ± 0.08
M 51 WT	—	—	5.70 ± 0.21	6.53 ± 0.28
M51 DDP	—	—	4.77 ± 0.13	7.60 ± 0.16
LAN-1 WT	—	10.10 ± 0.26	8.20 ± 0.28	—
LAN-1 ETO	—	>50	9.18 ± 0.22	—
LAN-1 CP	—	10.22 ± 0.21	8.44 ± 0.26	—
LAN-1 5-FU	—	8.22 ± 0.11	8.24 ± 0.31	—
PC-3 WT	10.0 ± 0.11	—	2.01 ± 0.07	—
LNCap WT	12.3 ± 0.11	—	2.12 ± 0.19	1.98 ± 0.74
LNCap ETO (MDR1$^+$)	11.1 ± 0.21	—	1.81 ± 0.68	1.72 ± 1.04
Jurkat WT	—	—	4.60 ± 0.08	5.28 ± 0.25
K-562 WT	—	—	4.20 ± 0.06	—
H460 WT	—	—	4.21 ± 0.91	13.43 ± 1.11
HEK293 WT	16.5 ± 0.12	2.5 ± 0.40	4.20 ± 0.28	32.1 ± 1.15
MCR-5	—	—	21.73 ± 6.47	15.70 ± 0.35
3T3	—	—	>50	11.97 ± 0.04
Ehrlich's ascites tumor (EAT)	12.1 ± 0.91	—	—	—

Overview of the in vitro cytotoxicity of Cuban propolis and isolated compounds in a panel of non-tumor and (wild-type and chemotherapy-refractory) tumor cell lines, as assessed by SRB and MTT assays. No cross-resistance was observed for whole propolis, nemorosone and plukenetione A; in contrast, mucronulatol did so in MDR1$^+$ systems. Fibroblasts were less sensitive to the compounds than the cancer cell lines. Values represent the means ± SD of at least three independent experiments. WT, wild-type.

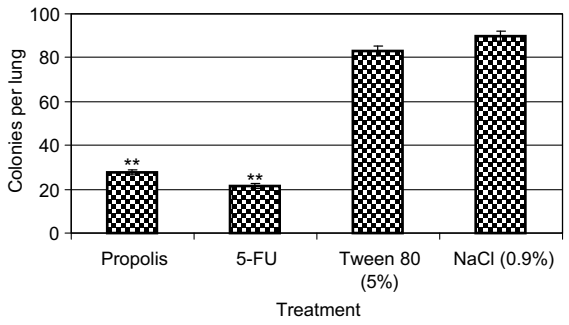

Figure 1. Anti-tumoral effect of Cuban propolis in vivo. Anti-metastatic effect of Cuban propolis in NMRI mice inoculated iv with 1×10^6 Ehrlich's ascites tumor cells in a volume of 0.1 ml and treated with 100 mg/kg of propolis, 20 mg/kg 5-FU, and the respective vehicles (Tween 80, 5% for propolis, NaCl, 0.9% for 5-FU) ip daily for nine days. Columns depict the mean ± SEM from three independent experiments. The comparison of means between the groups was performed by one-way analysis of variance using the Bonferroni post-hoc test corrections. A significant difference was found between the treatment with propolis dissolved in Tween 80 and the vehicle control Tween 80 (5%), $P < 0.001$, $n = 10$, difference of means = 55.4. Statistical comparison in the reference system 5-FU vs. NaCl (0.9%) revealed a significant difference $P < 0.001$, $n = 10$, difference of means = 68.5. **Highly significant.

olis revealed a great number of peaks (Fig. 2), our findings justified further screening of Cuban propolis for compounds that might contribute to its overall anti-cancer activity.

2.2. Chemical purification of the active compounds present in Cuban propolis

For this purpose, HPLC fractions were pooled into four areas (A, B, C, and D). We assayed the cytotoxic activity of each area in HCT8 wild-type colon carcinoma cells. In doing so, areas A, B, and C were found to be cytotoxic, while area D was ineffective. Applying successive purification procedures, we were ultimately able to isolate three compounds which were then subjected to studies on mechanisms of action.

The chemical structures of the active compounds were elucidated employing NMR and mass spectroscopy (data not shown), and identified using a chemical database. In area A, we found that mucronulatol, a heterocyclic compound of 302.3 Da was the most cytotoxic substance.[44] In area B, the polyisoprenylated benzophenone nemorosone (502.6 Da) was the major cytotoxic component present[43], while in area C, a polycyclic polyprenylated acylphloroglucinol of 500.6 Da, plukenetione A (1-benzoyl-8,8-dimethyl-3,5-bis(3-methyl-2-butenyl)-6-(2-methylpropenyl)tricyclo[3.3.1.1^{3,7}]-decane-2,4,9-trione) represented the most active compound (Fig. 2).[33]

Since mucronulatol and nemorosone have already been studied by our group[31,32], we focused our further investigations on plukenetione A also described in the scientific literature by other groups.[33]

2.3. Plukenetione A, an unusual adamantyl ketone with cytotoxic activity

Various metabolites from the Guttiferae family, many of which are biologically active compounds with an acylphloroglucinol moiety, were described in the past. Nevertheless, the biological or pharmacological activities of the plukenetiones are poorly studied so far.

In the very apolar area C of fractionated Cuban propolis (Fig. 2), we identified plukenetione A. This compound showed considerable cytotoxicity (IC_{50} values between 1.7 and 16.3 μg/ml) in a panel of human tumor cell lines (solid and hematological entities). No

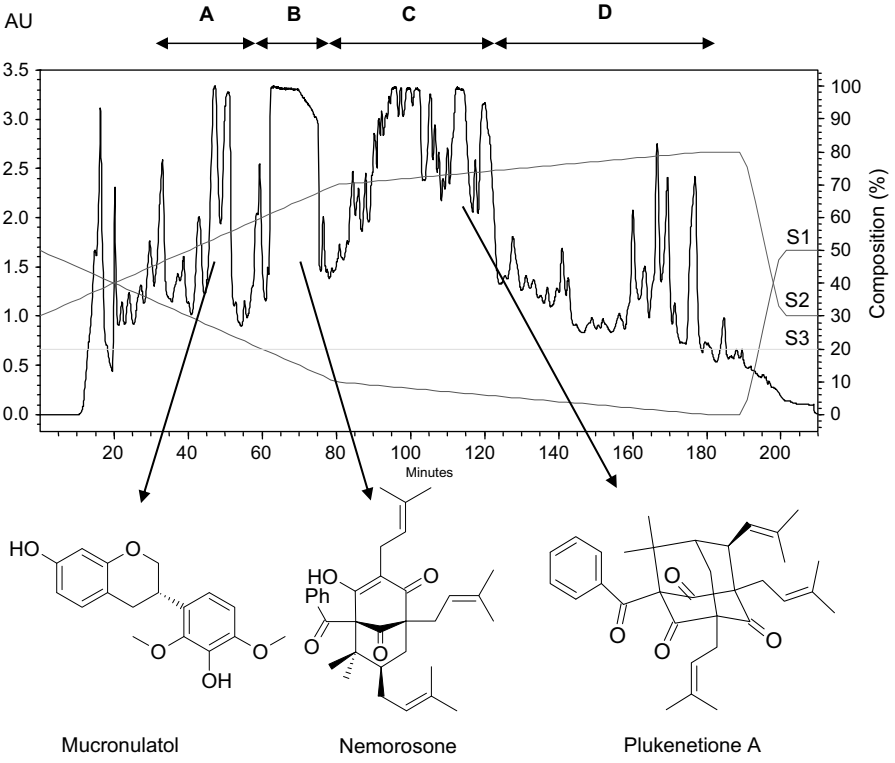

Figure 2. Chromatogram and active compounds present in Cuban propolis. A representative chromatogram of whole Cuban propolis extracted at 254 nm after injection of 150 mg of extract. The chromatogram reveals a considerable chemical complexity. After successive purification steps, we identified three substances cytotoxic in HCT8 colon carcinoma cells: mucronulatol, nemorosone, and plukenetione A. Areas A, B, C, and D are designated. Solvent 1 (S1), ammonium formiate; S2, methanol; S3, acetonitrile; AU, absorption units.

cross-resistance in chemotherapy-refractory sub-lines could be detected. Fibroblast control cell lines were equally sensitive, while non-tumorigenic HEK293 cells tolerated more than 30 μg/ml (Table 2). We conclude that plukenetione A is not a substrate for the elements involved in the mechanisms of resistance for adriamycin, cisplatin, etoposide, SN38, raltitrexed, and 5FU.

2.4. Influence on the cell cycle progression

To get a first hint about the mechanisms responsible for the anti-tumoral effect of plukenetione A, cell cycle analyses were performed in HCT8 wild-type colon carcinoma cells. Cytometric values revealed that plukenetione A enforced a G0/G1 arrest (37.4% control vs. 52.1% treated) accompanied with a considerable depletion in S-phase (14.7% control vs. 4.3% treated) in this cell line after the incubation with 1× IC_{50} (Fig. 3).

2.5. Plukenetione A induces DNA damage in colon carcinoma cells

Since several compounds leading to cell cycle arrest subsequently induce DNA fragmentation that is a hallmark of late apoptosis, the DNA integrity was examined after exposing HCT8 wild-type cells to increasing concentrations of plukenetione A for 8 h. Semi-quantitative measurement of radioactive DNA extracted from [^{14}C]-D-thymidine labeled cells exposed to this compound revealed DNA damage at 2.5× the IC_{50} in this cell line, which increased further at higher concentrations, as depicted in Figure 4.

2.6. Effects of plukenetione A on replicative enzymes

In order to identify possible mechanisms of action which might account for the disruption of the cell cycle in the cell system analyzed, studies on the activity of key enzymes implicated in cell replication were performed.

Cell cycle arrest, inhibition of the S-phase and DNA damage is inducible, amongst others, via inhibition of the topoisomerases, which upon formation of a putative cleavage complex with the DNA triggers its degradation.[45] Plukenetione A completely inhibited the unwinding activity of topoisomerase I from HCT8 nuclear extracts at 12.5 μg/ml, which represents 1× IC_{50} in this cell line (Fig. 5A). This finding clearly supports the classification of this compound as a topoisomerase inhibitor and could also explain the S-phase depletion observed in the cells.

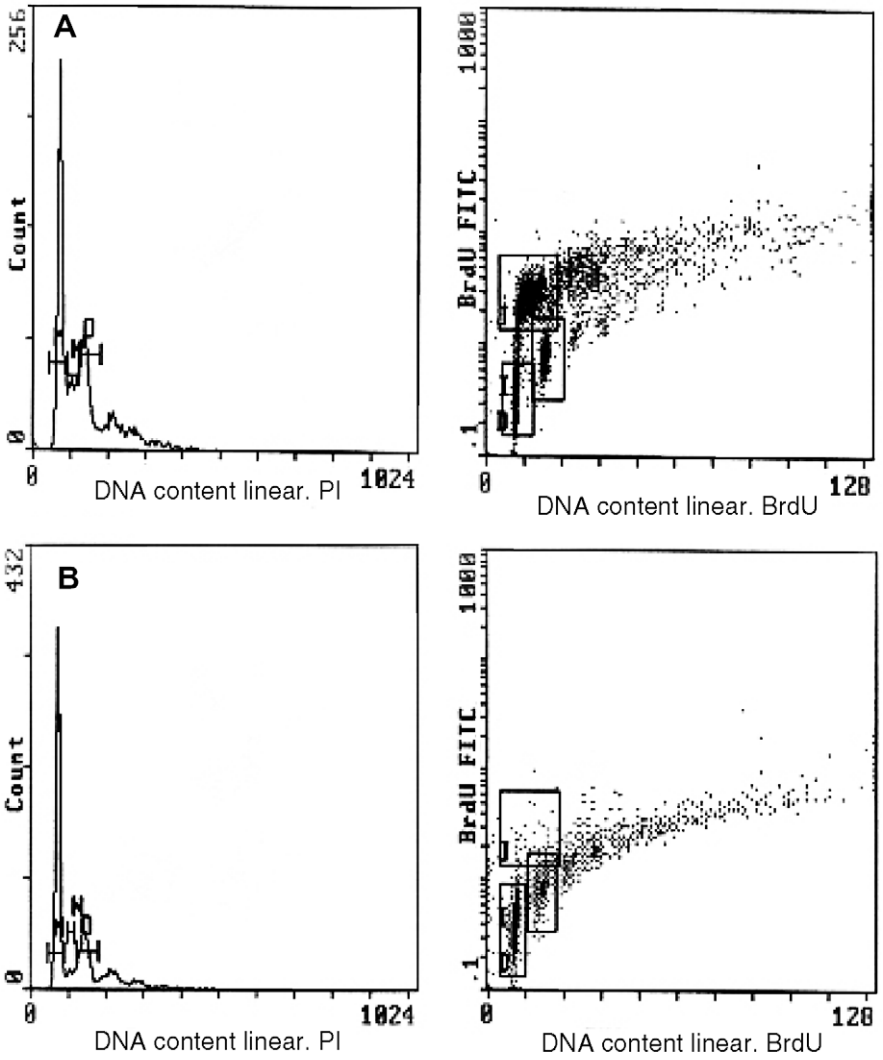

Figure 3. Influence of plukenetione A on cell cycle regulation in HCT8. A, untreated control; B, cells treated with 1× IC_{50}. Cell cycle distribution was measured after 24 h of exposure. Cytometric analyses were performed using PI staining and BrdU incorporation. In the control, 34.4% of the cells were in G0/G1 (M&D), 14.7% in S (N&J), and 21.5% in G2/M (O&I), while 52.1% of the treated cells were in G0/G1, 4.3% were in S, and 20.6% were in G2/M. The treated cells showed an increase in G0/G1-phase as well as a depletion of those in S-phase. The results depicted are representative for three independent experiments. BrdU FITC, bromo-deoxyuridine fluorescein isothiocyanate.

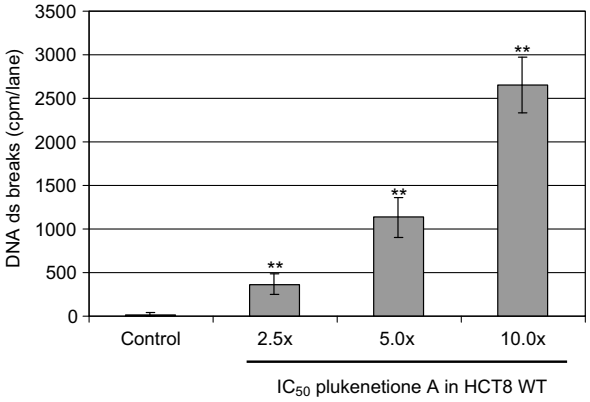

Figure 4. Apoptotic DNA double-strand breaks induced by plukenetione A in HCT8. Semi-quantitative DNA double-strand break analysis in [^{14}C]-D-Thy-labeled HCT8 WT cells after exposure to increasing concentrations of plukenetione A for 8 h. Maximum DNA fragmentation was observable after incubation with 10× IC_{50} of the drug in this cell line. Lane 1, control; lanes 2–4, cells incubated with plukenetione A at concentrations of 2.5× to 10× IC_{50}. The results depicted are represented as mean ± SD. Significant differences could be detected in all cases, $P < 0.001$, $n = 3$. ds, double-strand; **highly significant.

In the last decade, topoisomerase inhibitors were widely investigated by both the academia and the pharmaceutical industry. Some compounds with such properties, originally isolated from natural sources (camptothecine, topotecan, etc.), were successfully introduced into clinical practice as anti-cancer agents.[46] Thus, more attention should be paid to plukenetione A as a topoisomerase inhibitor, subjecting it to more detailed studies.

On the other hand, since a depletion of S-phase cells could result from the inhibition of DNA polymerase as well, the potential inhibition of this enzyme by plukenetione A was also taken into consideration. The compound efficiently inhibited DNA synthesis in vitro at concentrations of 6.25 μg/ml, which is well below the IC_{50} values in the majority of the cell lines studied (Fig. 5B). Critically, regardless of the highly conserved homology between polymerases from both eukaryotes and prokaryotes and their similar biological activity, the system employed to address this issue was biologically discordant with the replication events occurring in the cancer cells, as Taq polymerase is obtained from a prokaryote with life conditions very different from mammals. Therefore, it will be necessary to perform further experiments in order to define plukenetione A also as a mammal DNA polymerase inhibitor and delimit the isoforms that are most sensitive to this compound.

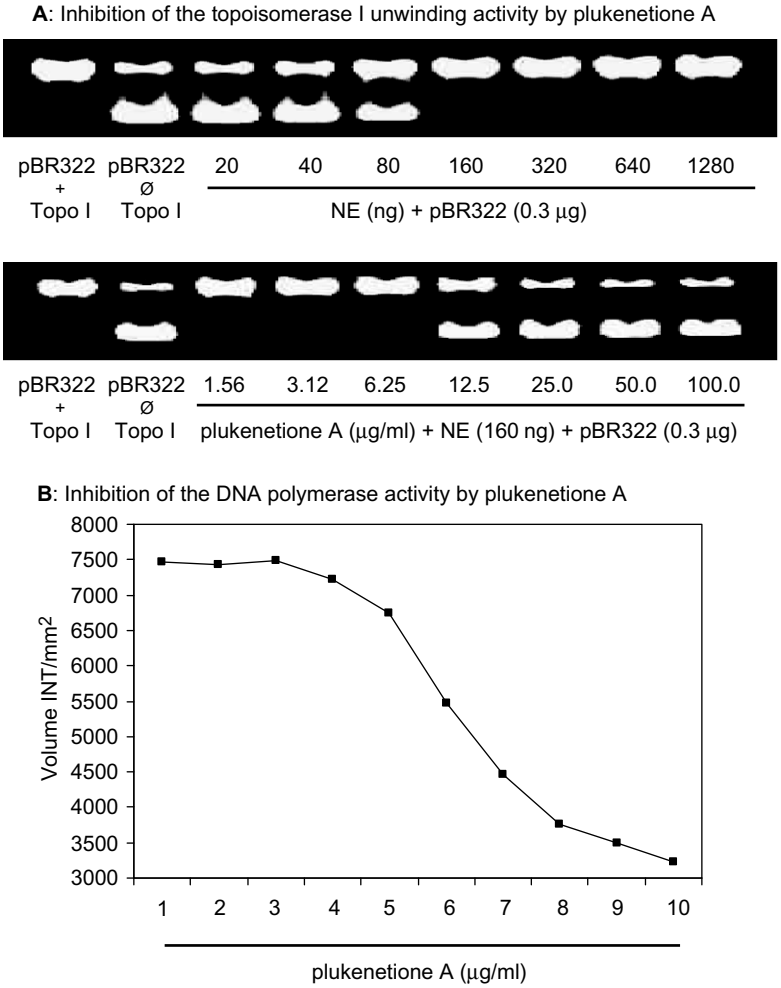

Figure 5. In vitro inhibition of replicative enzymes by plukenetione (A). A, upper: determination of nuclear extract amount necessary for the complete relaxation of the supercoiled pBR322 DNA plasmid (160 ng). A, lower: Inhibition of topoisomerase I unwinding activity on pBR322. Total relaxation was observable at up to 6.25 μg/ml and complete inhibition of topoisomerase I activity at 12.5 μg/ml, which represents approx. 1× IC_{50} in this cell line. (B) Inhibition of Taq DNA polymerase. The IC_{50} for its enzymatic activity was found to be 6.25 μg/ml. NE, nuclear extracts from HCT8 wild-type cells. The curve represents the mean of the light intensity of the GADPH PCR products in an invariable area as determined by Gel Doc Software (Volume INT mm^2). The results depicted are representative for three independent experiments.

Unfortunately, few works have been published in recent years describing the effectiveness of DNA polymerase inhibitors as anti-cancer agents.[47] The works focused on identifying novel DNA polymerase inhibitors were drastically reduced in the last years, while the emphasis is placed increasingly on reverse transcriptases inhibitors.

Interestingly, we found that plukenetione A inhibits both M-MuLV (Moloney Murine Leukemia Virus) and HIV reverse transcriptase in vitro, which are involved in the polymerization of nucleotides (enzymatic inhibition IC_{50} = 1.5 μg/ml, data not shown). Since it is well established that some T-cell leukemias are caused by the retroviruses HTLV I/II (human T-cell lymphotropic viruses), the inhibition of the aforementioned enzymes by plukenetione A might prove valuable in oncology as well. Furthermore, given that mammalian telomerase is a reverse transcriptase and frequently found activated in cancer, plukenetione A should be evaluated as a possible telomerase inhibitor as well.

2.7. Plukenetione A alters the gene expression in colon carcinoma cells

It is expected that a consequent alteration in the gene expression patterns might occur as result of the inhibition of DNA synthesis. The expression of some genes relevant for the molecular biology of the cancer cells was qualitatively analyzed by means of RT-PCR after exposing HCT8 wild-type cells to 2× IC_{50} of plukenetione A.

Expression of topoisomerase I, thymidylate synthase, housekeeping genes like GAPDH and β-actin did not show observable changes after the incubation of HCT8 cells with plukenetione A as depicted in Figure 6, while the genes which code for topoisomerase IIβ, EGR-R, and MRP appeared to be repressed. The unaltered gene expression of topoisomerase I is probably irrelevant, since we assume that its enzymatic activity is efficiently inhibited in the living cell. Interestingly, topoisomerase IIβ gene expression was completely repressed by this compound. This effect is also expected to support the G0/G1 arrest, according to the fact that topoisomerase IIβ protein levels are increased in this phase. On the other hand, the repression of EGF receptor gene expression by this drug might be useful in the treatment of tumors aberrantly expressing EGF-R which have deregulated malignant signal transduction pathways.

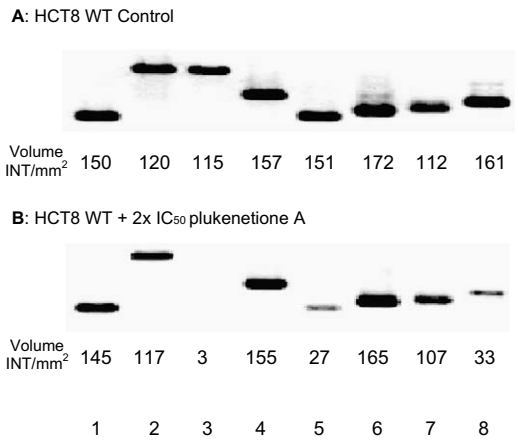

Figure 6. Influence of plukenetione A on gene expression in HCT8. Gene expression patterns in HCT8 wild-type cells untreated (A) and after the exposure to 2× IC_{50} of plukenetione for 24 h (B), analyzed by RT-PCR. Lane 1, topoisomerase I; lane 2, topoisomerase II alpha; lane 3, topoisomerase II beta; lane 4, GAPDH; lane 5, EGF receptor; lane 6, β-actin, lane 7, thymidylate synthase (TS); lane 8, multidrug resistance-related protein (MRP). Volume INT/mm^2 represents the means of the light intensities in an invariable area as determined using the Gel Doc Software. The results depicted are representative for three independent experiments.

HCT8 wild-type cells display constitutive MRP gene expression, probably because this cell line is derived from a tissue that is highly exposed to xenobiotics in the living organism. This gene is considerably down-regulated by the influence of plukenetione A. This could account for the lack of cross-resistance found in chemotherapy-refractory cell lines. Nevertheless, an in-depth study involving other MDR mechanisms must be carried out in order to corroborate this hypothesis.

In conclusion, considering the wide cytotoxicity profile and the lack of cross-resistance with cytostatics well established in clinical practice, plukenetione A appears to be a compound with promising therapeutic activities. We suggest that this substance exerts its cytotoxicity mainly by inhibiting replicative enzymes like topoisomerase I and probably DNA polymerase. It remains to be shown, though, how this inhibition is conferred in detail, for example, whether plukenetione A interacts directly with the enzymes, with the nucleotides, or intercalates with DNA, obstructing enzymatic activity leading to cell cycle disruption and/or DNA damage. Finally, the development of a chemical synthesis of plukenetione A is absolutely mandatory, because it's presence in propolis or other natural sources is extremely limited, forestalling more in-depth experiments. Hence, only chemical synthesis might provide the material necessary for a full pharmacological evaluation as anti-cancer and/or anti-viral drug, as well as for the production of derivatives that may further enhance its biological activity.

3. Conclusion

Cuban propolis displayed an anti-metastatic effect in EAT-bearing mice comparable to cytostatics employed in the clinical treatment of malignant diseases. It showed a complex chemical profile including two compounds with anti-tumoral activity that has been published previously by our group. Another cytotoxic substance, plukenetione A, was found in this source and was characterized from a pharmacological point of view for the first time.

The first report on plukenetione A was published in 1996 in a phytochemical study by Henry et al.[33] Here, we describe its isolation and for the first time its anti-cancer properties, elucidating a potential mechanism of action and possible targets.

Plukenetione A proved to be cytotoxic in a panel of cell lines from various cancer entities. Interestingly, this compound did not display cross-resistance in sub-lines expressing MDR1 phenotype. It efficiently inhibited the enzymatic activity of both topoisomerase I and DNA polymerase, suggesting them to be major physiological targets. As was revealed in cell cycle studies, plukenetione A enforced S-phase depletion and G0/G1 arrest. The aforementioned inhibitory effects were sufficient to induce drastic changes in the cellular biology, for example, DNA damage and the alterations in gene expression patterns of some genes relevant for cell replication and metabolism.

The properties of plukenetione A as an inhibitor of replicative enzymes seem to be promising for the application in cancer chemotherapy. Therefore, it is necessary to develop rational synthesis pathways for plukenetione A and derivatives to allow for more extended pharmacological studies.

4. Experimental

4.1. Cell lines and determination of cytotoxicity

All cell lines reflected in Table 1 were obtained from the tumor/cell line collection of the Medical School of the University Duisburg-Essen. All cells, comprising wild-type as well as chemotherapy-refractory sub-lines, were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Histology, resistance

factors (RF), and culture media with the appropriate standard supplements are listed in Table 1. The cytotoxicity was measured applying two different proliferation assays, the SRB (Sulforhodamine B, Sigma–Aldrich, Munich, Germany) for the adherent and the MTT [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide] (Sigma–Aldrich, Munich, Germany) for the suspension cell lines as described previously.[34,35] Cytotoxicity was analyzed after 24 h of exposition to concentrations of the respective compound ranging from 0.19 to 50 μg/ml. IC_{50} values were interpolated from semi-logarithmic dose–response plots and were reported as the mean and SDM of three independent experiments.

4.2. Generation of cell lines resistant to chemotherapeutic agents

Acquired resistance to cytostatics was developed in wild-type HT29, HCT8, MCF-7, M51, LAN-1, and LANCap cell lines by exposing cell cultures to increasing drug concentrations. Briefly, IC_{50} values for adriamycin, cisplatin, etoposide, SN38, raltitrexed, and 5FU were determined in these cell lines at first. Exponentially, growing cells were then exposed to 2× IC_{50} for 24 h. For recovery, cells were washed and incubated with drug-free culture medium until new colonies formed. This procedure was repeated several times, each time doubling the original IC_{50} up until 64× IC_{50} was reached. The surviving cells were subjected to a resistance selection by incubation with increasing concentrations of the respective drugs (4× to 512× IC_{50}) for 24 h. Cells which proliferated at higher drug concentrations within one week were considered chemotherapy-refractory. These resistant colonies were maintained in continuous presence of the respective drug at 10× the original IC_{50}. Thereafter, the resistance factor was determined as new IC_{50}/original IC_{50}. The expression of multi-drug resistance protein (MDR)1 was measured by fluorescence activated cell sorting (FACS) using anti-MDR1 monoclonal antibody (Novus Biologicals, Littleton, CO, USA) (Table 1).

4.3. Animals and experimental metastasis model

Specific pathogen-free female NMRI immunocompetent mice of 8–10 weeks were housed in semi-sterile cages, applying a regime of 12 h of light and 12 h of darkness with water and food supply ad libitum.

Mouse mammary carcinoma Ehrlich's ascites tumor (EAT) cells were washed in PBS twice and adjusted to 1×10^6 per 0.1 ml. One hundred microliters of cell suspension were inoculated into the lateral tail vein, and afterwards mice were randomized and split into three treatment groups each including 10 animals. Twenty-four hours after tumor cell injection, 100 mg/kg of propolis (dissolved in 5% Tween 80 purchased from Sigma–Aldrich, Munich, Germany), 20 mg/kg of 5-FU (Medac, Hamburg, Germany) and the respective vehicles were administered ip in daily doses for nine days. On day ten, animals were narcotized with isofluorane and sacrificed by cervical dislocation. Lungs were extracted and fixed with Bouin's solution for 24 h. Metastases on lungs were determined by counting the superficial colonies under a dissecting microscope. All experiments were carried out according to the institutional and legal ethical guidelines on animal care.

4.4. Chemical fractionation of propolis and structure elucidation

Ethanolic extracts (95%) of brown propolis collected in the Havana province, Cuba, during spring were fractionated by RP-HPLC, employing a Waters Separation Module Alliance 2690 HPLC system connected to a Waters 996 PDA detector, both controlled by the EmpowerTM2 software (Waters GmbH, Eschborn, Germany). One hundred milligrams were injected into a 250/21 mm preparative column packed with Nucleosil 100-7 C18 (Macherey–Nagel, Dueren, Germany) at a controlled temperature of 40 °C. Fractionation was performed applying a gradient system starting with a mixture of 0.01 M ammonium formiate pH 7.00:methanol:acetonitrile [50:30:20 (v/v/v)]. The composition of this mixture changed linearly within 210 min to a methanol:acetonitrile ratio of 80:20 (v/v) at a flow rate of 4 ml/min. The spectrum arrays were measured in the range from 200 to 400 nm and extracted at 254 nm for subsequent chromatographic analysis. Since the chromatogram revealed a high chemical complexity of this propolis species, we subdivided the fractions obtained into four main areas named A (fractions 30–58 min), B (60–80 min), C (82–140 min), and D (142–180 min), based on the respective cytotoxicity in HCT8 wild-type cells, and focused our further studies on area C. For the purification of the active component(s) in this area, an isocratic system consisting of ammonium formiate and methanol [15:85 (v/v)] was employed. The purity of the isolated compound was analyzed employing a Symmetry C18 column of 150 × 2.1 mm (Waters GmbH, Eschborn, Germany) under the same chromatographic conditions as described above at a flow rate of 0.5 ml/min.

The chemical structure of the active compounds was determined employing NMR and mass spectroscopy (data not shown). The modeling of the structures was performed using ACD/Chem Freeware software.

4.5. Cell cycle analysis

Cell cycle analyses were performed employing fluorescence activated cell sorting (FACS), combining propidium iodide (PI, Sigma–Aldrich, Munich, Germany) staining and 5-bromo-2′-deoxyuridine (BrdU, Sigma–Aldrich, Munich, Germany) incorporation as described by Tsugita et al.[36] 1×10^6 HCT8 wild-type cells in exponential growth phase were incubated with 1× IC_{50} of plukenetione A for 24 h. Anti-BrdU FITC-conjugated monoclonal antibody was purchased from Pharmingen, Heidelberg, Germany. BrdU incorporation and DNA content were measured using an EPICS XL flow cytometer (Beckman–Coulter, Krefeld, Germany) at an excitation λ = 488 nm.

4.6. DNA damage analysis

Semi-quantitative analyses to detect DNA damage were performed in HCT8 wild-type colon carcinoma cells. Exponentially growing cells were seeded at a density of 2.5×10^6 cells/125cm^2-flask. After allowing 24 h for anchorage, cells were labeled with 1 μCi/ml [^{14}C]-D-thymidine (Sigma–Aldrich, Munich, Germany) for an additional 24 h. Cultures were afterwards exposed to concentrations from 2.5× to 10× the respective IC_{50} of plukenetione A for 8 h. Cells were washed and incubated in drug-free medium for another 24 h. Subsequently, DNA isolation was performed according to the method of Chiao.[37] 50 μg of DNA of each sample were separated on a 1.8% agarose gel for 15 h at 1.2 V/cm in 0.5× TBE buffer. The gel was then stained with 0.5 μg/ml ethidium bromide for 20 min and destained for the same time in distilled water. DNA laddering was visualized using a transluminator, and the fragmentation lanes were cut out, melted in 2 ml of 1 N HCL at 50 °C and dissolved in 10 ml of liquid scintillation cocktail (Packard, Meriden, CT, USA). Radioactivity was counted on a TPICARB 2100RT scintillation counter (Packard, Meriden, CT, USA) and reported as cpm (counts per minute).

4.7. Topoisomerase I unwinding assay

To evaluate the effect of plukenetione A on the activity of topoisomerase I as reflected by the topologic changes of the plasmid

pBR322 *in vitro*, the Topo I unwinding assay described by Trask et al. was performed.[38] The required nuclear extracts were prepared from HCT8 wild-type cells according to the method of Sullivan as modified by Danks et al.[39] To determine the amount of nuclear extract that induced a total relaxation of pBR322, a precedent experiment was performed: 5 μl of 4× Reaction Mix (200 mM Tris–HCl, pH 7.5, 400 mM KCl, 40 mM $MgCl_2$, 2 mM EDTA, pH 8.0, and 120 μg/ml BSA), 5 μl of substrate (pBR322 0.06 μg/μl, Roche, Manheim, Germany), 5 μl of DEPC-treated water, and 5 μl of nuclear extracts at final concentrations from 20 to 1280 ng were mixed and placed on ice. After incubation at 37 °C for 30 min, the reaction was terminated by adding 2.5 μl of stop solution (0.5% SDS, 0.15 mg/ml proteinase K) and finally supplemented with 5 μl of running solution (0.1% bromophenol blue, 50% glycerol). The products were separated using pulsed field gel electrophoresis (pulse 0.1 s, angle 90°, 1.5 V/cm) on a 1.0% agarose gel in TBE buffer at 14 °C for 17 h. Gels were stained with 0.5 μg/ml ethidium bromide and documented using a BioRad Densitometer Gel Doc 2000 (BioRad, Munich, Germany). Once the optimal quantity of nuclear extract for the total relaxation of 0.3 μg of pBR322 was determined (160 ng), the same reactions were performed as described employing 160 ng of nuclear extract and plukenetione A at concentrations from 1.5 to 100 μg/ml diluted in water.

4.8. Gene expression analysis performed by RT-PCR

The amplification of cDNA fragments of genes of interest was carried out by reverse transcription, coupled with polymerase chain reaction (RT-PCR). Total RNA was extracted using Trizol® reagent (Invitrogen, Karlsruhe, Germany) from 1×10^7 HCT8 wild-type cells both untreated and previously incubated with 2× the IC_{50} of plukenetione A for 24 h, respectively. RT-PCR was performed using the GeneAmp® RNA PCR Core Kit purchased from Applied Biosystems (Foster City, CA, USA) in a GeneAmp System 2400 thermocycler (Perkin-Elmer, Waltham, MA, USA). One microgram of total RNA was employed for the synthesis of cDNA. The amplification of gene fragments was carried out with appropriately designed primers using the following NCBI Reference Sequence Data: topoisomerase I, ID: 19913404; topoisomerase II alpha, ID: 19913405; topoisomerase II beta, ID: 19913407; GADPH, ID: 83641890; EGF-R, ID: 41327737; β-actin, ID: 603381; thymidylate synthase, ID: 4507750, and MRP1, ID: 9955961. PCR conditions were denaturation at 94 °C for 50 s, annealing at 56 °C for 60 s, and extension at 72 °C for 90 s, over 30 cycles. The PCR products were resolved by agarose gel electrophoresis (1.8%) and visualized by ethidium bromide staining using a BioRad Densitometer Gel Doc 2000 system (BioRad, Munich, Germany).

4.9. Measurement of the DNA polymerase inhibition

The prospective inhibition of DNA polymerase by plukenetione A was measured performing a standard PCR carried out as described above. After the reverse transcription of total RNA, increasing concentrations of plukenetione A (0.1 to 25.6 μg/ml) were incubated with the PCR amplification of the cDNA for the GAPDH gene. The PCR products were resolved by agarose gel electrophoresis (1.8%), visualized by ethidium bromide staining, and the intensity was measured employing a BioRad Densitometer Gel Doc 2000 system (BioRad, Munich, Germany).

4.10. Statistical analysis

All data are given as means ± standard error of means (SEM). The comparison of means between the groups was performed by one-way analysis of variance (ANOVA) using the Bonferroni post-hoc test corrections. Statistical significance was accepted when $P < 0.05$. The results of FACS, RT-PCR for gene expression, topoisomerase, and DNA polymerase activities were descriptive and therefore not analyzed statistically.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2008.10.019.

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