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Cytotoxic effects of alkaloids on cervical carcinoma cell lines: a review

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ABSTRACT

Cervical cancer is the fourth type of women neoplasia, with thousands of new cases annually. It is closely related to human papillomavirus (HPV) infection, which has more than 13 oncogenic types, among them HPV 16 and 18 are implicated in 70% of cervical carcinoma cases. Alkaloids are nitrogenated and naturally occurring compounds, showing several uses in medical treatment, including cytotoxic and antineoplastic activities. In this work we aim to evaluate the cytotoxic and chemotherapeutic potential of alkaloids against cervical cancer. In order to accomplish this purpose, we have made a survey of potentially effective alkaloids with cytotoxic activities over HPV-16+ and HPV-18$^+$ cells (HeLa cells). Through a literature review between the years of 1980 and 2015, we described the major alkaloid sources, distribution in nature and also discussed the mechanisms of action for their cytotoxicity. We found that alkaloids showed efficacy as cytotoxic agents, inhibiting cell growth of the HPV-transformed cells in vitro and in vivo by means of activation of intrinsic and extrinsic pathways of apoptosis, which included the clivage of caspases and PARP-1 (Poli-Adenosyl-Ribose Protease 1), increase in p53 expression, release of cytochrome C and increase of cell death receptors expression like Fas, mainly observed in HeLa (HPV-18$^+$) cell lines. Moreover, these secondary metabolites helped in modulating the MDR (Multi-Drug Resistance) against the cell lines studied, which lead us to suggest their possible use as chemotherapeutic agents on the lesions caused by these viruses

Keywords: Cervical cancer. Alkaloids. HPV. Chemotherapy.

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INTRODUCTION

Cervical cancer is closely related to HPV (Human Papillomavirus), based on epidemiologic, virological and experimental evidences. HPV are viruses capable of infecting the skin and mucous membranes, with more than 150 of different types, between them 40 can infect the anogenital region (NCA, 2013).

Around 291 millions of women worldwide are infected with HPV, being 32% with HPV 16 and 18 or both. Considering an annual incidence of about 500 thousand cases of uterine cervix carcinomas, we can see that cancer is a rare outcome, even in the presence of HPV. Therefore, HPV is an important factor, but not sufficient itself, for the development of cervix cancer. Several factors like immunity, genetics and sexual behavior seem to interfere in the mechanisms, not yet completely known, that provides the regression or persistence of HPV infection. Others risk factors can be add like cigarette smoke, an early sexual initiation, contraceptive drugs, immunosuppressive and also the women's age, considering that patients under 30 years old may have a spontaneous regression in the infection, whereas those ones over this age may have a persistence in HPV lesions (Brasil, 2013).

Histologically, HPV lesions are classified as CIN-I (Cervical Intraepithelial Neoplasia grade I), CIN-II (Intraepithelial Neoplasia grade II), CIN-III (Intraepithelial Neoplasia grade III), microinvasive carcinoma and invasive carcinoma. Cytologically, the lesions are characterized as LSIL (Low Squamous Intraepithelial Lesion), HSIL (High Squamous Intraepithelial Lesion), microinvasive carcinoma and invasive carcinoma. LSIL occurs in women during the beginning of sexual activity and lead to a regression after 2 or 3 years, although around 14% may suffer a progress to CIN-II and CIN-III. High Squamous Intraepithelial Lesion grade III and microinvasive carcinoma must be treated because they are considered precursor lesions of invasive carcinoma. CIN-II lesions, commonly observed in young women, can be treated by means of chemical and physical methods (Silva-Neto, 2012).

In the later years, a vast number of natural products from plants, like the traditional Chinese medicinal herbs, have been an important source of potential agents used as inhibitors of cell proliferation, apoptosis inducers, angiogenesis suppressors, besides metastasis retarders. Also, they can be used as modulators of another chemotherapy drugs, helping their mechanisms of action and showing, therefore, a promising anticancer activity *in vitro* and *in vivo* (Tan *et al*., 2011). Among these natural products, we can mention the alkaloids, which are chemical compounds occurring in many natural sources with a growing interest as therapeutic agents. Alkaloids are commonly used in a great variety of treatments, pure or in association with other drugs, in derivative forms and also as raw basic material for the synthesis of many drugs. There are some examples of alkaloids properties like the anti-hypertensive activity of reserpine, anti-headache of ergotamine, analgesic of morphine, and antineoplasic drugs as paclitaxel (Sgadari *et al*., 2000).

Based on the promising therapeutic activities of alkaloids, this research aimed to describe the possible actions of these natural compounds as inhibitors of cell proliferation or cervix carcinoma formation, through a literature review, where we report the possible cellular mechanisms of action, the main targets and the methodological strategies used experimentally to evaluate their potential in chemotherapy.

METHODOLOGY

The present work consists of a literature review made between the years of 1980 and 2015, using some data base platforms like: PubMed, Medline, Sibi (USP), Portal Periódicos Capes, Bireme, ISI, SCIRUS, IBICT, Web of Science and Dissertation Abstracts. Throughout the search we have used the combined key words: alkaloids, cervical carcinoma, HPV, HeLa cells, SiHa cells and CaSki cells. We have found about 82 references, from which we selected 40, that were closely related to the subject of our study and still the most relevant.

General aspects on cancer chemotherapy

Cancer chemotherapy aims to generate a cytotoxic effect on the cancer cells inhibiting the tumor growth. The target consists in attacking an essential metabolic path to the proliferation like the disposal of purines and pyrimidines bases, which are needed to the RNA and DNA synthesis. The ideal effect of the antineoplastic drugs would be only over cellular metabolism, which is strictly related to malign cells. However, most of the drugs currently used are effective on all proliferation cells, normal and maligns, showing cytotoxic effects close to the therapeutics properties (Howland & Mycek, 2007).

The fast development of thousands of new drugs can offer a great hope to cancer patients. On the other hand, represents an enormous challenge for the evaluation of basic, preclinical and clinical researchers and possibly introduce these drugs in clinical routine. The possibility of a new drug shows effectiveness in cancer therapy can only be verified by means of clinical studies. However, due to ethical, economic and a limited number of patients able to be subjected to the clinical trials, the major part of researches needs to be done in experimental protocols. For this reason, researchers in the antineoplastic chemotherapy field have been developed safe and trustable methods to evaluate the efficacy of many drugs using *in vivo* and *in vitro* tests. Nowadays, there are standard and well established experimental methods for *in vivo* and *in vitro* evaluation of new anticancer drugs. Between many available compounds it is possible to choose the more active, which is submitted to clinical assays in later studies. The clinical application for which the drug will be used, such as palliative, curative and the type tumor over which the drug is more effective, should be considered when accomplishing the experimental study. For the evaluation of new antineoplastic agents, at least three different cell lines are preconized for *in vivo* and vitro experiments (Zips *et al*., 2005).

The acquisition of drug resistance to some agents used in chemotherapy is one of the major causes of failure in treatment of malign neoplasia, although the treatment can be effective in the beginning (Bakos & Romolya, 2006). Some resistant cell lines to a specific agent can become resistant to many others agents with different chemical structures. This phenomenon gave rise to what is called Multidrug Resistance (MDR). The classical form of MDR is due to an increase in P-glycoprotein (Pgp, MDR1), located in plasma membrane which is capable of extruding a great quantity of antineoplastic agents from the inside of the cell against a concentration gradient (Zalcman *et al*., 1997). Natural compounds might be able to revert MDR1 action, helping the cytotoxic activities of anticancer drugs (Ferreira *et al*., 2005).

Cell lines used in anticancer assays

Culture cells techniques play an important role in developing new anticancer drugs because they can be used in comprehension of mechanisms of cell intrusion and extrusion, interaction with cell receptors and cell metabolism. Assays which lead to observation of multiple steps of cell death are useful in measuring cell survival after treatment with cytotoxic drugs. Moreover, cell micro cultures combined with colorimetric methods used for measuring antiproliferative effects have established the necessary basis for the screening of cytostatic and cytotoxic drugs. Although there are some questions about the use of cell cultures to evaluate antitumor activity in clinical studies, they can be properly used for many purposes in cancer therapy studies like tumor angiogenesis, invasion and interactions between cells of the immune system (Baguley *et al*., 2013).

Normal cell lines undergo mitotic division in a determined number of times until lose its capacity of replication, in a way know as senescence. These cells are also called finite cells. Some cell lines can become

immortals in a transformation process, which can occur spontaneously or induced by a chemical substance or viral implantation. Conditions for cell culture may vary from one type to another, but an artificial medium where cells are cultivated must contain essential nutrients like amino acids, vitamins, carbohydrates and mineral and some nonessential like hormones, growth factors, gas (O2, CO2), besides physical-chemical conditions (pH, osmotic pressure and temperature (Life Technologies, 2013).

Cell lines commonly used for HPV studies are SiHa and CasKi (HPV-16 positive), HeLa (HPV-18 positive) C33-A (HPV-16 negative) and HT-3 (HPV-18 negative) (Diao *et al*., 2015). These cells can be treated with alkaloid samples, either in plant crude extract forms or pure and isolated compounds. Alkaloid activities can be evaluated by means of their capacity of inhibiting tumor and cell growth, inducing cell apoptosis and also the modulation of drug resistance to chemotherapy (Baguley *et al*., 2013).

An overview on the assays used to evaluate the cytotoxic activity

The discovery of new drugs for application in therapeutic systems against malign neoplasia has become a permanent need. Many public institutions, industries and laboratories have been researching many substances with potential antineoplastic activities. However, only a few prototypes can be used in clinical experiments due to the high costs and ethical considerations. A screening must be done to identify those products which can match specific criteria for antitumor drugs. Among them, the most active compounds are selected to proceed in the next stages, in a preclinical developing program of chemotherapeutic agents (Burger & Fiebig, 2004).

The assay for cell viability: MTT

3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide, also known as MTT, is a colorimetric assay used to evaluate the cell viability. It was first described by Mosmann (1983). MTT (a yellow salt, soluble in water), is reduced by mitochondrial dehydrogenase to an insoluble, dark purple product, named formazan. Formazan crystals are dissolved in organic solvents (isopropanol or DMSO) and the solution is measured spectrophotometrically. Since MTT can only be reduced by metabolically active cells, the level of activity is a measure of cell viability (Dias *et al*., 1999).

Flow cytometry assay: cell apoptosis and cell cycle blockage

Apoptosis consists in a meticulous regulated process of cell death, which helps in homeostasis and normal development. Its downregulation is associated with autoimmune and degenerative diseases and neoplasia. Flow cytometry is a multiparametric tool that allows the measure of many apoptosis characteristics in one single sample, which makes it a valuable method in studying the complex system of this type of cell death in its initial, intermediate and final stages (Bradford, 2013).

Flow cytometry and the use of BrdU (5-bromide-2'-deoxiuridine) allows the evaluation of a natural product activity as an inducer of cell cycle arresting in G0G1, S and G2-M phases (Luk *et al*., 2005; Crane *et al*., 2011).

Pgp ATPase assay for evaluation of drug resistance

MDR (Multidrug Resistance) is due to an increase in P-glycoprotein (Pgp) activity. This is located in plasma membrane and can extrude a variety of chemotherapy drugs from the cell against a concentration gradient, leading to drug resistance. Pgp ATPase consists in a method to evaluate the activity of P-glycoprotein during the drug resistance process. This assay uses insect cells (SF9 lines), which express human Pgp. Two ATP molecules are hydrolyzed for each transported drug (antitumoral drug tested) by Pgp, resulting in the release of two inorganic phosphates in the reaction medium. The level of phosphates released is measuared by a colorimetric reaction, in which phosphate reacts with ammonium molybdate, yielding a blue colored solution (Feng *et al*., 2007).

General aspects of alkaloids

Alkaloids (term derived from the arabian word al-quali, plant from which soda was obtained) are nitrogenated compounds, pharmacologically actives, which represent one of most vast secondary metabolites (i.e chemical substances) with specialized activities in nature, showing a great diversity of chemical structures, biosynthetic paths and biological activities. They are mainly encountered in angiosperms, but can also be isolated from insects, marine invertebrates and some micro-organisms (Simões, 2007).

The knowledge of these natural compounds is as old as the human civilization, with reports of their use as drugs, teas, remedies and poisons. In older times, the first civilizations, in their search for food and even in defense against enemies, poisoned the arrow heads with plants containing alkaloids. This use is probably the predecessor of therapeutic uses. Some alkaloids with poison activity are still being use in Africa and South Africa and from them some agents were made with therapeutic uses for human beings, like ouabain and k-strofantin, used for acute heart failure, fisostigmine for glaucoma treatment and miastenia gravis, *d*-tubocurarine as muscle relaxant in anesthesia, reserpine as antihypertensive and psychotropic drug and ajmaline, used for heart arrhythmia (Roberts & Wink, 1998).

Alkaloids vincristine and vinblastine are good examples of alkaloid compounds with antineoplastic properties, obtained from a natural source, *Vinca rosea* (*Catharanthus roseus*), a native plant from Madagascar island, widespread distributed, even in countries of hot weather (Shams *et al*., 2009). Vincristine is used for treatment of some myelomas, lymphomas and leukemia (Sajjad, 2012), whereas vinblastine is used against breast cancer (Ospovate, 2009) and Hodgkin lymphoma (Souza *et al*., 2010).

Review of the most active alkaloids on HPV cell lines

Alkaloids are natural compounds containing a ring and a nitrogen atom, classified according to biosynthetic pathways and chemical structures or carbon skeleton. Based on these chemical aspects, there are steroidal, indole, aporphine, tropane and isoquinoline alkaloids, among others. Differences in carbon skeleton may represent, in most of the cases, considerable changes in activity of these compounds on biological systems (Lu *et al*., 2013).

Among steroidal alkaloids, briofilin and O-methylsolanoscapine, isolated from the plants *Bryophyllum pinnata* (Lam.) Oken and *Solanum pseudocapsicum*, respectively, showed cytotoxic activities. Briofilin induced apoptosis in HeLa cells, which are epithelioid cell line from human cervical cancer (HPV-18^{+}), by increasing the expression of Bax protein, suppression of antiapoptotic molecules Bcl-2, caspase 3 activation and cleavage of Poli(ADP-Ribose) Polymerase-1 (PARP-1) (Mahata *et al*., 2011).

The isomeric alkaloids naucleficine and naucleactonine are subdivisions of indole alkaloids, known as biscoclaurines, isolated from *Nauclea orientalis*. These alkaloids have shown significant cytotoxicity against HeLa cell lines, with and IC_{50} of 4.0 and 7.8 µg/mL, respectively (Jirapast *et al*. 2010).

A β-carboline-derived alkaloid, harmalinane, largely distributed in plants, as well as alcoholic beverages and tobacco, significantly reduced HeLa cells viability in a dose-dependent manner, showing a good cytotoxic potential (Jiménez *et al*., 2008).

The ethanolic extract of the plant *Lycopodium clavatum* has been used in the alternative medicine for the treatment of several liver illnesses as for Alzheimer´s disease. The *in vivo* anticancer activity in mice treated with hepatic carcinogens has also been attributed to this plant. Licopodin, an active compound from *Lycopodium clavatum* induced apoptosis in HeLa cells through induction of reactive oxygen intermediates, cytochrome C release and caspase 3 activation (Mandal *et al*., 2010).

Isoquinoline alkaloids matrine and berberine showed cytotoxic activity over HeLa cell lines. Matrine, isolated from plants of the Sophora genus, inhibited cell adhesion to type I collagen, significantly reducing their migration when comparing with control cells. This suggests that matrine might have an antimetastatic activity and may be useful as a future antitumor drug. Berberine is an alkaloid isolated from plants of the traditional Chinese medicine, showing activities like inhibition of cell proliferation, apoptosis induction, angiogenesis suppression and delaying metastasis. This alkaloid, when combined with cisplatine, increased the toxicity over HeLa cell lines (Tan *et al*., 2000), by means of suppression of HPV E6 and E7 oncoproteins and expression of p53 and p21, which led to cell apoptosis (Mahata *et al*., 2011).

The indole alkaloid from *Vinca rosea*, vinblastine and its semisynthetic analogs vinorelbine and vinflunine showed significant cytotoxic activities over HeLa cell lines. Vimblastine reduced the antineoplastic drug resistance mechanism, besides blocking mitosis and showing IC_{50} of 0.45 nM. Vinflunine and vinorelbine showed IC_{50} of 1.8 and 1.24 nM, respectively (Ngan *et al*., 2001). The bis-indole alkaloids, 6-hydroxystaurosporine, sunguicine and isoguncine also showed cytotoxic activity over HeLa cell lines (Takahiro *et al*., 2005). A brominated indole alkaloid named eudistomin H, isolated from marine ascidian *Eudistoma viride* showed arrest of cells in G0/G1, S and G2/M phases and an increase in the sub G0/G1, with morphologic events such as cell shrinkage, membrane blebbing, chromatin condensation and formation of apoptotic bodies, leading to apoptotic death of HeLa cells (Rajesh & Annappan, 2014).

Aporphine alkaloids, like anonaine, are common in Annonaceae family, which also yield neolisitine, glaucine and normecamboline. These latter three alkaloids have shown promising significant cytotoxicity over HeLa cell lines (Tran *et al*., 2010).

The phenanthren-derived alkaloids chelerythrine, chelitudine, sanguinarine and sanguilutine are quaternary ammonium salts which showed toxicity over Hela cell, with IC_{50} ranging from 0.8 to 50 µg/mL. Sanguinarine showed a more toxic effect, with IC_{50} of 0,8 µg/mL, probably for showing a better penetration in plasma membrane, additionally causing reduction and depolarization of microtubules during cell interphase and mitosis (Slaninová *et al*., 2001).

Marine environment is also a promising source of natural products, such as algae and sponges. From the marine sponge *Aaptos* sp. a quinoline alkaloid named aaptamine and its derivatives demethyl(oxy)aaptamine and isoaaptamine were isolated. The demethyl(oxy)aaptamine showed higher anticancer activity over cervical carcinoma. The marine sponge *Theonella* sp. is good source of active metabolites such as peptides, cyclopeptides perthamides, glycopeptides *Theonella*mides and alkylpyridine alkaloids. From these latter compounds some derivates were synthesized and tested against HeLa cell lines, showing cytotoxic effect with IC_{50} ranging from 4.0 to 9.4 µM, probably by means of cell apoptosis induction (Gonçalves *et al*., 2014). *Eudistoma cf. rigida*, a tunicate found near Okinawa and New Guinea is the source of marine alkaloids rigidins A, B, C, D and E. Even though these alkaloids have shown a weak cytotoxicity over HeLa cell lines, some of their synthetic derivatives, mainly the 7-deazahypoxanthine-like compounds have displayed antiproliferative activities against HeLa cells by pronounced effects on microtubule organization in a manner similar to the known compound colchicine (Frolova *et al*., 2013).

Luotonin A is a pyrroloquinazolinoquinoline alkaloid isolated from the Chinese herbal medicinal plant *Peganum nigellastrum* (Cagir *et al*., 2003). It has been described as a topoisomerase 1 inhibitor, although not showing a high potency. However, this compound serves as model for the synthesis of more potent analogues, providing a new opportunity for drug discovery in the anticancer area (González-Ruiz *et al*., 2014).

Other alkaloids classes also showed cytotoxicity activity over HeLa cell lines, such as acridone alkaloids, from imidazole class, naamidine H, a pyrano quinolone, and a microbial alkaloid, isolated from *Streptomyces staurosporeus*, named staurosporine, which showed strong activity on HeLa cell line growing, with IC_{50} of 4 x 10(-12) M22. Indigenous people have been used plants from Amaryllidaceae family in traditional medicinal practices. This family is well known for its alkaloid constituents with potent cytotoxic activities like crinane alkaloids. Among them distichamine showed the most potent cytotoxic activity, with IC_{50} 2.2 µM over HeLa cells (Nair *et al*., 2014).

In this work we did not find a significant number of articles relating to cytotoxic properties of alkaloids over HPV-16+ (SiHa cells), maybe because HeLa cells are the most assayed cell line and possess well-established protocols. Moreover, HeLa cell lines divide indefinitely in laboratory and also do not undergo cell aging, wich allows researchers to use them in many medical studies. For these reasons HeLa cell lines are broadly used in investigations like the mechanisms involved with programmed cell death, treatment of diseases, mapping of genes, nature of cancer and development of vaccines (AccessScince Editors, 2014).

CONCLUDING REMARKS

A critical evaluation of the present work led us to think about the need of introducing new alternatives in chemotherapeutic area of cervical cancer treatment. We can conclude that natural products from several sources, such as microbial, aquatic or ground plants, animals, etc., may serve as a vast source of new drugs with cytotoxic potential and promising activities against the lesions caused by HPV. In this context, alkaloids are very effective as inhibitors of HPV cells growth, mainly HPV-18$^+$ (HeLa cells). Knowing the many sources for obtaining a great variety of alkaloid classes and with the considerations made in this study, it is possible to raise optimists perspectives to believe that natural products are a rich field of chemotherapeutic agents against cervical cancer, even as isolated and pure compounds or as adjuvants in association with well-known cytotoxic drugs commonly used in clinical procedures, improving their efficacy over malign cells.

RESUMO

Efeitos citotóxicos de alcaloides sobre linhagens de células do câncer cervical: uma revisão

O câncer cervical é a quarta neoplasia incidente em mulheres, com o surgimento de milhares de novos casos anualmente. Está altamente relacionado à infecção pelo papilomavírus humano (HPV), que apresenta mais de 13 tipos oncogênicos, dentre os quais os tipos 16 e 18 são encontrados em 70% dos casos de câncer do colo do útero (câncer cervical). Alcaloides são substâncias naturais nitrogenadas que apresentam diversos usos na terapia, incluindo atividades antineoplásica e citotóxica. Neste sentido, objetivamos neste trabalho avaliar o potencial citotóxico e quimioterápico de alcaloides sobre o câncer cervical. Para tanto, relacionamos os alcaloides com potencial citotóxico sobre HPV-16+ e HPV-18$^+$ (células HeLa), bem como mostramos suas principais fontes de obtenção, distribuição na natureza e discutimos os mecanismos de ação pelos quais realizam seu efeito citotóxico, através de uma revisão bibliográfica realizada entre o período de 1980 e 2015. Os alcaloides mostraram-se eficazes como drogas citotóxicas, inibindo o crescimento de células alteradas pelo HPV tanto *in vitro* quanto *in vivo*, com ativação de mecanismos intrínsecos e extrínsecos de apoptose, tais como ativação das caspases, clivagem de PARP-1 (Poli-ADP-Ribose Protease 1), aumento da expressão de p53, liberação de citocromo C e aumento da expressão de receptores de morte como o Fas, principalmente em células das linhagens HeLa (HPV-18$^+$). Adicionalmente, esses metabólitos secundários auxiliaram na modulação da resistência a múltiplas drogas pelas linhagens de células estudadas, o que nos leva a sugerir o seu possível uso na quimioterapia das lesões provocadas por estes vírus.

Palavras-chave: Câncer cervical. Alcaloides. HPV. Quimioterapia.

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