Cryptolepine consolidates its anticancer potential by increasing the anti-cancer pathway genes and decreasing that of pro-cancer pathway genes

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Abstract

Background: Cancers are one of the commonest causes of deaths globally. Reports indicate that greater than sixty percent of cancers in the world occur in low and middle-income countries with about seventy percent of all cancer deaths occurring in these regions. Conventional cancer treatments involve surgery, radiotherapy, chemotherapy, etc. However, the negative side effects such as high cost and toxicity associated with these treatment options have increased the demand for less toxic and less expensive anti-cancer drugs from natural sources. One of such natural products believed to have anti-cancer potential is cryptolepine (CRYP), an alkaloid extracted from the roots of Western and Central African plant *Cryptolepis sanguinolenta*. In addition to its anti-cancer potential, CRYP has been reported to possess a myriad of pharmacological activities. However, the mechanisms underlying the anti-cancer and pharmacological activities of CRYP have not been fully explored.

Methods: We screened 45 immune and cancer signalling pathways for their regulation following treatment with CRYP using the dual-luciferase based Cignal Finder Multi-Pathway Reporter Arrays to pinpoint which pathways are regulated by CRYP. Additionally, the effects of CRYP on the transcript levels of interferon regulatory factor 1 (IRF-1), progesterone receptor (PR), hypoxia-inducible factor-1 alpha (HIF-1 α) and signal transducer and activator of transcription 3 (STAT 3) were assessed by real-time quantitative polymerase chain reaction (RT-qPCR).

Results: We observed that of the 45 immune and cancer signalling pathways screened, nine were up-regulated while twenty-seven were down-regulated by CRYP. However, CRYP had no effect on nine of the pathways screened. We also observed that CRYP induced an increase in the transcript levels of IRF1 and PR but decreased that of HIF1- α and STAT3.

Conclusion: The upregulation of human anti-cancer pathway genes including IRF-1 and PR and concomitant down-regulation of pro-cancer pathway genes including HIF1- α and STAT3 suggest additional mechanisms through which CRYP could exhibit its anti-cancer potential.

Background

Cancers have been identified as one of the commonest causes of deaths globally. Estimates from the International Agency for Research on Cancer (IARC) predicts that the global incidence of cancer is expected to surge upwards from 14 million per year to about 22 million per year by the year 2030 (1). During the same period, cancer-induced deaths are expected to rise from an estimated 8.2 million to 13 million per year. It has been reported that greater than 60% of all cancer cases in the world occur in low and middle-income countries with about 70% of all cancer deaths occurring in these regions (1). Cancers are mostly caused by lifestyle behaviours such as abuse of tobacco and alcohol, inadequate intake of fruit and vegetable on a regular basis, obesity and lack of physical activity. In addition, certain chronic infections from hepatitis B virus (HBV), hepatitis C virus (HCV) and some types of human papillomavirus (HPV) may also increase the risk of cancer (2).

Traditional interventions that are employed in the treatment of cancers include surgery, radiotherapy, chemotherapy, hormone therapy, immune therapy, targeted therapy and psychosocial support(2). Aside from the high costs that are usually associated with these treatment options, toxic side effects associated with cancer chemotherapy and radiation therapy have opened the avenues for research into the discovery of new anticancer agents from plants and other natural sources. Making cancer treatments more effective and less expensive especially in resource-limited countries including Ghana will greatly reduce cancer-induced deaths even in situations where health-care services are not well developed. In view of that natural products from plant sources that are basically used in traditional medicines are being explored as one of the major sources of cancer chemo-preventive drug discovery. One of such plant products that is being considered as a potential anticancer agent is CRYP.

CRYP is an alkaloid that is extracted from the roots of a plant called *Cryptolepis sanguinolenta*. This plant is common in the West and Central parts of Africa. CRYP has been widely reported to exhibit a myriad of pharmacological and biological properties which include anti-malarial (3), anti-bacterial(4), anti-fungal(5) and anti-hyperglycaemic(6, 7) under different *in vitro* and *in vivo* conditions. In addition to these many pharmacological and biological activities, CRYP has also been reported to have anti-inflammatory activity in different animal model systems (8, 9). The anti-inflammatory activity of CRYP has led some researchers to evaluate the anticancer potential of CRYP.

Some studies have reported that CRYP and its derivatives could exhibit their anticancer properties by directly binding to DNA and inhibiting DNA replication and also inhibiting the functions of topoisomerase II (10-12). In fact, studies have shown that several anti-cancer agents mechanistically work by inhibiting topoisomerase II enzyme and that inhibition of topoisomerase II induces apoptosis (13). CRYP has also been reported to have significant effects on the functional ability of p53 one of the key tumour suppressor proteins (14). For example, Zhu and Gooderham (2006) reported that CRYP induces the accumulation of p53 protein in human lung adenocarcinoma A549 cell lines (15). The increase in the levels of p53 protein following CRYP treatment has been reported to enhance DNA repair mechanisms of the affected cells (16, 17). Caspases are the key architects of apoptosis and CRYP has been reported to significantly activates caspase 3 but CRYP-induced activation of caspase 8 has been reported to be minimal suggesting that CRYP may induce apoptotic cell death partly via the mitochondrial pathway (15, 18). Bcl-2 is a protooncogene and reports have indicated that treatment of cells with CRYP results in inhibition of Bcl-2 activity resulting blockage of apoptotic cell death (15, 18).

In spite of the these reported anti-cancer potential, how CRYP regulates various genes including the oncogenic ones and those with anti-cancer activity have not been fully explored. We therefore, used the Signal Finder 45 Pathway Reporter Array plate (Qiagen and SA Biosciences, USA) to simultaneously assess the effects of CRYP on 45 different signalling pathways that cover research areas such as cancer, immunology, development and toxicology to pin-point which pathways are affected by CRYP. Based on the screening data, IRF1, PR, HIF1- α and STAT3 target genes were selected and the effects of CRYP on the transcript expression of these selected genes were confirmed using RT-qPCR.

We report that treatment of human embryonic kidney 293 (HEK 293) cells with CRYP increased the transcript expression levels of IRF-1 and PR but decreased that of HIF1- α and STAT3. The outcome of the current study could offer promising avenues for further research into the development of CRYP as a novel anticancer agent. Achieving this feat would improve the treatment success of cancers and increase the chances of survival of cancer patients in Ghana and other low- and middle-income countries.

Materials And Methods

Reagents and chemicals

The CRYP used in the study was donated by Prof. Kwesi Mensah Boadu, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. It was dissolved in phosphate buffered saline (PBS) to a stock concentration of 43050 μ M, filter sterilized and divided into aliquots, wrapped in aluminum foil and stored frozen at -20 °C until used. The CRYP stock solution was diluted to the desired concentration in normal growth medium when necessary. The foetal bovine serum (FBS) (cat # F2442) was purchased from Sigma-Aldrich, USA. Dulbecco's Modified Eagles Medium (DMEM) (high glucose, L-glutamine, sodium pyruvate) (cat # 1-26F58-1) was purchased from BioConcept Ltd, Switzerland. Minimum essential medium (MEM) non-essential amino acids (NEAA) (cat # 0823) was purchased from ScienCell, USA. Opti-MEM reduced serum medium (cat #31985-070) was purchased from Gibco Life Technologies, USA while penicillin-streptomycin (cat #15140) was purchased from Gibco by Invitrogen, UK. Thiazolyl blue tetrazolium bromide powder (MTT powder) (cat # M5655-1G) was purchased from Sigma Aldrich, USA. The MTT powder was dissolved in PBS to a stock concentration of 5 mg/ml, filter sterilized and divided into working aliquots, wrapped in aluminum foil and stored at -20°C until used. Isopropyl alcohol (> 99.5% purity, cat # 67-63-0) was purchased from Dae-Jung Chemicals and Metals, Siheung-Si, Gyeongii, Korea. Signal Finder Reporter Array Plates (cat # CCA-901L) were purchased from SA Bioscience, USA. Attractene Transfection Reagent (cat # 301005) was purchased from Qiagen, USA. The Dual Luciferase Reporter Assay System (cat# E1960) was purchased from Promega, USA.

Cell culture

The HEK 293 cells (cat # CRL-1573) used in the study was purchased from the American Type Culture Collection (ATCC), USA. The cells were grown in DMEM high glucose-containing L-glutamine, sodium pyruvate supplemented with 10% v/v heat-inactivated FBS, 1% v/v MEM non-essential amino acids, 100 IU/ml of penicillin and 100 μ g/ml of streptomycin. The cultures were maintained at 37 °C in 5% carbon dioxide (CO₂) under humidified condition.

Cytotoxicity assay

HEK 293 cells were grown to about 60% confluence and then treated with increasing concentrations of CRYP (0-10 μ M). The cytotoxic effect of CRYP was evaluated using MTT assay at 24, 48- and 72-hours post-treatment following the manufacturer's instruction. Briefly, the cell culture media containing the

CRYP was carefully removed. Fifty microliters (50 µl) of serum-free media and 50 µl of MTT solution were added into each well. Solvent control wells or blank wells containing 50 µl of the MTT reagent and 50 µl of cell culture media (no cells) were included in the assay. The plates were incubated for 3 hours at 37°C. After incubation, 150 µl of isopropyl alcohol (MTT solvent) was added into each well. The plates were wrapped in aluminum foil and shaken on Edmund Buhler orbital shaker (Edmund Buhler GmbH, Germany) for 15 minutes. The optical density (OD) of the wells was read at 590 nm using iMark Microplate Reader (Bio-Rad, USA).

Signal Finder 45-Pathway Reporter Array and Reverse Transfection

We used the Signal Finder 45 Pathway Reporter Array Plate (Qiagen and SA Biosciences, USA) to simultaneously assess the effects of CRYP on 45 different signalling pathways. These 45 signalling pathways cover research areas such as cancer, immunology, development and toxicology. The Signal Finder 45-Pathway Reporter Array has 45 pathway reporters dried and coated down in duplicate wells of the 96-well plate with the remaining 6-wells containing positive and negative controls. Each reporter consists of an inducible transcription factor-responsive construct and a constitutively expressing *Renilla* luciferase construct. The inducible transcription factor-responsive construct encodes the firefly luciferase reporter gene and monitors both the increase and decrease in the activity of the coupled transcription factor in a said signalling pathway while the *Renilla* construct encodes the *Renilla* luciferase reporter gene which serves as an internal control to which firefly activity is normalized. To determine the effects of CRYP on the 45 signalling pathways, the reporter constructs were reverse transfected into HEK 293 cells following the manufacturer's instruction. Briefly 50 µl of Opti-MEM[®] was added into each well of the Signal Finder Array plate to resuspend the reporter constructs. The plate was incubated at room temperature for 5 minutes. The Attractene transfection reagent was diluted in Opti-MEM after which 50 µl of diluted Attractene was added into each well containing 50 µl of the diluted nucleic acids. Cells were suspended to a density of 8×10^5 cells/ml in Opti-MEM[®] containing 10% of FBS and 0.1mM NEAA and 50 µl of the cell suspension was added to each well of the Signal Finder Array plate containing the reporter constructs and the transfection reagent. The cells were incubated at 37°C in a 5% CO₂ incubator for 18 hours. Following reverse transfection, the cells were treated with assay medium (Opti-MEM[®] supplemented with 0.5% FBS, 0.1mM NEAA, 100 U/ml Penicillin and 100 µg/ml Streptomycin) containing 5 µM CRYP for another 18 hours. A control experiment in which the cells were reverse transfected but not treated with cryptolepine was done in parallel with the treated experiment. The cells were lysed passively and dual luciferase expression was determined using the dual luciferase reporter assay system (Promega, USA, cat no E1960) following the manufacturer's protocol. After preparing the cell lysates, 20 µl of the aliquot was employed for luminescence measurement using Berthold Orion luminometer (Berthold Detection Systems, Germany).

Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR)

HEK 293 cells were cultured and treated with increasing concentrations of CRYP (0-5 µM) for 24 hours. Total RNA was then extracted using the Gene JET RNA purification kit (Thermo Scientific, Germany)

following the manufacturer's instruction. The quantity and the purity of the total RNA was verified by spectroscopy (Nano Drop 1000, Thermo Scientific). The purity was later confirmed by 1% agarose gel electrophoresis using ethidium bromide as a stain. The total RNA was converted to cDNA as we previously described (19). The cDNA was stored frozen at -80 °C until used in the qPCR. IRF1, PR, HIF1- α and STAT3 target genes were amplified using the Maxima Probe/Rox qPCR master mix (Thermo Scientific, Germany). The primers and probes used were designed and synthesized by Biomers, Germany (Table 1). The probes of the target genes and the endogenous control Beta-actin (B-actin) were labelled with different fluorescent reporter dyes at the 5' end and quencher dyes at the 3' end and this allowed the target genes to be amplified in the same tube in a duplex qPCR reaction. After optimizing the primer and probe PCR conditions, a duplex qPCR was performed in a 20 μ l reaction volume that contained 0.3 μ M forward and reverse primers of the target genes, 0.2 μ M of the target probes, 0.2 μ M forward and reverse primers of the B-actin, 0.2 μ M of the B-actin probe and 2.0 μ l of 1:5 dilution of the cDNA samples. The qPCR cycling conditions were as we previously described(19). The qPCR reaction products were analyzed using the Applied Biosystems StepOne Plus Manager Software. The relative quantification of the target genes was calculated using the Relative Standard Curve method.

Statistical Analysis

All experiments were conducted three (3) times at different times in duplicate or triplicate wells where applicable. Data were entered into an excel spread sheet and then analysed using excel and the appropriate data management software. One-way Analysis of variance (ANOVA) and *student t*-tests were used to analyse the level of significance between the treated and untreated groups where applicable. P-value $\leq$ 0.05 was considered as statistically significant.

Table 1: Primers and probe sequences

Target genes	Sequences of primers & probes	Fluorophores
IRF-1	Forward primer:5'-TTTGTATCGGCC TGTGTGAATG-3' Reverse primer:5'-AAGCATGGCTGG GACATCA-3' Probe:5'-CAGCTCCGGAACAAACAG GCATCCTT-3'	5'FAM-3'BHQ1
PR	Forward primer:5'-AGAAATGACTGC ATCGTTGATAAAATC-3' Reverse primer:5'-GGACCATGCCAG CCTGAC-3' Probe:5'-TCTGCCCAGCATGTCTGCC TTAGAAAGTGC-3'	5'FAM-3'BHQ1
HIF1-α	Forward primer:5'-CAGAGCAGGAAA AGGAGTCA-3' Reverse primer:5'-AGTAGCTGCATGA TCGTCTG-3' Probe:5'-ACTAGCTTTGCAGAATGCT CAGAGAA-3'	5'FAM-3'BHQ1
STAT3	Forward primer:5'-GGAGCAGAGATG TGGAATG-3' Reverse primer:5'-GTGGGTCTCTAG GTCAATCTTG-3' Probe:5'-AGTCTCGAAGGTGATCAG GTGCAG-3'	5'FAM-3'BHQ1
B-actin	Forward primer: 5'-TCACCCACACTG TGCCCATCTACGA-3' Reverse primer:5'-CAGCGGAACCGC TCATTGCCAATGG-3' Probe:5'-ATGCCCCCCCCCATGCCATC CTGCGT-3'	5'HEX-3'TAMRA

Results

Cytotoxicity of CRYP

We first evaluated the cytotoxic effects of different concentrations of CRYP on HEK 293 cells using MTT assay. It was observed that CRYP decreased the viability of HEK 293 cells in a dose-dependent manner after exposure of HEK 293 cells to CRYP concentrations of up to 10 μ M for 24, 48 and 72 hours (Fig. 1).

For the following succeeding experiments, CRYP concentrations of up to 5 μ M were used and with an incubation period of 24 hours. These concentrations were selected based on the facts that more than 80% of the cells survived at those concentrations at 24-hour post-incubation (Fig.1). An additional Table file 1 containing mean ($\pm$ standard deviation) values from all experimental replicates gives more details (see Additional file 1) as error bars are too small to show on Figure 1.

Fig. 1: HEK 293 cells and cytotoxicity of CRYP. CRYP cytotoxicity was evaluated by MTT assay on HEK 293 cells treated with CRYP (0-10 μ M) for 24, 48 and 72 hours. The viability of cells was calculated as the ratio between CRYP-treated cells and non-treated cells (equal volume of PBS replacing CRYP). Data are presented as mean and standard deviation of three independent experiments each performed in triplicate wells, *p-value* = 0.03 as determined by one-way ANOVA. The error bars represent the standard deviation among the three independent experiments but are too small to show.

CRYP differentially regulates 45 signaling pathways

We next utilized the Signal Finder 45 Pathway Reporter Array to simultaneously screen for the effects of CRYP on the relative activity of 45 signaling pathways following CRYP treatment of HEK 293 cells after reverse transfection of the reporters into the HEK 293 cells. The relative fold change values were obtained by dividing the normalized luciferase activities of each treated pathway-focused reporter by the normalized luciferase activity of the untreated pathway reporter. Of the 45 signaling pathways screened, nine (first 9 bars on the left; Fig.2) were upregulated by CRYP while twenty-seven (the last 27 bars on the right; Fig.2) were downregulated by CRYP. CRYP had no detectable effects on nine (9 bars at the middle between the upregulated and the downregulated bars; Fig.2) of the pathways screened. The criteria for categorization of the signaling pathways into up-regulated, down-regulated and no effects were as follows: signaling pathways that had relative fold change values > 1.5 , < 0.7 and $= 0.7-1.5$ were considered to be up-regulated, down-regulated or unaffected respectively (Fig. 2). An additional Table file 2 containing the reporters, signaling pathways, transcription factors and the fold change values shows more details (see Additional file 2).

Fig. 2: Effects of CRYP on 45 signaling pathways. The relative expression levels of the 45 signaling pathways in HEK 293 cells after treatment with 5 μ M of CRYP was evaluated using the dual luciferase reporter gene assay. The results were expressed as log2 of the fold change of the expression of transcription factors between CRYP-treated cells and non-treated cells. The error bars represent standard deviation of three independent experiments conducted in duplicate wells.

CRYP induces an increase in the transcript expression of IRF-1 and PR but decreases the transcript expression of HIF-1 α and STAT3

Having demonstrated at the luciferase reporter level that CRYP differentially regulates the 45 signaling pathways, two each of the upregulated pathways (namely IRF-1 and PR) and the downregulated pathways (namely HIF1- α and STAT3) were selected and further investigated using RT-qPCR. These genes were selected based on the fact that the anti-cancer therapeutic potential of CRYP targeting these

genes have not been fully exploited. RT-qPCR analysis of the transcript levels of IRF-1, PR, HIF-1 α and STAT3 in HEK 293 cells treated with increasing concentrations (0, 2.5 and 5 μ M) of CRYP was performed. It was observed that CRYP induced an increase in the transcript levels of IRF1 and PR in a dose-dependent fashion. At 5 μ M CRYP, the transcript level of IRF-1 was increased by 9.4 folds (Fig. 3a) while the transcript level of PR was increased by 20.9 folds (Fig. 3b). On the other hand, CRYP inhibited the transcript levels of HIF-1 α and STAT3. At 2.5 μ M and 5 μ M CRYP, the transcript levels of HIF-1 α decreased by 70% and 60% respectively (Fig. 4a). With reference to STAT3, 2.5 μ M of CRYP induced a 60% decrease in the transcript expression level. However, at 5 μ M, CRYP had no significant detectable effects on the transcript expression level of STAT3 (Fig. 4b).

Fig. 3. CRYP induces an increase in the relative mRNA expression levels of (a) IRF1 and (b) PR in HEK 293 cells after treatment with increasing concentrations of CRYP (0, 2.5 and 5.0 μ M). The relative mRNA expression levels of IRF1 and PR after normalization to B-actin (endogenous control) were expressed as means $\pm$ standard deviations. The relative levels of IRF1 and PR were calculated using the relative standard curve method. The untreated cells (0.0 μ M) was used as reference group to estimate relative gene expression changes. For IRF-1, ** P -value <0.007, **** P -value <0.0001. For PR, ** P -value < 0.02, **** P -value < 0.0001. The error bars represent standard deviations of triplicate wells in an experiment.

Fig. 4. CRYP induces a decrease in the relative mRNA expression levels of (a) HIF-1 α and (b) STAT3 in HEK 293 cells after treatment with increasing concentrations of CRYP (0, 2.5 and 5.0 μ M). The relative mRNA expression levels of HIF-1 α and STAT3 after normalization to B-actin (endogenous control) were expressed as means $\pm$ standard deviations. The relative levels of HIF-1 α and STAT3 were calculated using the relative standard curve method. The untreated cells (0.0 μ M) was used as reference group to estimate relative gene expression changes. For HIF-1 α , ** P -value <0.001. For STAT3, ** P -value < 0.0004. The error bars represent standard deviations of triplicate wells in an experiment.

Discussion

Cancer treatment is highly expensive and unaffordable by many especially in the low- and middle-income countries of the world. In addition to the high cost of treatments, toxic side effects associated with chemotherapy and radiation therapy have resulted in huge demand for cheaper and less toxic alternatives from natural sources (2). Making cancer treatments more effective and affordable especially in resource-limited countries including Ghana will greatly reduce cancer-induced deaths even in situations where health-care services are not well developed. In view of that natural products from plant sources that are basically used in traditional medicines especially in the resource-limited countries are being explored as one of the major sources of cancer chemo-preventive drug discovery. One of such plant products which is gaining much popularity as a potential anticancer agent is CRYP an alkaloid that is extracted from the roots of a plant called *Cryptolepis sanguinolenta* in the Western and Central parts of Africa. CRYP has been reported to exhibit anti-inflammatory activity and cytotoxic potential that is mediated through its direct and indirect interactions with DNA (9, 12, 14, 20, 21). In the current study, we observed that treatment of HEK 293 cells with low concentration of CRYP induced a significant increase

in the transcript expression levels of IRF-1 and PR but decreased transcript expression levels of HIF-1 α and STAT 3.

IRF1 was initially discovered as a transcriptional activator of the interferon (IFN) system in response to viral infection (22, 23). However, evidence gathered after its initial discovery suggest that IRF1 also exhibits a myriad of cellular functions such as antitumor and immune regulatory properties (24-29). The antitumor properties of IRF1 are exhibited through its transcriptional regulation of antitumor genes and oncogenes (30). For example, IRF1 has been reported to induce an increase in the expression of antitumor genes, such as major histocompatibility complex I and II (MHC I & II) (31, 32) and p53 (33, 34) but induces a reduction in the expression of tumour-promoting genes such as Cyclin D1(35) and survivin (36). Studies have also shown that the dysfunctioning of IRF1 has been linked to the development of several different types of cancers in humans (26). In fact, it has been reported in breast cancer patients that IRF1 expression is decreased in neoplastic breast tissues compared to normal counterparts (37). In addition, the mRNA expression level of IRF1 has been reported to be inversely linked to the grade of the tumour, risk of recurrence and death of breast cancer patients (38, 39). It has also been reported that the IRF1 expression level is reduced or completely lost in lymphoma and colon cancer (40, 41). Taken together, the above evidence strongly indicates anti-tumour roles of IRF1. It is a common knowledge that majority of drugs that are used in the treatment of human diseases including cancers have more than one target through which they exhibit their effects. Therefore, demonstration that CRYP induces an increased expression of the transcript level of IRF1 in the current study raises the possibility that IRF1 signalling could be one of the possible several ways through which CRYP could exhibit its anticancer mechanism and that CRYP or its analogues may be considered and developed as new anticancer agents.

The biological actions of PR signalling depend highly on the context. While some studies have reported that PR signalling promotes proliferation or differentiation, others have reported that PR signalling inhibits proliferation or differentiation depending on the conditions. In fact, some studies have reported that the role of PR signalling in breast tumour may be dependent on the stage of disease progression or the tumour type (42). Mohammed et al., (2012) reported that PR signalling can antagonize the proliferative effects of oestrogen in breast carcinomas (43). In addition, the presence of PR in primary breast carcinomas has been reported to be a marker of a very positive prognosis and is associated with a less aggressive cancer than PR negative tumours and that the presence of PR in the primary tumour is also associated with better overall survival (44, 45). Therefore, the ability of CRYP to induce an increase in the transcript expression level of PR could be targeted and exploited as a possible mechanism through which breast cancer can be treated.

HIF-1 α is a major regulatory gene that is involved in the cells' response to a reduced oxygen supply. It plays a significant role in the progression and spread of tumour through activation of genes that are linked to the regulation of angiogenesis, cell survival, energy metabolism, and apoptotic and proliferative responses (46-48). Studies have shown that tumours that lack HIF-1 α , tend to exhibit a significant reduction in vascularization and growth rates compared with normal cells (49, 50). In fact, HIF-1 α has been reported to be overexpressed in many human malignancies, such as colon, breast, pancreas,

prostate, kidney, stomach, and oesophageal cancers compared to their respective normal tissues (51-53). Thus, reports indicate that overexpression of HIF-1 α is associated with poor clinical outcomes in patients with various cancers and that expression of HIF-1 α is associated with poor survival in cervical cancer (54), endometrial carcinoma (55), oligodendroglioma (56), ovarian cancer (57) and different breast cancer subtypes (58-60). All these pieces of information point to the fact that therapeutic targeting of HIF-1 α has the potential to improve cancer treatment efficacy and survival of cancer patients. Therefore, the demonstration that CRYP inhibits HIF-1 α even at the transcript level suggests that CRYP could be exploited and developed as emerging and novel anti-cancer agents.

The multitude of evidence in the literature has shown that *STAT3* plays a significant role in the development, progression and maintenance of many tumours in humans (61-65). An increase in the level of activated *STAT3* has been shown to correlates with recurrent tumours and poor prognosis of many human cancers (66-68). Studies have shown that STAT-3 activated genes block apoptosis, promote cell proliferation and survival, enhance angiogenesis and metastasis and inhibit anti-tumour immune response (62-65, 69). On the other hand, available evidence suggests that any process or approach that disrupts *STAT3* signalling may result in inhibition of growth and apoptosis in tumour cell lines and can inhibit tumour growth in mouse xenograft cancer models (64, 65, 70-72). Therefore, the ability of CRYP to inhibit the transcript expression level of *STAT3* at low concentration in the current study suggest that CRYP can be exploited further and developed as a cheaper and novel anti-cancer drug agent.

As to why the lower concentration of CRYP (2.5 μ M) induced a significant decrease in the transcript expression level of *STAT3* but a relatively higher concentration (5 μ M) had no significant detectable effects on the transcript expression level of STAT3 is a subject for further research.

Conclusions

The current study identified some novel human pro- and anti-cancer signalling pathways regulated by CRYP in line with scientists' suspicion that CRYP has anti-cancer potential. The outcome of the current study offers promising avenues for further research into the development of CRYP as a novel anticancer agent. Achieving this feat would improve the treatment success of cancers and increase the chances of survival of cancer patients in low- and middle-income countries. Also, further studies are required to confirm the protein levels of all the genes reported in Fig. 2 of the current study.

Abbreviations

CRYP: Cryptolepine; IRF-1: Interferon regulatory factor-1; PR: Progesterone receptors; HIF-1 α : Hypoxia inducible factor-1 alpha; STAT3: Signal transducer and activator of transcription 3; IARC: International agency for research on cancer; HBV: Hepatitis B virus; HCV: Hepatitis C virus; HPV: Human papillomavirus; DMEM: Dulbecco's modified eagles' medium; FBS: Foetal bovine serum; MEM: Minimum essential medium; NEAA: Non-essential amino acids; MTT: Thiazolyl blue tetrazolium bromide powder;

HEK 293 cells: Human embryonic kidney cells; MHC I & II: Major histocompatibility complex I and II; KNUST: Kwame Nkrumah University of Science and Technology

Declarations

Data availability

The raw datasets analyzed during the current study will be made available from the corresponding author on a reasonable request.

Conflicts of interest

The authors declare that they have no competing interests regarding the publications of this paper.

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Authors' contributions

MM and GA conceived the idea and designed the experiments. PWN and SAD performed the experiments, analyzed the data and wrote the manuscript. GA provided the funding for the study. All authors read and approved the final manuscript.

Consent for publication

Not applicable

Ethics approval and consent to participate

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Figures

Figure 1

HEK 293 cells and cytotoxicity of CRYP. CRYP cytotoxicity was evaluated by MTT assay on HEK 293 cells treated with CRYP (0-10μM) for 24, 48 and 72 hours. The viability of cells was calculated as the ratio between CRYP-treated cells and non-treated cells (equal volume of PBS replacing CRYP). Data are presented as mean and standard deviation of three independent experiments each performed in triplicate wells, p-value = 0.03 as determined by one-way ANOVA. The error bars represent the standard deviation among the three independent experiments but are too small to show.

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Figure 2

Effects of CRYP on 45 signaling pathways. The relative expression levels of the 45 signaling pathways in HEK 293 cells after treatment with 5μM of CRYP was evaluated using the dual luciferase reporter gene assay. The results were expressed as log2 of the fold change of the expression of transcription factors between CRYP-treated cells and non-treated cells.

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Figure 3

CRYP induces a decrease in the relative mRNA expression levels of (a) HIF-1α and (b) STAT3 in HEK 293 cells after treatment with increasing concentrations of CRYP (0, 2.5 and 5.0μM). The relative mRNA expression levels of HIF-1α and STAT3 after normalization to B-actin (endogenous control) were expressed as means ± standard deviations. The relative levels of HIF-1α and STAT3 were calculated using the relative standard curve method. The untreated cells (0.0μM) were used as reference group to estimate relative gene expression changes. For HIF-1α, **P-value <0.001. For STAT3, **P-value < 0.0004. The error bars represent standard deviations of triplicate wells in an experiment.

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