In vivo Study of a Newly Synthesized Chromen-4-one Derivative as an Antitumor Agent against HCC

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Abstract
Background Chromenes are a wide group of natural compounds that can be synthesized chemically. The chromen-4-one nucleus acts as a skeleton for varieties of additional active groups that makes the chromene activity vary between antioxidant and anti-inflammatory agents. In the present study, a newly synthesized chromene compound exhibits different behaviors other than anti-inflammatory and antioxidant activities that it is the first time that a member of chromen-4-one compound can control the cancer progress. Inflammation is the first step in tumor development where the severity grade can potentiate tumor growth and progression. In many tumors, pro-inflammatory genes record high expression level such as tumor necrosis factor (TNF-α) and vascular endothelial growth factors (VEGF). These pro-inflammatory factors act as rate limiting steps in tumor initiation, and controlling its expression acts as an early therapeutic way to control the tumor proliferation. The chromone derivatives have biological activities such as anti-inflammatory and anti-tumor activity.

Methods In the present study, hepatocellular cancer (HCC) induced by diethylnitrosamine (DEN) in rats and then treated with the new chromone derivative and the parameters TNF-α, VEGF, p53, Cyt C, MMP-9, Bcl2, and Bax were measured.

Results The treatment strategy Ch compound is to downregulate pro-inflammatory gene expression of early genes as TNF-α as well as VEGF and subsequently control other factors such as p53, Cyt C, and MMP-9. Also, retrieve the balance between Bcl2 and Bax proteins in DEN-induced HCC in rats.

Conclusion The ability of the new Ch derivative to control the primary initiators of HCC such as TNF-α offers this derivative an anti-tumor activity and encourages further researches to follow and monitor its effect on the molecular level.

Keywords HCC · Chromene derivative · TNF-α · VEGF · p53 · Cyt C · MMP-9 · Bcl2Bax

Background
Chromones (benzopyran-4-one) are known as bioactive agents due to their anti-inflammatory properties. Moreover, some of chromones are natural such as UMB, 7-hydroxycoumarin, in golden apples with its antioxidant property and can be used as modulator to protect against gamma radiation [1].

Also, one of the 5,7-dihydroxy-chromen-4-one (DCO-6) inhibits (NO) is produced by lipopolysaccharide and considered as an anti-inflammatory agent [1].

Palifermin and amifostine are only two compounds used as radioprotectors in spite of many compounds of several classes that were studied as radioprotectors [2]. A novel chromone derivative ((2E) 2-(4-oxo-4H-chromen-3-yl)methylene amino-4-nitrobenzoic acid) shows anti-tumor activity towards EAC (Ehrlich Ascites carcinoma) cell line [3] and was tested as a promising radioprotector agent [4].
The majority problems of health facing the world recently are tumor and infectious diseases [5]. In developing countries, cancer is a main cause of death and new therapeutic strategies take great attention in biomedical researches [6]. Hepatocellular cancer (HCC) is a third fatal cancer leading to death [7].

HCC is related to many risk factors such as HCV [8] which leads to a multistep cascade related to cytokines initiation of inflammation and activation of oxidative stress leading to chronic hepatitis, cirrhosis, fibrosis, and regeneration hepatic tissues [9]. In addition, carcinogenesis in hepatic tissues may be related to HCV gene overproduction causing liver proliferation [10].

Diethylnitrosamine (DEN) is known as powerful hepatocarcinogen causing malignant transformation present in tobacco, water, some fried foods, cheddar cheese, chemical fertilizers of plants, cosmetics, as well as some pharmaceutical products [11].

DEN induces damage of DNA repair hepatic enzymes; such damage leads to liver tumors of modeling in experimental animal [12] through hepatocellular accumulation of reactive oxygen species (ROS) [13]. The overload of DEN causes many dramatic changes such as induction of lipid peroxidation, which can be detected by the levels of MDA (malonaldehyde), as well as elevation of circulating AFP levels [14] and disturbance of p53 as tumor suppressor and pro-apoptotic protein, which subsequently shifts the balance between the anti-apoptotic Bcl-2 and apoptotic Bax proteins to tumorigenesis [15]. In addition, the MMP (metalloproteinase) which is involved in invasion and metastasis via disturbance of the mitochondrial permeability. Also, signals of mitochondrial Cyt c release to the cytosol. C (Cyt c) leads to activation of caspase-9. This event activates caspase-3 cleavage [16]. Treatment of tumors is related to several mechanisms of action of chemotherapeutic agents that should take place in the treatment of cancer [17].

The aim of this work is to study the previous chromone derivative to treat the HCC and study its mode of action.

Methods

The present study has been done at the Biology of Radiation Department, National Center of Radiation Research and Technology (NCRRRT), Cairo, Egypt.

Chemical Compound

Diethylnitrosamine (DEN) as well as chemicals were bought from Sigma-Aldrich Company, St. Louis, MO. The molecular formula of DEN is (C₂H₅)₂ NNO, and the molecular weight is 102.1

Chromene

The chromone derivative was previously prepared, designed, and characterized [3]. Its chemical structure is illustrated in Fig. 1.

In Vitro Studies

In the present study, the anti-tumor activity of triplicate samples of serially diluted Ch compound was determined against hepatocytocarcinoma cell line Hep G2 by MTT method by colorimetric change of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to blue product of formazan in mitochondria of viable cells. The color is measured by spectrophotometer at 595 nm by 990-win 6 software of ELISA microplate reader (DV990BV4), Roma, Italy [18].

Experimental Design and Animal Grouping

Male Wister rats (adult) weighting 120–150 g were purchased from the El-Nile Pharmaceutical and Chemical Industries Company, Cairo, Egypt. The dealing conditions and treatment were approval of the Institution of Animal Care and Uses Committee (IACUC) [19].

Forty male rats were selected randomly into four groups, with (10) animals in group as mentioned in Table 1.

Blood Samples

After completion of experiment, rats were incubated for 24 h and sacrificed under diethyl ether anesthesia. Collection of blood was done by heart puncture; the samples were centrifuged for 10 min at rate of 3000 rpm to obtain plasma for biochemical analysis.

Fig. 1 Chemical structure of Ch compound ((2E)-2-(4-oxo-4H-chromen-3-yl)methylene amino-4-nitrobenzoic. acid)
Liver Tissue

The liver tissue of experimental animals was dissected out and divided into 2 parts: one of them was embedded in 10% formalin for histopathology, and the second part was homogenized (10% weight/volume) in phosphate-buffered saline (0.02 M sodium phosphate buffered with 0.15 M of NaCl, pH = 7.4) in a glass tissue homogenizer with a Teflon pestle [21].

Experimental Parameters

Activities of L-alanine-aminotransferase (ALT) (ab105134) and aspartate-amino-transferase (AST) (ab105135) were estimated in plasma using colorimetric assays as described by Reitman and Frankel [22]. Serum samples were tested for alpha-fetoprotein (AFP) using (ELISA) kit of AFP from Cloud, Clone Corp. in the USA (catalog number MBS700622) while the Rat C-Reactive Protein ELISA is from Abcam, Canada (ab108827).

Homogenates of hepatic tissue samples were used to assay for malondialdehyde (MDA) (ab118970) that was spectrophotometrically determined according to Tsikas [23]. The TNF-α (DY510), VEGF (RRV00), cytochrome C (DCTC0), and MMP-9 (NBP2-13,173) concentrations were assayed by ELISA kit for rats (Novus Biological, USA).

Apoptotic Markers

Evaluation of Bcl2 and Bax gene expressions in HCC tissue samples. The anti-apoptotic (Bcl2) and Bax apoptotic gene expressions were quantified upon RNA extraction and synthesis of cDNA.

Fifty milligrams of EC tissues was used to isolate total RNA by TRizol reagent [24]. Then, the enzyme reverse transcriptase is used to synthesize standard cDNA from 1 mg of RNA as a template. The mentioned primers are in Table 2.

Quantitative real-time polymerase chain reaction (qPCR) RT-PCRs are assayed according to Pfaffl [25]. Expression mRNA of Bcl2 and Bax relative and the normalization average by reference β-actin.

Histopathological Examination

At the time of sacrifice, the liver tissues were excised from the animals. Samples from each liver tissue were fixed in 10% formalin and embedded in paraffin. Section of 5 microns thickness were cut and stained with hematoxylin and eosin [21] and examined by light microscope for histopathological investigation.

Statistical Analysis

Statistical analysis between differences of means was figured out using one-way analysis variance (ANOVA). In case of a significant (F-ratio), post hoc the least significant difference (LSD) test of multiple comparisons for evaluation of the statistical significance between treated groups at p < 0.05 significance level. Cross-tabulation analysis was carried out, and the significance (χ² [2]) was calculated at p < 0.01 to evaluate percentage of survival of rats. All of the statistical analysis was calculated using Statistical Package of Social Science (SPSS) version 20.0 (SPSS Inc., Chicago, IL, USA).

| Table 1 | Groups of rats |
|-----------------|----------------|
| Group 1 (control): | Animal receive intraperitoneal injection of saline |
| Group 2 (Ch): | Four weeks; animals were received (ip) injection with Ch in saline suspension (5 times per week); the dose is 20 mg/kg b.wt. [4] |
| Group 3 (DEN): | DEN dissolved in 0.9% saline, with a dose of 20 mg per kg b.wt per day, 5 times per week for 6 weeks [20] |
| Group 4 (DEN + Ch): | Rats were received DEN like group 3 after that treated with chromene for 4 weeks like group 2 after DEN gavage |

| Table 2 | Primer sequence of all studied genes |
|-----------------|-----------------|
| Gene symbol | Primers sequence | GenBank accession number |
| Bax | F: 5'-AGGGTGGCTGGGAAGGC-3' R: 5'-TGAGCCAGGGGTTAGG-3' | XM_039087751.1 |
| Bcl2 | F: 5'-ATCGCTCTGATGGATGAGTAC-3' R: 5'-AGGAGACGGCGGAGAATCAAAC-3' | NM_016993.2 |
| β-actin (rat) | F: 5'-TGT TGG AGA CCT TCA ACA CC-3' R: 5'-TAG GAG CCA GGG CAG TAA TC-3' | NM_031144.3 |
Results

In Vitro Study

The anti-tumor activity of Ch was estimated by using [HepG2] cell lines. The Ch derivative was tested at different concentrations, and results are plotted in Fig. 2.

In Vivo Studies

Effect of Ch Derivative Treatment on Liver Function Enzymes

Induction of HCC by DEN uptake significantly raise the concentrations of ALT and AST enzymes with respect with control group, but the levels of such enzymes decrease upon treatment with Ch derivative near the normal ranges (Fig. 3).

Effect of Ch Derivative on MDA of the Induced HCC in Rats

A significant induction of MDA in the HCC-induced group in the concentration with respect to control group and Ch group. While CH treatment lowers the concentration of MDA in a significant manner in treated group in comparison with HCC group (Fig. 4).

Effect of Ch Derivative on TNF-α and VEGF of the Induced HCC in Rats

The gavage of DEN induces the overexpression of TNF-α and VEGF as a pro-inflammatory initiator, but the ip injection of Ch derivative downregulates the TNF-α and VEGF significantly with respect to HCC-induced group by 48.58% and 55.46%, respectively (Fig. 5).

Effect of Ch Derivative Treatment on Cytochrome C

The exposure to DEN leads to lowering in the level of cytochrome c in comparison with normal control group. The Ch treatment with derivative elevates the level of cytochrome c significantly in comparison with DEN group (Fig. 6).

Effect of Ch Derivative Treatment on MMP-9

The gavage of DEN increases the level of MMP-9 in DEN group with respect to control group while the level of MMP-9 decreased significantly upon Ch treatment in group DEN+Ch group with respect to DEN group (Fig. 7).

Effect of Ch Derivative Treatment on p53

Figure 8 mentions the increase of p53 in DEN group and significantly exceeds the control group, and the Ch treatment

Fig. 2 Different concentrations of Ch derivative and activity against HCC cell line

Fig. 3 The changes of the liver function enzymes due to treatment with the Ch derivative a, b, and c denote significant change at $P<0.05$ versus control, chromene, and DEN groups, respectively

Fig. 4 Mean of values of MDA concentration in different treated groups compared with controls a, b, and c denote significant change at $P<0.05$ versus control, chromene, and DEN groups, respectively
compound leads to inhibition in the level of p53 in comparison with DEN group.

Effect of Ch Derivative Treatment on AFP and CRP

The treatment of rats with DEN elevates the AFP and CRP respectively significant with to control group while the treated HCC rats in (HCC + Ch) group with Ch derivative lead to decrease the levels of AFP and CRP (13% and 28%, respectively) in comparison with HCC group Fig. 9.

Effect of Ch Derivative on Apoptosis

The balance between the Bcl2 and Bax proteins is very important in normal cases, but a balance shift appears upon DEN gavage by increasing Bcl2 protein and decreasing Bax to keep the viability of tumor cells while this balance is returned again near the normal by treatment with Ch derivative (Fig. 10).

Histopathological Examination

Studying the histological section of male rat liver under light microscope showed that there was no histological alteration observed and the normal controls histological structure. Liver tissue section of male rats treated with chromene under light microscope shows spotty necrosis. Treatment with diethylnitrosamine under light microscope showed that, showing hepatocellular carcinoma formation (note the trabecular and pseudoacinar pattern). The histological section of HCC-induced male rat liver treated with chromene showed markedly dilated congested blood vessels and areas

![Graph](image1.png)  
**Fig. 5** Mean values of TNF-α and VEGF concentrations in different treated groups compared with controls a, b and c denote significant change at $P<0.05$ versus control, chromene, and DEN groups, respectively

![Graph](image2.png)  
**Fig. 6** Mean values of cytochrome C concentration in different treated groups compared with controls a, b, and c denote significant change at $P<0.05$ versus control, chromene, and DEN groups, respectively

![Graph](image3.png)  
**Fig. 7** Mean values of MMP-9 concentration in different treated groups compared with controls a, b, and c denote significant change at $P<0.05$ versus control, chromene, and DEN groups, respectively
of hemorrhage as well as tumor tissue degenerative changes and apoptosis as recorded in Fig. 11.

Discussion

Hepatocarcinogenesis is a complicated process including several steps, with a plenty of signals, leading to a diversified molecular profile [26].

DEN generates ROS which result in DNA damage, mutations, and cancer [27]. Application of DEN is accompanied by overexpression of inflammatory cytokines such as TNF which is the rate limiting step of carcinogenesis cascade. The oxidative stress produced upon DEN application can activate NOX1 (NADPH oxidase) axis which subsequently activate Kupffer cells and newly recruited macrophages to express TNF in abnormal concentration to start the inflammation and carcinogenesis [28].

In the present study, the Ch compound exerts an ability to inhibit TNF-α molecules. Two docking studies were carried out to deduce that chromen-4-one nucleus has special behavior to inhibit TNF-α. They mentioned that the TNF-α molecule has pharmacophoric features that can act as hydrogen bond acceptors as the chromen-4-one has aromatic rings and (C=O) in aromatic ring which can donate electrons to the residues Leu 120 and Gly 121 in the TNF-α in addition the two aromatic rings that can donate electrons to Leu 57, Gly 122, and Ile 58 [29, 30].

The data of the present study revealed that the level of TNF-α was increased in the DEN induced HCC but decreased upon chromone treatment; our finding was supported by Afzal et al. [31] who mentioned that, once the expression of TNF-α increased, the cascade of inflammation starts to activate tumor promotion, angiogenesis, proliferation, and survival via activation of NF-kB.

The angiogenesis is initiated by upregulation of VEGF as a step next to NF-kB activation [31]; VEGF is known as an angiogenic agent which has a motivated facility for angiogenesis and growth of tumors and metastasis in the conditions like HCC [32].

Saleem et al. [33] observed that levels of VEGF in the DEN group were significantly elevated implying advanced pace of angiogenesis which supports our results of increasing VEGF level in the group treated with DEN.

Also, the activation of NF-kB initiates overexpression of other tumor promotors such as MMP-9 and COX-2 [34].
In the present study, MMP-9 level is increased in the group treated with DEN while the treatment with chromene derivative decreases the MMP-9 level.

Among them, MMP-2 (72 kDa) and MMP-9 (92 kDa) are found to regulate tumor invasion and metastasis. Researches in malignant tumors reported the overexpression and enhanced activity of both of these MMPs [35]. Enhanced metastasis is evidently linked with enhanced MMP-2 and MMP-9 expressions as a tumor progressor. On the other hand, COX-2 enzyme enhances tumor through overproduction of MDA as a biproduct of COX-2 catalyzed breaking down of PGH 2 (prostaglandin H2) [36].

Malondialdehyde is considered as a mutagen in mammalian cells and carcinogen for rats [37] due to its affinity toward DNA to form adducts with deoxyguanosine to yield pyrimidopurinone–deoxyguanosine; both MDA carboxyls react with nitrogen, forming the pyrimido[1,2-α]purine-10(3H)-one-2′-deoxyribose, or malondialdehyde-2′-deoxyguanosine adduct (M1dG) [38] and N6-(3-oxoprenyl) deoxyadenosine (M1dA) and N4-(3-oxoprenyl) deoxyxycytidine (M1dC) [39]. Although, formation of DNA adducts is a fateful step in cancer development, which can be exerted by another pathway in addition to the effect of MDA. That said, DEN itself is not a carcinogen but it exerts its carcinogenic effect after metabolism in hepatic cytochrome P450 (CYP) enzymes to yield alkylated guanine [40] while Kang et al. [41] revealed that the CYP-deficient mice show less tumor incidence in comparison with the wild type. Also, as the CYP enzymes decrease, the bioactivation of DEN is declined leading to less availability of formation of DNA adduct and less tumorigenesis [42]. As a normal defense response, the hepatic cells start to express the protein p53 to obligate the mutated cells for entrance the apoptotic pathway [15]. The mode of action of p53 involves stimulation of reactive oxygen species so that the overproduction of p53 leads to more inflammatory response, accumulation of ROS, and initiation of tumorigenesis in the surrounding environment as mentioned by Yan et al. [43] who recorded the accumulation of p53 in wild-type rates treated with DEN with histopathological evidence of malignant hepatic tumors while less p53 filtration in hepatic p53+/- rats with lower risk of tumor formation.

Chiu et al. [44] studied two groups of humans with HCC: the first group is negative immune staining of p53, and the second group is positive immune staining p53. They found an increase in the level of Bax in both groups, but the expression of Bcl2 decreased in the first group and increased in the second group with more advanced histopathological of tumor grade. They explain their results by finding out the ratio of anti-apoptotic and pro apoptotic proteins Bcl2 and Bax where this ratio decreased in the first group immune negative p53 leading to apoptotic fate of the tumor cells but increased in the second group immune positive p53 to exceed the percentage 88% with respect to controls; that means that the tumor cells have more tendency to an anti-apoptotic behavior in group 2.

It has been demonstrated extensively that translocation of Bax from cytosol to mitochondria facilitates the release of the cytochrome c [45] because Bax can form channels, which allow direct cytochrome c release [46].

The Bcl2 itself binds to pro apoptotic members such as Bax, preventing pore formation and cytochrome c release [47]. In contrast, increase in expression of Bax induces cell death eliminating tumor cells [48].

It has been suggested that a high ratio of Bax to Bcl2 can lead to collapse of mitochondrial membrane potential, resulting in the release of cytochrome c and consequently causes cell apoptosis [49]. Our data also confirm decreased Bcl2 protein expression; its inhibitory effect on Bax and caspase-9 was removed and leads to overexpression of Bax and finally activation of caspase-9. Although, activation of caspase-9 also leads to loss of mitochondrial membrane potential by cleaving anti-apoptotic members of Bcl2 family including Bcl-xL and Bcl2 [50].

However, it has been recently reported that ethyl 2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14-1) binds to Bcl2 protein and blocks its anti-apoptotic function in HL-60 cells [51].

The newly synthesized Ch derivative can control the cross talk between the signals in the tumor cells to diminish its progress as it started with inhibition of TNF-α which down-regulates the NF-kB. The central role of NF-kB [32] is to control many signals such as VEGF [33] and MMP-9 [34] as well as controlling the enzymes of cytochrome P450 [52].
All these events rebalanced the ratio of disturbed Bcl-2 and Bax to sustain the normal status [50].

Our results revealed that increase in the Bcl2 in the DEN treated group accompanied by drop in the level of the Bax leading to anti-apoptotic behavior appears in pathological observation while the treated group with DEN + Ch shows rebalanced Bcl2 and Bax levels near the control group.

The previous events are concerned with the cell proliferation which appeared as elevation in alpha fetoprotein (AFP) to develop the proliferation in HCC [53]. This finding supports our data where the treatment with DEN leads to elevation of AFP parallel to overexpression of p53. In addition, after the inflammatory event and DNA disturbance affected by DEN administration, the inflammatory cascade reaching HCC, the hepatic function is disturbed as shown in the elevation of liver function enzymes ALT and AST [43] as well as increase in the expression of CRP in the HCC-induced group because hepatocyte is the main origin of the CRP and its synthesis is increased due to inflammatory stimulation [54, 55].

The administration of Ch derivative treats with the inflammatory event by deactivation of NF-κB which in turn controls the cascade of inflammation; this finding is supported by Elshawi and Nabeel [4].

Conclusion

Tumor is one of the inflammatory response drawbacks, but the mentioned Ch derivative has the ability to downregulate TNF-α and VEGF which, subsequently, cut off the crosstalk between other inflammatory, proliferation, and angiogenesis mediators to decrease the severity of tumor development and the anti-apoptotic behavior of the tumor cells that can be rebalanced again upon Ch administration. Other studies should be done to detect more about the chromene-based compound behavior and their ability to sustain the normal status.

Abbreviations  TNF-α: Tumor necrosis factor alpha; VEGF: Vascular endothelial growth factors; HCC: Hepatocellular cancer; DCO-6: (E)-5,7-dihydroxy-3-(3-oxo-3-phenylprop-1-en-1-yl)-4H-chromen-4-one; NO: Nitric oxide; EAC: Ehrlich ascites carcinoma; HCV: Hepatitis C virus; MDA: Malonaldehyde; ROS: Reactive oxygen species; MMP: Metalloproteinase; Cyt c: Cytochrome C; NCRRT: National Center for Radiation Research & Technology; DEN: Diethylnitrosamine; Ch: [(2E)-2-(4-oxo-4H-chromen-3-yl)methylene amino-4-nitrobenzoic acid; IACUC: Institutional Animal Care and Use Committee; ALT: L-alanine aminotransferase; AST: Aspartate amino transferase; ELISA: Enzyme-linked immunosorbent assay; AFP: Alpha-fetoprotein; RT-PCR: Real-time polymerase chain reaction; cDNA: Cloned deoxyribonucleic acid; RNA: Ribonucleic acid; qPCR: Quantitative real-time polymerase chain reaction; LSD: Least significant difference; SPSS: Statistical Package for Social Science

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Author Contribution  All authors have read and approved the manuscript. A. I. Nabeel donated the chromen-4-one new derivative and wrote the manuscript. S. Z. Mansour shared the laboratory and animal house for work. E. M. E. Mahdy contributed the idea and plan of work. H. A. El-Mezayen revision role. S. A. Mohamed apply the practical work and share the writing of manuscript.

Availability of Data and Material  Available.

Declarations

Ethics Approval and Consent to Participate  Animal dealing conditions and treatment were guided as per the National Institute of Health Guide for Animal and approved by the Institutional Animal Care and Use Committee (IACUC). Reference: National Research Council (US) Committee for the Update of the Guide. Guide for the care and use of laboratory animals. 8th ed. Washington, DC: National Academies Press, 2011.

Consent for Publication  Not applicable

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