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Anticancer activity of new coumarin substituted hydrazide–hydrazone derivatives

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ABSTRACT

Drug resistance is a major impediment for cancer treatment, to overcome it we designed and synthesized sixteen coumarins bearing hydrazide–hydrazone moiety and evaluated them against human drug-resistant pancreatic carcinoma (Panc-1) cells and drug-sensitive (hepatic carcinoma; Hep-G2 and leukemia; CCRF) cell lines *in vitro*. The 6-brominated coumarin hydrazide–hydrazone derivatives (BCHHD) **7c**, **8c** and **10c** were more potent than doxorubicin (DOX) against resistant Panc-1 cells. BCHHD **7c** showed significant cytotoxicity against all tested cells (IC₅₀: 3.60–6.50 μM) on comparison with all other coumarin hydrazide–hydrazone derivatives (CHHD), whereas BCHHD's **8c** and **10c** showed significant antiproliferative activity only against resistant Panc-1 cells with IC₅₀ of 2.02 μM and 2.15 μM, respectively. All the investigated BCHHD's were able to activate caspases 3/7 and they could induce apoptosis in resistant Panc-1 cells. Microarray analysis showed that BCHHD **7c** induced the expression of apoptotic- and cell cycle arrest (G2/M)- genes in resistant Panc-1 cells. Moreover, BCHHD **7c** induced the up-regulation of CDKN1A, DDIT4, GDF-15 and down-regulation of CDC2, CDC20, CDK2 genes. Based on our results, we conclude that **7c** could be a potent anticancer drug to overcome drug resistance in cancer and it could be highly beneficial for patients in the clinic.

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1. Introduction

Drug resistance constitutes lack of response to many chemically and mechanically unrelated anticancer agents by cancer cells. It is one of the main causes for failure of chemotherapy and can lead to recurrence of disease or even death [1]. Clinical administration of high doses of anticancer drugs to overcome resistance leads to drug-induced toxicities [2]. Hence newer anticancer agents need to be synthesized and tested for its efficacy both *in vitro* and *in vivo* to overcome drug resistance.

The natural and synthetic coumarins attract great attention due to their wide range of biological properties, including anticancer [3], anti-HIV [4], anti-inflammatory [5] and antibacterial [6] activities. Furthermore, their cancer chemopreventive properties have been recently emphasized [3,7]. The apoptosis and differentiation-induced activities of coumarins extend to several different cell line

models *in vitro*, and they appear to be the most promising in terms of cancer treatment [8].

Coumarins could exert their anticancer activity by different mechanisms; either by inhibiting the telomerase enzyme [9], inhibiting protein kinase activity and down regulating oncogene expression [10] or by inducing the caspase-9 mediated apoptosis. Additionally, researchers showed that coumarins are able to suppress cancer cell proliferation by arresting cell cycle in G0/G1 [9], G2/M phases [11], and through affecting the p-gp of the cancer cells [12,13]. It was also reported that hydroxycoumarins might exert their anticancer activity by generating free radical species in cancer cells producing oxidative stress leading to pro-apoptotic effect [7]. It was proven that the δ -lactone ring of the coumarinic system has a fundamental role in both the generation and stabilization of such species as well as in the pro-apoptotic action of hydroxycoumarins [7]. Moreover, the antiproliferative activity of 7-hydroxycoumarin derivatives could be due to their effect on the mitochondrial thiol compounds of cancer cells [14].

Literature survey revealed that the hydrazide–hydrazone (–CO–NH–N=CH–) moiety has significant role as antitumor

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agent [15–18]. On the other hand, Nerkar et al. reported that the *in vitro* anticancer activity of some carbohydrazone derivatives is due to their ability to inhibit dihydrofolate reductase enzyme [19]. It was also reported that furan, thiophene, pyrrole and isatin derivatives have cytotoxic activities against several cancer cell lines [18].

Based on the afore-mentioned findings, and in an attempt to find new potent anticancer agents; novel hybrid compounds having coumarin hydrazide–hydrazone backbone have been designed to evaluate their cytotoxic activity against several tumor cell lines (Fig. 1). Moreover, the possible underlying mechanisms of action have been also investigated.

2. Results and discussion

2.1. Chemistry

The synthesis of the target compounds is summarized in Scheme 1. To synthesize the CHHD's 7–10, several 2-cyanoacetohydrazones 2–5 were prepared by condensing 2-hetaryl aldehydes with 2-cyanoacetohydrazide (1) or by combining isatin with 2-cyanoacetohydrazide (1) [20–23]. The chemical structure of the newly synthesized *N'*-((1*H*-pyrrol-2-yl)methylene)-2-cyanoacetohydrazide (4) was established on the basis of analytical and spectral data. Its IR spectrum displayed absorption bands at 3325, 3265, 2260, 1665, 1635 cm^{-1} due to the presence of two NH, CN, amidic C=O, and C=N groups, respectively. The ^1H NMR spectrum of 4 revealed four singlet signals at δ 3.67, 6.75, 8.43, 8.93 ppm assigned to the methylene group, amidic NH, azomethine CH=N, and pyrrole NH, besides three aromatic protons centered around 7.22 ppm due to the pyrrole ring residue. The Knoevenagel condensation of 2-hydroxybenzaldehydes 6a–d with 2-cyanoacetohydrazones 2–5 in refluxing ethanol containing a catalytic amount of piperidine followed by treating the product with dilute HCl afforded CHHD's 7–10 in high chemical yield. The structures of the latter CHHD's 7–10 were elucidated on the basis of their spectra (IR, ^1H NMR, ^{13}C NMR, and MS) and elemental analyses. For example, their IR spectra showed the absence of CN absorption bands present in IR spectrum of their precursors 2–5, and revealed the presence of coumarin C=O absorption bands in the region 1679–1690 cm^{-1} . Their ^1H NMR spectra, in addition to the expected signals due to the aromatic protons, exhibit three singlet signals near δ 8.54, 8.63, and 9.23 ppm assignable to the protons of azomethine (CH=N), chromene-H₄ and amidic NH, respectively. Analysis of the ^{13}C NMR spectra of the

coumarins revealed the presence of a new signal for the carbonyl group of coumarin at approximately 160 ppm. CHHD's 7–10 exhibited peaks corresponding to their molecular ions $[\text{M} + \text{H}]^+$ in the ESI-mass spectrum.

2.2. Biology

2.2.1. Antitumor evaluation

Currently, we are studying the synthesis and antiproliferative activity of compounds having coumarin and/or hydrazide–hydrazone pharmacophores. The initial antiproliferative screening showed that compounds having coumarin hydrazide–hydrazone pharmacophore were more potent than compounds having coumarin or hydrazide–hydrazone pharmacophores (data not shown).

All CHHD's 7–10 were screened for their *in vitro* cytotoxic and growth inhibitory activities against three different tumor types, namely resistant Panc-1, Hep-G2 and CCRF, in comparison with the activity of the known anticancer reference drug DOX. The cytotoxic activities of our tested compounds were expressed as IC_{50} μM value (the dose that reduces survival to 50%) (Table 1).

Regarding the activity of CHHD's 7–10 against resistant Panc-1 cell line, the results in Table 1 showed that BCHHD's 8c and 10c possessed the highest degree of cytotoxicity. They were three times more active than DOX (Fig. 2). On the other hand, BCHHD 7c was almost equipotent to DOX (IC_{50} : 6.50 μM). CHHD's 8a (IC_{50} : 7.87 μM) and 7b (IC_{50} : 8.75 μM) were quite less potent than DOX. The activity of the tested CHHD's against resistant Panc-1 cell line had the following descending order: (8c > 10c > 7c > 8a > 7b > 8b > 7a > 10a > 10b > 9c > 9a > 7d > 8d > 9b > 10d > 9d).

Concerning Hep-G2 cell, it is evident that all of the tested CHHD's 7–10 showed antitumor activities with IC_{50} values ranging from 3.60 to 40.30 μM . Interestingly, BCHHD's 7c (IC_{50} : 3.60 μM) and 9c (IC_{50} : 4.16 μM) were more potent than reference drug DOX (IC_{50} : 5.43 μM) (Fig. 3). On the other hand, the CHHD 9b had moderate activity (IC_{50} : 9.29 μM). The activity of the tested compounds against Hep-G2 cell line had the following descending order: (7c > 9c > 9b > 10c > 9d > 7b > 8b > 8d > 9a > 10d > 7a > 10a > 10b > 7d > 8c > 8a).

The CHHD's 7–10 were also screened against CCRF cancer cell line. The results showed that most of the tested CHHD's were weaker than DOX and BCHHD 7c was the most active. Although, 7c is fairly less potent than DOX against CCRF cell line (IC_{50} 5.15 μM vs 1.05 μM respectively), it is still promising antitumor agent against leukemia since the chemotherapeutic DOX has known cardiotoxic side effect [24].

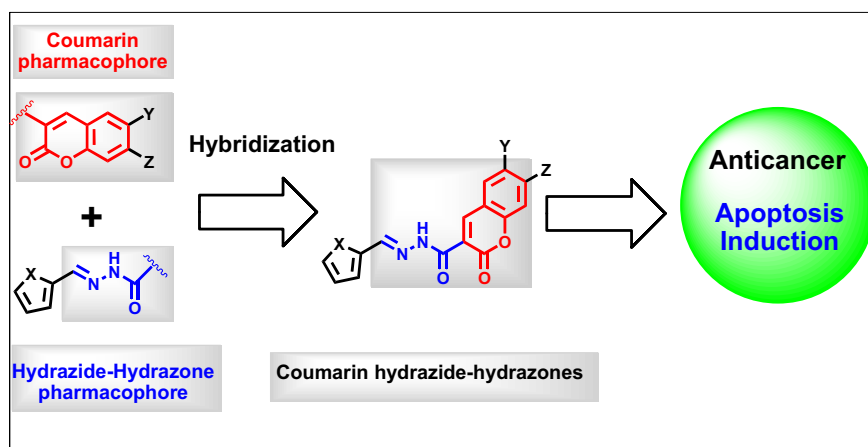
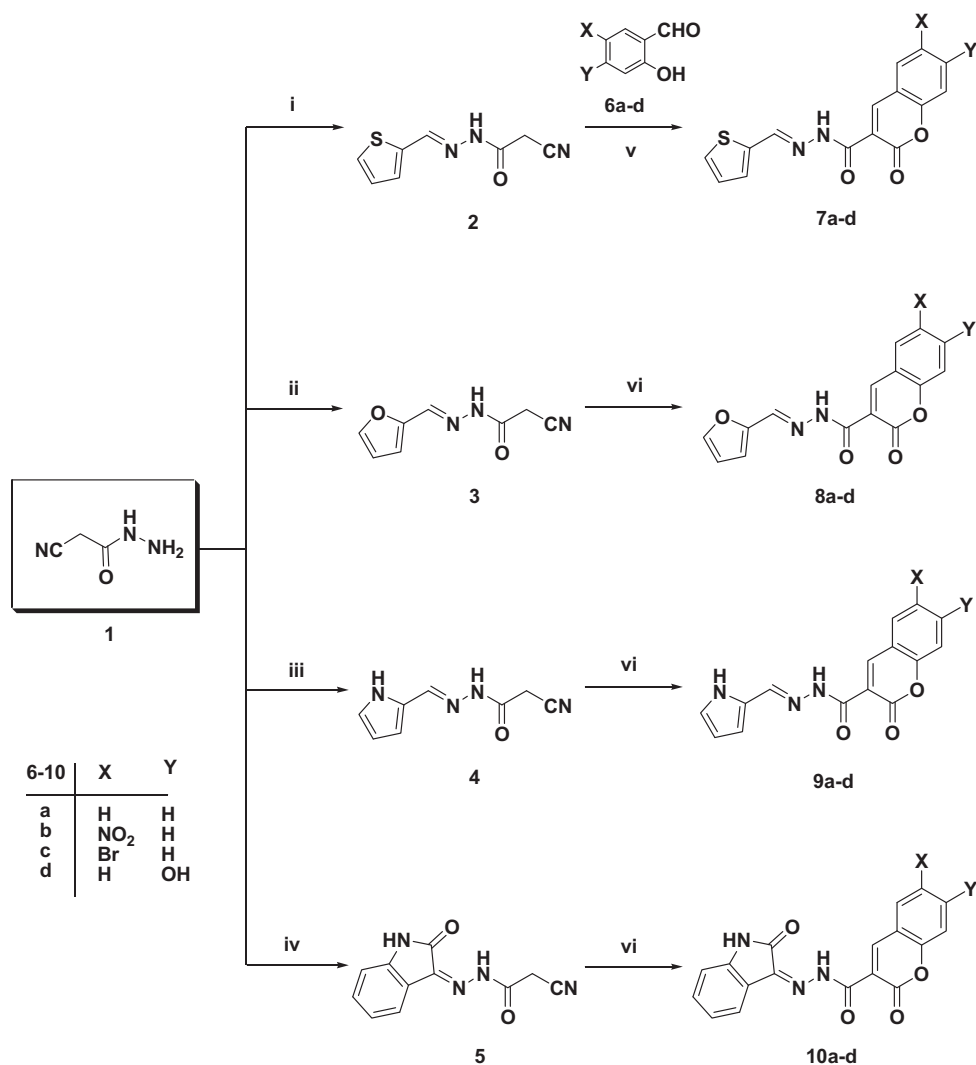


Fig. 1. Design of CHHD's as anticancer agents.



i) Thiophene-2-carbaldehyde, ethanol, acetic acid, reflux, 30 min.; ii) furan-2-carbaldehyde, ethanol, acetic acid, reflux, 15 min.; iii) 1*H*-pyrrole-2-carbaldehyde, ethanol, acetic acid, reflux, 30 min.; iv) isatin, ethanol, acetic acid, reflux, 30 min.; v) ethanol, piperidine, reflux, 6 h.; vi) compound **6a-d**, ethanol, piperidine, reflux, 6 h.

Scheme 1. Synthesis of substituted CHHD's **7–10**.

The most potent compounds against CCRF are illustrated in Fig. 4. The activity of the tested compounds against CCRF had the following descending order: (**7c** > **9a** > **9c** > **7b** > **10c** > **8c** > **8b** > **7a** > **9d** > **8a** > **10a** > **8d** > **7d** > **7b** > **9b** > **10d**).

The results displayed in Table 1 showed that all the synthesized CHHD's **7–10** had broad spectrum antitumor activity against all screened cancer cell lines regardless of tumor type. Some of our CHHD's exhibited very potent antiproliferative activity against resistant Panc-1 and Hep-G2 cell lines. These results suggest that the coumarin hydrazide–hydrazone backbone is an interesting antitumor pharmacophore. Moreover, some CHHD's were even more potent than the standard drug Dox. The most potent compounds in this study were BCHHD's **7c**, **8c**, **9c** and **10c**. This could be explained by the electronegative effect of the bromine atom on the coumarin hydrazide–hydrazone backbone (Fig. 5).

BCHHD's **7–10** were the most promising anticancer agents as they were highly potent against all the tested cell lines. Interestingly, BCHHD's **8c** and **10c** showed very high potency against

resistant Panc-1 cell line. On the other hand, 7-hydroxy CHHD's had the weakest anticancer activity in this study (Fig. 6).

Regarding the structure activity relationship (SAR) of our compounds; the thiophene and pyrrole substituted CHHD's were the most active against Hep-G2 and CCRF cancer cell lines. Furan and isatin substituted CHHD's were the most active against Panc-1 cell line; while, pyrrole derivatives were the least active against the same cell line. The sensitivity of the tested cell lines to CHHD's **7–10** were in the following descending order: resistant Panc-1 > CCRF > Hep-G2.

2.2.2. Caspase 3/7 assay

At the cellular level, apoptosis is a programmed cell death and is an important controller of physiological growth. In addition, it regulates tissue homeostasis. The stimulation of apoptosis signal transduction pathways in cancer cells is the main mechanism for the activity of currently available chemotherapy and/or immunotherapy [25,26].

Table 1

In vitro anticancer activity of CHHD's **7–10** against resistant Panc-1, Hep-G2, and CCRF cancer cell lines.

Compounds	IC ₅₀ (μ M)		
	Panc-1 cells	Hep-G2 cells	CCRF cells
7a	10.95 \pm 0.31	24.00 \pm 2.80	15.19 \pm 4.39
7b	8.75 \pm 3.06	20.10 \pm 2.09	21.90 \pm 2.25
7c	6.50 \pm 0.16	3.60 \pm 0.63	5.15 \pm 0.25
7d	14.65 \pm 5.20	29.47 \pm 2.40	21.87 \pm 1.25
8a	7.87 \pm 0.94	40.34 \pm 1.35	16.14 \pm 0.07
8b	9.70 \pm 1.46	21.29 \pm 3.19	13.58 \pm 2.86
8c	2.02 \pm 0.64	31.14 \pm 1.71	11.73 \pm 1.64
8d	15.44 \pm 2.45	22.22 \pm 0.68	18.37 \pm 1.35
9a	14.63 \pm 3.19	22.48 \pm 1.15	8.45 \pm 0.82
9b	17.95 \pm 5.66	9.29 \pm 1.58	25.27 \pm 0.83
9c	12.61 \pm 0.21	4.16 \pm 0.21	9.02 \pm 4.62
9d	22.16 \pm 1.79	16.47 \pm 1.38	15.23 \pm 2.26
10a	11.77 \pm 1.79	24.31 \pm 0.76	17.86 \pm 5.33
10b	11.78 \pm 3.80	26.90 \pm 4.29	10.15 \pm 0.95
10c	2.15 \pm 1.14	16.10 \pm 0.72	11.29 \pm 2.85
10d	19.92 \pm 0.29	23.95 \pm 0.59	26.62 \pm 2.19
DOX	6.90 \pm 0.32	5.43 \pm 0.24	1.05 \pm 0.01

To determine whether the chemosensitizing effect of our BCHHD's demonstrated above is secondary to their ability to activate the apoptotic cascade, the resistant Panc-1 cell line was treated with coumarin samples ($2 \times \text{IC}_{50}$; IC_{50} ; $\frac{1}{2} \times \text{IC}_{50}$) or DMSO (solvent control) using Caspase-Glo 3/7 assay. Fig. 7 shows that BCHHD's **7c**, **8c**, **9c** and **10c** caused significant increase in activation of caspase-3/7 in a dose dependent manner ($p < 0.05$). These results suggest that samples-induced apoptosis was, in part, due to activation of caspases 3/7.

2.2.3. Microarray analysis

The results of caspase 3/7 assay encouraged us to search for other molecular targets for BCHHD's in resistant Panc-1 as well as in Hep-G2 cell lines. For that purpose we performed gene

expression profiling using microarray technology. Differentially expressed genes were organized into functionally annotated networks.

2.2.3.1. Identification of molecular targets and pathways involved in the growth inhibitory effect of BCHHD 7c in resistant Panc-1 and HepG2 cells. For the identification of molecular targets, networks and pathways mediating the growth inhibitory effect of BCHHD's **7–10**; resistant Panc-1 and Hep-G2 cells were treated with BCHHD **7c** in DMSO or with DMSO (solvent control). Then, gene expression analysis has been carried out as previously described [27–29]. Microarray results showed that treatment of cells with **7c** significantly up-regulated 741 and 92 genes and down-regulated 549 and 43 genes in resistant Panc-1 and Hep-G2 cells, respectively ($P < 0.05$). Significantly regulated genes included apoptotic, cell cycle, growth factors, and tumor suppressor genes. Table 2 represents the top thirty up and down-regulated genes.

2.2.3.2. Pathway analysis and classification of significantly regulated genes. In order to get insight into canonical pathways and mechanisms involved in the modulatory effect of BCHHD's **7–10** on pancreatic and hepatic cancer cell growth, the Ingenuity Pathway Analysis (IPA) tool (www.ingenuity.com) was utilized as previously described [28,29]. The top ten canonical pathways, regulated after treatment of resistant Panc-1 cells included Mitotic Roles of Polo-Like Kinase, Estrogen-mediated S-phase Entry, Cell Cycle: G2/M DNA Damage Checkpoint Regulation (Fig. 1, supplementary data), Role of CHK Proteins in Cell Cycle Checkpoint Control and EIF2 Signaling (Fig. 2, supplementary data).

Additionally, DNA replication, recombination, repair, molecular transport, RNA trafficking, cell cycle, cellular assembly and organization were among the top ten regulated networks after **7c** treatment (data not shown).

Among the significantly up-regulated genes in resistant Panc-1 cell treated with BCHHD **7c** are growth differentiation factor-15

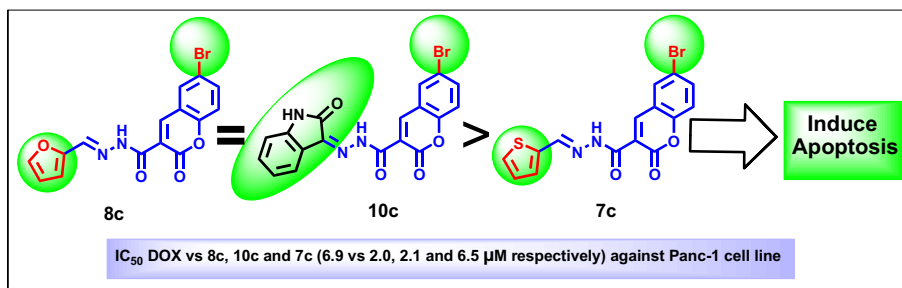


Fig. 2. The most potent CHHD's against resistant Panc-1 cancer cell line.

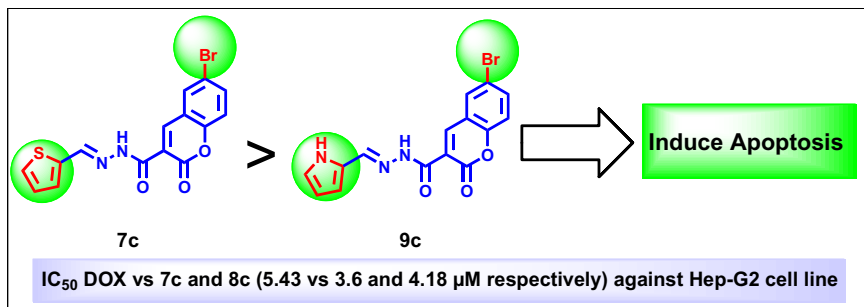


Fig. 3. The most potent CHHD's against Hep-G2 cancer cell line.

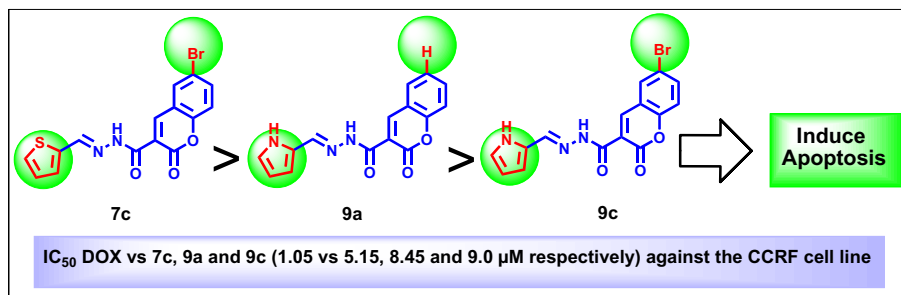


Fig. 4. The most potent CHHD's against DOX sensitive CCRF cancer cell line.

(GDF-15), cyclin-dependent kinase inhibitor 1A (CDKN1A), and DNA-damage-inducible transcript 4 protein (DDIT4).

Yim et al. [30] reported the isolation of the natural coumarin product, decursin, and showed that it had an antiproliferative effect against human prostate carcinoma cells. Decursin up-regulated CDKN1A and down regulated CDK2 genes as well as differentiating other genes. Our BCHHD **7c** regulated CDKN1A and CDK2 in a similar manner as decursin does. Also, it was reported that the antiproliferative effect of deferasirox against myeloid leukemias could be due to the up-regulation of the CDKN1A and GDF-15 [31]. Zimmers et al. [32] reported that loss of GDF-15 expression eliminated the chemopreventive effects of sulindac in animal models of intestinal cancer. Additionally, the cyclin-dependent kinase inhibitor PHA-848125 impaired melanoma cell growth; it significantly up-regulated 4 genes (PDCD4, SESN2, DDIT4, DEPDC6) [33]. Androutsopoulos et al. [34] reported the synergistic cytostatic effects of diosmetin and luteolin against human hepatoma Hep-G2 cells. They induced G2/M arrest as well as up-regulated phospho-extracellular-signal-regulated kinase (p-ERK), phospho-c-jun N-terminal kinase, p53 and CDKN1A (p21) proteins.

Our results were consistent with the previous reports suggesting that the induced up-regulation of CDKN1A, DDIT4 and GDF-15 genes in Panc-1 cells treated with BCHH **7c** could be an acceptable mechanism of action.

On the other hand, cell-division cycle protein 2 homolog (CDC2), cell-division cycle protein 20 (CDC20), (CDC3), Cyclin-dependent kinase 2 (CDK2) and DNA replication licensing factor (MCM4) were significantly down regulated genes in resistant Panc-1 cells treated with BCHH **7c**.

It was reported that Daphnoretin, natural coumarin product, induced G2/M phase arrest accompanied by down-regulation of CDC2 in human osteosarcoma (HOS) cells [35]. Similarly, the natural coumarin product, Osthole, induced apoptosis and arrested the cell cycle of human lung cancer A549 cells in the G2/M phase and down regulated CDC2 gene expression [36]. Co-administration of TPA and silibinin induced growth arrest by the down-regulation of CDC2 and the up-regulation of P21 expression in MDA-MB231 human breast cancer cells [37]. Nomura et al. [38] reported that geldanamycin produced G2 arrest in U87MG glioblastoma cells through down-regulation of CDC2. It is well known that the

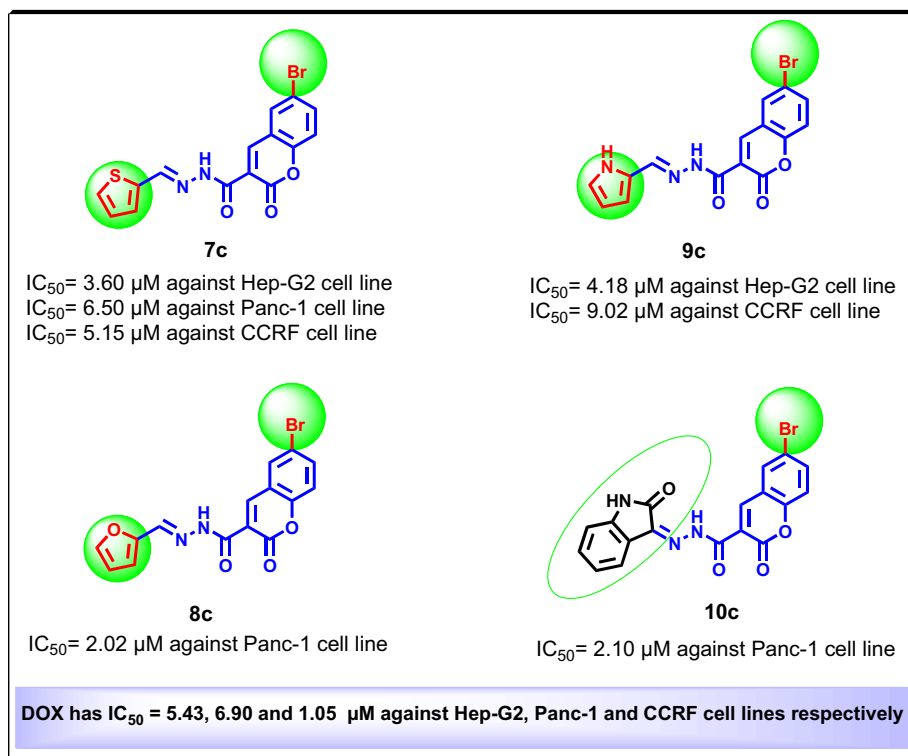


Fig. 5. The most potent CHHD's as anticancer agents.

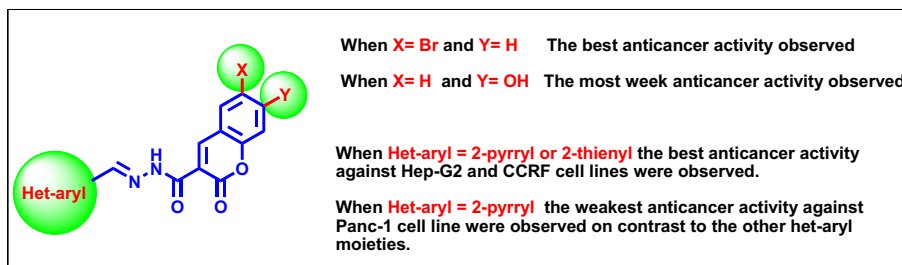


Fig. 6. Anticancer structure activity relationship of the studied CHHD's.

antitumor effect of ganodermanontriol against breast cancer cells is through the down-regulation of CDC20 and uPA [39]. Grape polyphenols have antitumor activity because it was able to down-regulate many genes; CDK2 is one of these genes [40]. Inhibition of CYP1B1 suppressed the growth of the endometrial carcinomas due to down-regulation of minichromosome maintenance complex component 4 (MCM4) as well as some other genes [41]. It is worthwhile to mention that our results were consistent with all previous studies.

Regarding hepatic cancer cells, the results indicated that NRF2-mediated oxidative stress response, VDR/RXR activation, xenobiotic metabolism signaling and HER-2 signaling in breast cancer and PDGF signaling were among the top ten canonical pathways, regulated after treatment of Hep-G2 cells with our newly synthesized BCHHD **7c** (Fig. 3, Supplementary data). Additionally, cell cycle, cellular movement, developmental disorder, cellular development, cellular growth and proliferation, skeletal and muscular system development and function were among the top ten regulated networks after BCHHD **7c** treatment (data not shown).

The present analysis is a starting point for the generation of hypotheses on candidate genes and for a more detailed dissection of the functional role of individual genes for the activity of our compounds in tumor cells. Moreover, our results provide new insights into signaling activities, which may facilitate the development of anticancer strategies and/or combination therapies.

3. Conclusion

Sixteen CHHD's **7–10** were prepared and their chemical structures were proved by spectral and elemental analyses. The antitumor activity of these compounds was screened against resistant Panc-1 cells as well as Hep-G2 and CCRF cell lines using DOX as the

positive control. CHHD's **7–10** had broad spectrum antiproliferative activity against all tested cell lines. The most potent compounds were BCHHD's **7c**, **8c**, **9c** and **10c**. They induced apoptosis in drug-resistant Panc-1 and -sensitive Hep-G2 cell lines and this could be, in part, due to the activation of caspases 3/7. The microarray result showed that BCHHD **7c** could induce apoptosis and cell cycle arrest in G2/M phase. Moreover, growth factors and tumor suppressor genes were regulated. BCHHD **7c** antiproliferative effect could be mediated by the up-regulation of CDKN1A, DDIT4, GDF-15 and/or down-regulation of CDC2, CDC20, CDK2 genes. Furthermore, it was shown that BCHHD **7c** is able to regulate multiple signaling pathways in human resistant pancreatic cancer cells as well as hepatic cancer cells. Our results presented here could be used as a starting point for development of powerful coumarin anticancer therapies.

4. Experimental

4.1. Chemistry

Melting points were determined on digital Gallen-Kamp MFB-595 instrument using open capillary tubes and are uncorrected. IR spectra were recorded on Shimadzu FTIR 440 spectrometer using KBr pellets. Mass spectra were performed on Shimadzu Qp-2010 plus mass spectrometer at 70eV. The ESI-Mass spectra (ESI-MS) were taken in positive mode on Agilent 6320 triple quadrupole mass spectrometer (Agilent technologies, USA) equipped with an electrospray ionization interface (ESI) coupled to an Agilent 1200 HPLC (Agilent Technologies, USA). ¹H NMR and ¹³C NMR spectra were recorded on a Bruker model 500 MHz Ultra Shield NMR spectrometer in DMSO-d₆ using tetramethylsilane (TMS) as an internal standard; chemical shifts are reported as δ_{ppm} units. The elemental analyses were done at the Microanalytical Center, Cairo University, Cairo, Egypt. 2-Cyano-*N'*-((thiophen-2-yl)methylene)acetohydrazide (**2**) [20], 2-cyano-*N'*-((furan-2-yl)methylene)acetohydrazide (**3**) [21], 2-cyano-*N'*-(2-oxoindolin-3-ylidene)acetohydrazide (**5**) [22], 2-oxo-*N'*-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (**10a**) [23] and 7-hydroxy-2-oxo-*N'*-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (**10d**) [42] were prepared according to the literature procedure. Salicylaldehyde derivatives (**6a–d**) were purchased from Sigma–Aldrich.

4.1.1. Synthesis of *N'*-((1H-pyrrol-2-yl)methylene)-2-cyanoacetohydrazide (**4**)

Pyrrole-2-carbaldehyde (1.90 g, 0.02 mol) was added to a hot solution of cyanoacetic acid hydrazide **1** (1.98 g, 0.02 mol) in ethanol (30 mL). To the obtained solution, 3 drops of acetic acid were added. The reaction mixture was allowed to reflux for 30 min. The desired product obtained as solid on hot. The reaction flask was allowed to cool down and the product was filtered off and recrystallized from ethanol/DMF (5:1) to give compound **4**.

White powder, yield (77%), mp 205–206 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 3325 (NH), 3265 (NH), 2260 (CN), 1665 (C=O), 1635 (C=N); ¹H NMR

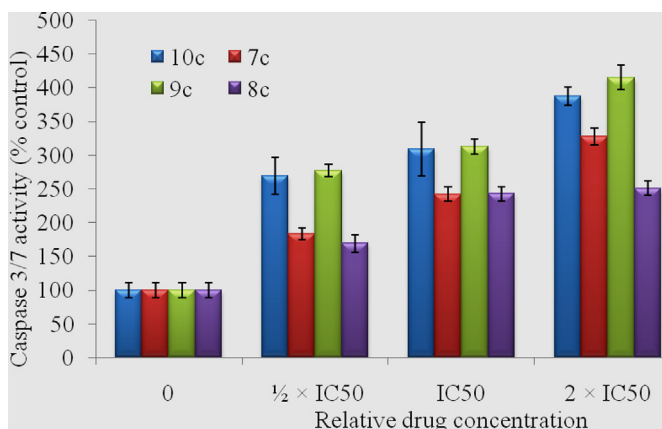
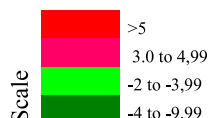


Fig. 7. Caspase 3/7 assay results of BCHHD's **7c**, **8c**, **9c** and **10c** against resistant Panc-1 cancer cell line (24 h incubation). The results were significant; $p < 0.05$.

Table 2The most significant thirty up and down-regulated genes after treating Panc-1 cancer cell line with BCHHD **7C** for 48 h. (F)^a, Fold changes.

Gene Name	F ^a	Gene Name
ASNS		Homo sapiens asparagine synthetase (ASNS), transcript variant 1, mRNA.
ATP5G1		Homo sapiens ATP synthase, H ⁺ transporting, mitochondrial F0 complex.
C10RF112		Homo sapiens chromosome 1 open reading frame 112 (C10rf112), mRNA.
CCDC99		Homo sapiens coiled-coil domain containing 99 (CCDC99), mRNA.
CDC2		Homo sapiens cell division cycle 2, G1 to S and G2 to M (CDC2).
CDC20		Homo sapiens cell division cycle 20 homolog (S. cerevisiae) (CDC20), mRNA.
CDC4		Homo sapiens cell division cycle 2, G1 to S and G2 to M (CDC2).
CDK2		Homo sapiens cyclin-dependent kinase 2 (CDK2), transcript variant 1, mRNA.
CDKN1A		Homo sapiens cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A).
DDIT4		Homo sapiens DNA-damage-inducible transcript 4 (DDIT4), mRNA.
FANCD2		Homo sapiens Fanconi anemia, complementation group D2 (FANCD2).
GDF15		Homo sapiens growth differentiation factor 15 (GDF15), mRNA.
IGFBP1		Homo sapiens insulin-like growth factor binding protein 1 (IGFBP1), mRNA.
IGFBP1		Insulin-like growth factor binding protein 1 (IGFBP1), transcript variant 2.
ISG20		Homo sapiens interferon stimulated exonuclease gene 20kDa (ISG20), mRNA.
KIF18A		Homo sapiens kinesin family member 18A (KIF18A), mRNA.
LOC149501		PREDICTED: Homo sapiens misc_RNA (LOC149501), miscRNA.
LOC392437		PREDICTED: Homo sapiens misc_RNA (LOC392437), miscRNA.
LOC392437		PREDICTED: Homo sapiens misc_RNA (LOC392437), miscRNA.
LOC728873		PREDICTED: Homo sapiens misc_RNA (LOC728873), miscRNA.
MCM3		Homo sapiens minichromosome maintenance complex component 3 (MCM3)
MCM4		Homo sapiens minichromosome maintenance complex component 4 (MCM4).
MMP7		Homo sapiens matrix metalloproteinase 7 (matrilysin, uterine) (MMP7), mRNA.
NDRG1		Homo sapiens N-myc downstream regulated gene 1 (NDRG1), mRNA.
PLAT		Homo sapiens plasminogen activator, tissue (PLAT), transcript variant 1.
PTTG1		Homo sapiens pituitary tumor-transforming 1 (PTTG1), mRNA.
TM4SF18		Homo sapiens transmembrane 4 L six family member 18 (TM4SF18), mRNA.
TOP2A		Homo sapiens topoisomerase (DNA) II alpha 170kDa (TOP2A), mRNA.
TRIB3		Homo sapiens tribbles homolog 3 (Drosophila) (TRIB3), mRNA.
VRK1		Homo sapiens vaccinia related kinase 1 (VRK1), mRNA.



(DMSO-d₆): δ_{ppm} = 3.67 (s, 2H, CH₂), 6.75 (s, 1H, NH), 7.16–7.35 (m, 3H, Ar-H), 8.43 (s, 1H, CH=), 8.93 (s, 1H, pyrrole-NH); Anal. Calcd. for C₈H₈N₄O (176.18): C, 54.54; H, 4.58; N, 31.80%, Found: C, 54.57; H, 4.63; N, 31.84%.

4.1.2. General procedure for the synthesis of BCHHD's (**7–10**)

A mixture of cyanoacetohydrazones **2–5** (0.005 mol) and salicylaldehyde derivatives **6a–d** (0.005 mol) in absolute ethanol (20 mL) in the presence of piperidine (0.5 mL) was stirred under reflux for 6 h. The reaction mixture was poured onto ice water (50 mL) and acidified with diluted HCl. The precipitated solid was filtered off, washed with water (2 × 30 mL), air dried and then recrystallized from the ethanol/DMF (5:1) to afford the desired BCHHD's **7–10**.

4.1.2.1. 2-Oxo-N'-((thiophen-2-yl)methylene)-2H-chromene-3-carbohydrazide (7a). Orange powder, Yield (78%), mp 190–191 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 3329 (NH), 3065 (CH-Ar), 1685 (C=O), 1646 (C=O), 1619 (C=N); ¹H NMR (DMSO-d₆): δ_{ppm} = 7.16 (dd, *J* = 4.0, 5.0 Hz, 1H, Thiophene-H₄), 7.25 (d, *J* = 8.5 Hz, 1H, Chromene-H₈), 7.31 (t, *J* = 7.5 Hz, 1H, Chromene-H₆), 7.50 (d, *J* = 4.0 Hz, 1H,

Thiophene-H₃), 7.59 (t, *J* = 8.0 Hz, 1H, Chromene-H₇), 7.72 (d, *J* = 5.0 Hz, 1H, Thiophene-H₅), 7.84 (d, *J* = 7.0 Hz, 1H, Chromene-H₅), 8.54 (s, 1H, CH=N), 8.63 (s, 1H, Chromene-H₄), 9.23 (s, 1H, NH); ¹³C NMR (DMSO-d₆): δ_{ppm} = 114.9 (Chromene-C₃), 116.3 (Chromene-C₈), 119.7 (Chromene-C₁₀), 124.2–129.4 (3 carbons, Chromene-C₆, methylene carbon, Thiophene-C₅), 130.0–133.2 (3 carbons, Chromene-C₅, Thiophene-C₃, Thiophene-C₄), 138.6 (Chromene-C₇), 141.7 (Chromene-C₄), 144.2 (Thiophene-C₂), 153.4 (Chromene-C₉), 155.3 (CO), 158.0 (CO). MS *m/z* (%): 298 (M⁺, 38.0), 202 (38.0), 153 (22.5), 145 (14.7), 110 (32.3), 96 (51.4), 57 (100.0); Anal. Calcd. for C₁₅H₁₀N₂O₃S (298.32): C, 60.39; H, 3.38; N, 9.39%, Found: C, 60.35; H, 3.39; N, 9.36%.

4.1.2.2. 6-Nitro-2-oxo-N'-((thiophen-2-yl)methylene)-2H-chromene-3-carbohydrazide (7b). Brown powder, Yield (81%), mp 181–182 °C; IR (KBr) $\nu_{\text{max}}/\text{cm}^{-1}$: 3331 (NH), 3077 (CH-Ar), 1681 (C=O), 1644 (C=O), 1613 (C=N), 1526 and 1340 (NO₂); ¹H NMR (DMSO-d₆): δ_{ppm} = 7.11 (t, *J* = 4.0 Hz, 1H, Thiophene-H₄), 7.39–7.74 (m, 3H, Chromene-H₈, Thiophene-H₃, Thiophene-H₅), 8.56 (s, 1H, CH=N), 9.02–9.24 (m, 3H, Chromene-H₇, Chromene-H₄, Chromene-H₅), 9.43 (s, 1H, NH); MS *m/z* (%): 345 (M⁺ + 2, 30.3), 343 (M⁺, 6.4), 297

(12.9), 260 (2.7), 247 (11.2), 233 (5.1), 218 (11.9), 189 (99.9), 170 (100.0), 153 (8.8), 110 (89.0), 96 (29.3), 82 (6.1); Anal. Calcd. for $C_{15}H_9N_3O_5S$ (343.31): C, 52.48; H, 2.64; N, 12.24%, Found: C, 52.46; H, 2.65; N, 12.22%.

4.1.2.3. 6-Bromo-2-oxo-*N'*-((thiophen-2-yl)methylene)-2H-chromene-3-carbohydrazide (7c). Orange brown powder, Yield (86%), mp 175–176 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3322 (NH), 3074 (CH-Ar), 1679 (C=O), 1649 (C=O), 1607 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 7.16 (d, J = 5.0 Hz, 1H, Thiophene- H_3), 7.21 (d, J = 8.5 Hz, 1H, Chromene- H_8), 7.50 (m, 1H, Thiophene- H_4), 7.72 (m, 1H, Thiophene- H_5), 8.00 (m, 1H, Chromene- H_7), 8.39 (s, 1H, Chromene- H_5), 8.50 (s, 1H, CH=N), 8.62 (s, 1H, Chromene- H_4), 9.36 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 115.5 (Chromene- C_3), 117.2 (Chromene- C_6), 120.4 (Chromene- C_8), 120.9 (Chromene- C_{10}), 127.9 (C=N), 129.4 (Thiophene- C_5), 131.7 (Thiophene- C_3), 135.3 (Thiophene- C_4), 138.6 (Chromene- C_5), 140.4 (Chromene- C_7), 144.4 (Chromene- C_4), 152.5 (Thiophene- C_2), 154.7 (Chromene- C_9), 159.6 (CO), 161.8 (CO); m/z (+ESI) 378.0 ($[\text{M} + \text{H}]^+$, 100); Anal. Calcd. for $C_{15}H_9\text{BrN}_2\text{O}_3\text{S}$ (377.2): C, 47.76; H, 2.40; N, 7.43%, Found: C, 47.73; H, 2.39; N, 7.40%.

4.1.2.4. 7-Hydroxy-2-oxo-*N'*-((thiophen-2-yl)methylene)-2H-chromene-3-carbohydrazide (7d). Brownish red powder, Yield (81%), mp 233–234 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3413 (OH, broad peak), 3214 (NH), 3074 (CH-Ar), 1681 (C=O), 1639 (C=O), 1603 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.30–7.80 (m, 6H, Ar-H), 8.13 (s, 1H, CH=N), 8.19 (s, 1H, Chromene- H_4), 10.30 (s, 1H, NH), 11.90 (s, 1H, OH); MS m/z (%): 315 ($\text{M}^+ + \text{H}$, 82.7), 314 (M^+ , 69.3), 297 (77.5), 231 (5.1), 230 (100.0), 189 (52.0), 153 (32.6); Anal. Calcd. for $C_{15}H_{10}N_2\text{O}_4\text{S}$ (314.32): C, 57.32; H, 3.21; N, 8.91%, Found: C, 57.35; H, 3.24; N, 8.95%.

4.1.2.5. *N'*-((Furan-2-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (8a). Brown powder, Yield (79%), mp 199–200 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3322 (NH), 3078 (CH-Ar), 1693 (C=O), 1641 (C=O), 1598 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.63–6.67 (m, 1H, Furan- H_4), 6.90–6.96 (m, 2H, Furan- H_3 , Chromene- H_8), 7.18–7.31 (m, 2H, Chromene- H_6 , Chromene- H_7), 7.55–7.61 (m, 1H, Chromene- H_5), 7.81–7.89 (m, 1H, Furan- H_5), 8.46 (s, 1H, CH=N), 8.61 (s, 1H, Chromene- H_4), 8.93 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 111.2 (Furan- C_3), 111.9 (Furan- C_2), 114.9 (Chromene- C_3), 120.6 (Chromene- C_8), 122.1 (Chromene- C_4), 124.9 (Chromene- C_6), 126.6 (Chromene- C_5), 128.4 (Chromene- C_7), 134.7 (C=N), 138.6 (Chromene- C_{10}), 143.6 (Furan- C_5), 148.7 (Furan- C_2), 150.2 (Chromene- C_9), 157.3 (CO), 162.1 (CO). MS m/z (%): 282 (M^+ , 16.5), 215 (1.2), 202 (2.4), 123 (100.0), 109 (13.1), 94 (4.6), 67 (5.8); Anal. Calcd. for $C_{15}H_{10}N_2\text{O}_4$ (282.25): C, 63.83; H, 3.57; N, 9.92%, Found: C, 63.86; H, 3.62; N, 9.95%.

4.1.2.6. *N'*-((Furan-2-yl)methylene)-6-nitro-2-oxo-2H-chromene-3-carbohydrazide (8b). Orange brown powder, Yield (82%), mp 220–221 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3311 (NH), 3072 (CH-Ar), 1683 (C=O), 1643 (C=O), 1615 (C=N), 1523 and 1339 (NO_2); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.66 (t, J = 3.5 Hz, 1H, Furan- H_3), 6.88 (m, 1H, Furan- H_4), 6.96 (d, J = 3.5 Hz, 1H, Furan- H_5), 7.87 (s, 1H, CH=N), 8.18 (m, 1H, Chromene- H_8), 8.31 (s, 1H, Chromene- H_4), 8.38 (m, 1H, Chromene- H_7), 8.46 (s, 1H, Chromene- H_5), 9.02 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 110.2 (Furan- C_3), 110.8 (Furan- C_4), 114.5 (Chromene- C_3), 120.9 (Chromene- C_7), 121.2 (Chromene- C_5), 122.3 (Chromene- C_8), 123.7 (Chromene- C_{10}), 134.7 (C=N), 139.1 (Chromene- C_4), 144.1 (Furan- C_5), 145.2 (Chromene- C_6), 149.7 (Furan- C_2), 156.6 (Chromene- C_9), 160.3 (CO), 161.8 (CO); m/z (+ESI) 328.30 ($[\text{M} + \text{H}]^+$, 100); Anal. Calcd. for $C_{15}H_9N_3\text{O}_6$ (327.25): C, 55.05; H, 2.77; N, 12.84%, Found: C, 55.11; H, 2.82; N, 12.87%.

4.1.2.7. 6-Bromo-*N'*-((furan-2-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (8c). Brown powder, Yield (89%), mp 205–206 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3332 (NH), 3077 (CH-Ar), 1681 (C=O), 1646 (C=O), 1601 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.65 (m, 1H, Furan- H_4), 6.97 (m, 1H, Furan- H_3), 7.22 (d, J = 9.0 Hz, 1H, Chromene- H_8), 7.73 (d, J = 9.0 Hz, 1H, Chromene- H_7), 7.88 (m, 1H, Furan- H_5), 8.29 (s, 1H, Chromene- H_5), 8.39 (s, 1H, CH=N), 8.51 (s, 1H, Chromene- H_4), 9.34 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 109.8 (Furan- C_3), 110.4 (Furan- C_4), 113.9 (Chromene- C_3), 118.9 (Chromene- C_6), 122.4 (Chromene- C_8), 123.9 (Chromene- C_{10}), 128.9 (Chromene- C_5), 130.7 (Chromene- C_7), 133.9 (CH=N), 137.8 (Chromene- C_4), 143.3 (Furan- C_5), 148.9 (Furan- C_2), 148.6 (Chromene- C_9), 158.9 (CO), 161.6 (CO); m/z (+ESI) 362.2 ($[\text{M} + \text{H}]^+$, 100); Anal. Calcd. for $C_{15}H_9\text{BrN}_2\text{O}_4$ (361.15): C, 49.89; H, 2.51; N, 7.76%, Found: C, 49.92; H, 2.53; N, 7.81%.

4.1.2.8. *N'*-((Furan-2-yl)methylene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (8d). Brownish powder, Yield (65%), mp 270–271 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3418 (OH, broad peak), 3221 (NH), 3089 (CH-Ar), 1691 (C=O), 1642 (C=O), 1599 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.25–7.20 (m, 5H, aromatic protons), 7.58 (d, J = 8.5 Hz, 1H, aromatic proton), 7.83 (s, 1H, CH=N), 8.08 (s, 1H, Chromene- H_4), 10.25 (s, 1H, NH), 11.65 (s, 1H, OH); MS m/z (%): 298 (M^+ , 30.3), 297 (77.5), 231 (100.0), 230 (9.3), 189 (52.0); Anal. Calcd. for $C_{15}H_{10}N_2\text{O}_5$ (298.25): C, 60.41; H, 3.38; N, 9.39%, Found: C, 60.45; H, 3.41; N, 9.42%.

4.1.2.9. *N'*-((1H-Pyrrol-2-yl)methylene)-2-oxo-2H-chromene-3-carbohydrazide (9a). Brown powder, Yield (71%), mp 227–228 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3384 (NH), 3310 (NH), 3045 (CH-Ar), 1682 (C=O), 1652 (C=O), 1607 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.75 (m, 1H, Pyrrole- H_3), 6.88 (m, 1H, Pyrrole- H_4), 6.98 (s, 1H, Pyrrole-NH), 7.08–7.61 (m, 4H, Aromatic protons), 7.78 (d, J = 7.5 Hz, 1H, Chromene- H_5), 8.26 (s, 1H, CH=N), 8.77 (s, 1H, Chromene- H_4), 10.03 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 115.6 (Pyrrole- C_4), 116.1 (Pyrrole- C_3), 116.6 (Chromene- C_3), 119.3 (Pyrrole- C_5), 123.9 (Chromene- C_8), 129.9 (Chromene- C_{10}), 130.3 (Chromene- C_6), 132.1 (Chromene- C_5), 133.4 (Chromene- C_7), 134.8 (Pyrrole- C_2), 152.8 (Chromene- C_4), 153.2 (CH=N), 155.1 (Chromene- C_9), 156.0 (CO), 157.7 (CO); m/z (+ESI) 282.2 ($[\text{M} + \text{H}]^+$, 100); Anal. Calcd. for $C_{15}H_{11}N_3\text{O}_3$ (281.27): C, 64.05; H, 3.94; N, 14.94%, Found: C, 64.11; H, 3.97; N, 14.95%.

4.1.2.10. *N'*-((1H-Pyrrol-2-yl)methylene)-6-nitro-2-oxo-2H-chromene-3-carbohydrazide (9b). Orange powder, Yield (88%), mp 211–212 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3324 (NH), 3200 (NH), 3085 (CH-Ar), 1690 (C=O), 1633 (C=O), 1618 (C=N), 1526 and 1342 (NO_2); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.16 (s, 1H, NH), 6.53 (m, 1H, Pyrrole- H_4), 6.88 (m, 1H, Pyrrole- H_3), 6.95–6.97 (m, 2H, Pyrrole- H_5 and Chromene- H_8), 8.20 (d, J = 6.0 Hz, 1H, Chromene- H_7), 8.37 (s, 1H, CH=N), 8.43 (s, 1H, Chromene- H_4), 8.99 (s, 1H, Chromene- H_5), 11.58 (s, 1H, NH); MS m/z (%): 326 (M^+ , 39.1), 260 (8.8), 247 (22.5), 233 (3.1), 218 (11.9), 189 (100); Anal. Calcd. for $C_{15}H_{10}N_4\text{O}_5$ (326.26): C, 55.22; H, 3.09; N, 17.17%, Found: C, 55.27; H, 3.13; N, 17.19%.

4.1.2.11. *N'*-((1H-Pyrrol-2-yl)methylene)-6-bromo-2-oxo-2H-chromene-3-carbohydrazide (9c). Brown powder, Yield (86%), mp 210–211 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3393 (NH), 3282 (NH), 3107 (CH-Ar), 1682 (C=O), 1632 (C=O), 1612 (C=N); ^1H NMR (DMSO- d_6): δ_{ppm} = 6.05 (m, 1H, Pyrrole- H_3), 6.43 (m, 1H, Pyrrole- H_4), 6.85 (m, 1H, Pyrrole- H_5), 7.11 (d, J = 9.0 Hz, 1H, Chromene- H_7), 7.60 (d, J = 9.0 Hz, 1H, Chromene- H_8), 8.09 (s, 1H, Chromene- H_5), 8.41 (s, 1H, CH=N), 9.22 (s, 1H, Chromene- H_4), 11.49 (s, 1H, NH), 13.18 (s, 1H, NH); ^{13}C NMR (DMSO- d_6): δ_{ppm} = 109.3 (Pyrrole- C_4), 114.4 (Pyrrole- C_5), 117.1 (Pyrrole- C_3), 121.1 (Chromene- C_3), 123.0

(Chromene-C₆), 126.7 (Chromene-C₈), 130.0 (Chromene-C₁₀), 131.8 (Chromene-C₅), 135.1 (Chromene-C₇), 139.9 (Pyrrole-C₂), 142.0 (Chromene-C₄), 152.4 (CH=N), 154.8 (Chromene-C₉), 156.9 (CO), 157.2 (CO); MS *m/z* (%): 360 (M⁺, 3.8), 342 (1.3), 292 (6.8), 280 (3.0), 279 (1.8), 265 (15.0), 250 (42.9), 222 (38.0), 136 (2.72), 108 (7.9), 93 (91.3), 79.9 (100), 78 (60.2), 66 (73.6); Anal. Calcd. for C₁₅H₁₀BrN₃O₃ (360.16): C, 50.02; H, 2.80; N, 11.67%, Found: C, 50.06; H, 2.83; N, 11.70%.

4.1.2.12. N'-((1H-Pyrrol-2-yl)methylene)-7-hydroxy-2-oxo-2H-chromene-3-carbohydrazide (9d). Brownish powder, Yield (62%), mp 254–255 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3421 (OH, broad peak), 3379 (NH), 3222 (NH), 3077 (CH-Ar), 1699 (C=O), 1639 (C=O), 1604 (C=N); ¹H NMR (DMSO-d₆): δ_{ppm} = 6.10–7.54 (m, 6H, aromatic protons), 7.82 (s, 1H, CH=N), 8.21 (s, 1H, Chromene-H₄), 11.51 (s, 1H, NH), 11.62 (s, 1H, NH); 14.15 (s, 1H, OH); MS *m/z* (%): 297 (M⁺, 10.9), 296 (46.4), 280 (5.4), 231 (5.3), 218 (1.3), 204 (7.2), 189 (16.2), 161 (7.7), 136 (9.4), 108 (22.7), 93 (41.7), 79 (50.7), 67 (100.0); Anal. Calcd. for C₁₅H₁₁N₃O₄ (297.27): C, 60.61; H, 3.73; N, 14.14%, Found: C, 60.65; H, 3.75; N, 14.19%.

4.1.2.13. 6-Nitro-2-oxo-N'-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (10b). Yellow powder, Yield (53%), mp 211–212 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3299 (NH), 3188 (NH), 3073 (CH-Ar), 1691 (C=O), 1665 (C=O), 1654 (C=O), 1601 (C=N), 1529 and 1339 (NO₂); ¹H NMR (DMSO-d₆): δ_{ppm} = 6.85–8.90 (m, 8H, Ar-H), 10.86 (s, 1H, NH), 11.23 (s, 1H, NH); *m/z* (+ESI) 379.10 ([M + H]⁺, 100); Anal. Calcd. for C₁₈H₁₀N₄O₆ (378.30): C, 57.15; H, 2.66; N, 14.81%, Found: C, 57.18; H, 2.71; N, 14.84%.

4.1.2.14. 6-Bromo-2-oxo-N'-(2-oxoindolin-3-ylidene)-2H-chromene-3-carbohydrazide (10c). Brown powder, Yield (49%), mp 209–210 °C; IR (KBr) $\nu_{\max}/\text{cm}^{-1}$: 3302 (NH), 3195 (NH), 3071 (CH-Ar), 1684 (C=O), 1661 (C=O), 1648 (C=O), 1612 (C=N); ¹H NMR (DMSO-d₆): δ_{ppm} = 6.70–8.10 (m, 7H, Ar-H), 8.91 (s, 1H, Chromene-H₄), 10.61 (s, 1H, NH), 10.93 (s, 1H, NH); ¹³C NMR (DMSO-d₆): δ_{ppm} = 114.9, 117.9, 120.1, 122.2, 123.9, 124.8, 125.2, 129.8, 130.7, 131.5, 131.8, 133.1, 139.0, 146.9, 149.7 (15 Ar-H), 159.9 (C=O), 161.4 (C=O), 166.2 (C=O); MS *m/z* (%): 411 ([M – H]⁺, 0.2), 332 (0.1), 280 (0.2), 266 (0.2), 251 (0.6), 223 (0.3), 188 (0.2), 160 (0.3), 145 (0.8), 131 (1.0), 78 (2.1), 69 (100.0); Anal. Calcd. for C₁₈H₁₀BrN₃O₄ (412.19): C, 52.45; H, 2.45; N, 10.19%, Found: C, 52.49; H, 2.51; N, 10.21%.

4.2. Biology

4.2.1. Cell culture and treatment

All reagents were handled in a sterile fume hood. DMEM medium, and fetal bovine serum (FBS) were purchased from Gibco; phosphate-buffered saline pH 7.4 (PBS) and trypsin-EDTA were obtained from Sigma–Aldrich. Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay was used to assess the cytotoxicity of the studied samples. The growth medium (DMEM medium with 10% FBS, 100 U/mL penicillin, and 100 mg/L streptomycin), and alamar blue were stored at 48 °C, while trypsin–EDTA and FBS were stored frozen at –208 °C and thawed before use; PBS was stored at room temperature. The Hep-G2, Panc-1 and CCRF were obtained from the German Cancer Research Center (DKFZ). Cells were cultured in 50 cm² culture flasks (Corning) using DMEM medium supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (100 mg/mL). The culture was maintained at 37 °C in an atmosphere of 5% CO₂ and 95% relative humidity. The cells were transferred to a new flask every 2 days and treated with trypsin–EDTA to detach them from the flask. Cells were counted under a microscope using a hemacytometer (Hausser Scientific). Cell solutions were diluted with growth medium to a concentration

of 1×10^5 cells/mL and transferred to a 96-well plate, and treated with gradient concentrations of test compounds.

4.2.2. Resazurin cell growth inhibition assay

Alamar Blue or Resazurin (Promega, Mannheim, Germany) reduction assay was used to assess the cytotoxicity of the studied samples. The assay tests cellular viability and mitochondrial function. Briefly, adherent cells were grown in tissue culture flasks, and then harvested by treating the flasks with 0.025% trypsin and 0.25 mM EDTA for 5 min. Once detached, cells were washed, counted and an aliquot (5×10^3 cells) was placed in each well of a 96-well cell culture plate in a total volume of 100 μ L. Cells were allowed to attach overnight and then treated with samples. The final concentration of samples ranged from 0 to 100 μ M. After 48 h, 20 μ L Resazurin 0.01% w/v solution was added to each well and the plates were incubated at 37 °C for 1–2 h. Fluorescence was measured on an automated 96-well Infinite M2000 Pro™ plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. For leukemia cells, aliquot of 5×10^4 cells/mL (obtained from overnight suspension) were seeded in 96-well plates, and test samples were added immediately. After 48 h incubation, plates were treated with resazurin solution as above mentioned. Doxorubicin was used as positive control. Each assay was done at least three times, with two replicates each. The viability was compared based on a comparison with untreated cells. IC₅₀ (on cancer cells) were the concentration of sample required to inhibit 50% of the cell proliferation and were calculated from a calibration curve by a linear regression using Microsoft Excel.

4.2.3. Caspase-glo 3/7 assay

The influence of our test samples on caspase 3/7 activity in pancreatic cancer resistant cells (Panc-1) was detected using Caspase-Glo 3/7 Assay kit (Promega). Cells cultured in DMEM were seeded in 96-well plates and treated with the sample ($2 \times \text{IC}_{50}$; IC_{50} ; $\frac{1}{2} \times \text{IC}_{50}$) or DMSO (solvent control). After 24 h treatment, 100 μ L of caspase 3/7 reagent were added to each well, mixed and incubated for 1 h at room temperature. Luminescence was measured using well Infinite M2000 Pro™ instrument (Tecan). Caspase 3/7 activity was expressed as percentage of the untreated control.

4.2.4. Transcript profiling using microarray techniques

Cells were either treated with 25 μ M of the BCHHD 7–10 or with solvent (control) for 48 h. RNA extraction, purification and microarray processing has been carried out as previously described [27,28]. Briefly, total-RNA was isolated and the RNA integrity was checked on an Agilent Bioanalyser 2100 (Agilent Technologies, Palo Alto, USA). Fluorescently labeled cDNA samples were then prepared and a well-defined subset of some 7000 genes highly associated with the occurrence of pancreatic carcinoma was spotted on our microarray chips. Spotted genes included those related to apoptosis, cell cycle, growth factors, angiogenic, and housekeeping genes. After hybridization, fluorescence signals detection was done with a confocal ScanArray 5000 scanner (Packard Bioscience, USA). GenePix Pro 6 (Axon Instruments, Union City, USA) software was used for signal analysis.

4.2.5. Identification of canonical pathways and networks of interacting genes

Here, we used the Ingenuity Pathway Analysis software (IPA) (Mountain View, USA) to identify networks of genes and other canonical pathways modulated after treating pancreatic cancer cells with BCHHD 7c. Using IPA tool, the microarray result genes were grouped and classified based on their biological functions and

pathways involved in their effects. A cut-off value of 1.5 was used to define genes. Only genes with expression level equal to or more than 1.5 were included in our pathways and networks [27–29].

4.2.6. Data analysis

Microarray quality, normalization and correspondence cluster analysis were performed as described in previous studies [27–29].

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.ejmech.2014.02.026>.

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