DOI: 10.29409/ijcmg.v4i2.61

Iraqi Journal of Cancer and Medical Genetics

Novel Synthetic 3-Amino-2-Methylquinazolin-4(3H)-one (L) Induce Apoptosis and Modulate Antioxidant Enzyme Activity in Some Cancer Cell Lines

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Abstract :

Aquinazoline ring possess interesting biological activities, we study the combined cytotoxic activity, induction of apoptosis and antioxidant enzyme modulation of new synthetic aquinazoline compound in several cancer cell lines. This compound is 3-amino-2-methylquinazolin-4(3H)-one (L), it was synthesized in the laboratories of Department of Applied Science - University of Technology – Iraq. Its showed potent cytotoxic and growth inhibition effect on all cancer cell lines tested. Cells death was due to the induction of apoptosis through modulation of superoxide dismutase (SOD), glutathione S transferase (GST) and glutathione (glutathione L-glutamyl-L-cysteinylglycine, GSH). These results indicate that this compound has desirable drug-like properties; it's of great significance to understand its full anti-proliferative mechanism.

keyword: *Quinoline, Anti Oxidant enzyme, Apoptosis*

Introduction:

Chemically synthesized organic compounds are attracting more attention recently. Different approaches were invested to study the biological importance and therapeutically activities of such compounds (1). Quinazoline ring is an aromatic benzopyrimidine system; many of its derivatives possess interesting biological activities, such as analgesic, anti-inflammatory, anti-microbial, and anti-tumor (2, 3).

Quinazoline nucleus has attracted the attention of medicinal chemists due to its well known anticancer activity and many substituted quinazoline derivatives have recently earned great interest in chemotherapy as antitumor drugs (4, 5) These compounds are widely used in agrochemicals as plant pesticides, antifungal agents and herbicides (6). Compounds containing the 4(3H)-quinazolinone ring have been reported to possess different biological activities. Its activity depends on the substituents in the ring system (7). The quinazoline compound was distinguished as kinase inhibitors, the activity which potentiates them to become new class of anticancer drugs.

The quinazoline derivative represents the first generation of HER1 targeted small-molecule anticancer agent such as Lapatinib, which is the only U.S. FDA approved HER1 tyrosine kinase inhibitor available for cancer treatment. An effort to test other quinazoline compound as kinase inhibitors or EGFR inhibiters is an active field of reaserch (8). 4-anilinoquinazolines are the most promising small molecule EGFR tyrosine kinase inhibitors (9). Synthetic analog of 5, 8-disubstituted quinazolines, was characterized as a novel cancer cell mitotic blocker and inducer of apoptosis by inhibiting microtubule polymerization (10).

As an early knowledge, persistent oxidative stress at sub-lethal levels makes cancer cell resistance to apoptosis.

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Sublethal oxidative stress promotes cell proliferation in vitro, with both superoxide and hydrogen peroxide stimulating growth (11, 12).

Increasingly, the role of free radicals having consciousness of high position in carcinogenesis, researchers has been more interest in antioxidants role in the prevention, treatment, and alleviation of therapy-related side effects of cancer (13).

Inherent oxidative stress can affect several functions in cancer cells or tumor tissues, such as cell proliferation, promotion of mutations and genetic instability, alterations in cellular sensitivity to anticancer agents, invasion, and metastasis.

Oxygen radicals increase tumor cell production of the angiogenic factors IL-8 and VEGF and secretion of MMP-1, a collagenase that aids vessel growth within the tumor microenvironment (14). Oxidative stress can stimulate angiogenesis of the tumor. Oxygen radicals may also augment tumor cell migration, increasing the risk of invasion and metastasis (15).

Many researchers investigate the activity of different types of compound as antioxidant agents, compound from natural sources, medicinal plants or even foods were tested to explore their usefulness as ROS Scavengers to alleviate oxidative stress and prevent the related diseases (16, 17). In this report we tried to investigate the combined cytotoxic activity and antioxidant enzyme modulation ability of a new synthetic aquinazoline compound that is 3-amino-2-methylquinazolin-4(3H)-one (L) in different types and origin of cancer cell lines.

Methods:

Synthesis of 3-amino-2-methylquinazolin-4(3H)-one (L)

The compound 3-amino-2-methylquinazolin-4(3H)-one (L) under investigation was synthesized in the Department of Applied Science University of Technology-Iraq. Detailed synthesis, physical, chemical, structural, and purity analysis of the synthetic organic compound was previously published (7).

The compound was obtained as brown crystalline powder, it dissolved in DMSO (500 µg / mL stock solution) and stored at 4°C. In the current study this compound will be shortly named as R1.

Cancer cell lines

Seven types of different origin cancer cell lines were used, cervical cancer cell line (Hela) obtained from ICCRMG. AMN3; is a mammary adenocarcenoma of BALB/c mice origin, established at the Iraqi Center for Cancer Research and Medical Genetics (ICCRMG) (18).

Primary rat embryo fibroblast cells (REF) with normal Karyotype and no tumor genesis properties, established in ICCRMG (17). Vero (NCI-DCTD, USA) cell line is a normal adult green African monkey kidney cells initiated in 1962, Japan (19).

Human epidermoied larynx carcinoma cells (HEp2) originally described by Moore et al., (1955) (20) and obtained as a cell line from ICCRMG. Rabdomyosarcoma cells (RD) is humane cell line derived from biopsy specimen of a 7 years old Caucasian girl as described by and Johnston and Siegel (1990) (21) and obtained as a cell line from ICCRMG.

Glyoblastoma cells (GB) is humane cell line derived from biopsy specimen of 70 years old Iraqi male and established as cell line in ICCRMG (17). AMN3, GB, HEp2 and REF cells were grown in RPMI-1640 medium supplemented with 10 % fetal calf serum (FCS) RD, Vero and Hela cells were grown in MEM medium supplemented with 10 % FCS.

Cell viability assay

Cancer cells were seeded in a 96-well plate (1×105 cells / well) and cultured overnight, then treated with various concentrations of R1 (0, 15.63, 31.25, 62.5, 125, 250, 500 µg/mL) and incubated for additional 72 hours.

Cell viability was assayed with MTT (3- 4, 5- dimethylthiazol–2-yl) -2, 5-diphenyl tetrazolium bromide) by adding 20 µl of MTT solution (5 mg/mL, Sigma Chemical Co.) to each well and incubated for 3 hours at 37˚ C. The MTT-formazan crystals formed by metabolically viable cells were dissolved in 150 µl of DMSO.

The absorbance was measured by a micro titer plate reader (Asays, Austria) at a wavelength of 570 nm.

Staining with 4, 6-Diamidino-2-phenylindole (DAPI)

Cells were cultured over microscopic cover slips in 2 cm diameter Petri dishes. After monolayer formation old media was discarded and 2 mL of RPMI-1640 or MEM serum free media containing the IC50 final concentration of R1 (500 µg/mL for cell lines that don't have IC50) for each cell line or vehicle control (DMSO) for 24 hr.

The cover slips were lifted and cells were fixed with solution of 4% formaldehyde for 10 min and then in 1 mL of methanol, cells were washed three times with PBS, Fixed cells were stained with 4 µg/mL DAPI for 10 min. The nuclear morphology of apoptotic cells was observed by fluorescent microscopy and counted in 25-30 microscopic fields.

DNA fragmentation assay

The cellular DNA extraction was carried out with KAPA Express Extract Kit (KAPA Biopsystems, Boston, Massachusetts, USA) as described by manufacturer.

Cells treated with the IC50 final concentration of R1 for each cell line or vehicle control (DMSO) for 24 hr. Agarose gel electrophoresis was conducted as described by Maniates and coworker (1989) (22) using 0.7% agarose in Tris Boric acid EDTA (TBE) buffer. Blue/orange loading dye (Promega, USA) was used, 2 µl of 1X dye solution was mixed with suitable amount of sample DNA. Lamda DNA/ EcoRI + Hid III (Promega, USA) ranging in size between 125-21 bp marker was used to indicate the DNA fragments length.

Intracellular antioxidant enzymes activity assay

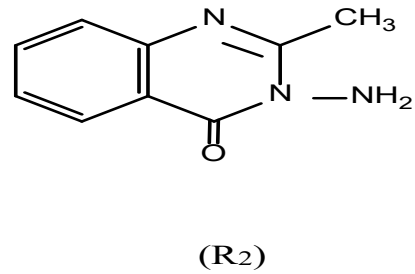
This assay was conducted as described by Al-Hilli (17). The IC50 (for cell lines that R1 did have inhibition concentration fifty) and 500 µg/mL (for cell lines R1 did not have IC50) or vehicle control (DEMSO). After exposed for 72 hr, 37˚C, cells were lysed and the activity of intracellular super oxide dismutase (SOD) (SOD determination Kit, Fluka, USA), intracellular reduced glutathione (GSH) (GSH determination Kit, US biological, USA) and intracellular glutathione-S-Transferase (GST) (GST determination Kit, US biological, USA) was assayed according to kit manufacturer.

Results:

Chemical formula of the compound under investigation 3-amino-2-methylquinazolin-4(3H)-one(L) is C9N3OH9 giving a molecular weight of 145 (Figure 1).

We give this compound a nick name as R1. To analyze the cell specificity of this compound against cancer cells we used different types and origins of cell lines. Our data indicate that R1 inhibited the growth of Hela, AMN3, HEp2, Vero, REF, RD and GB cell lines in different manner.

it bear different significant ($p<0.01$) growth inhibition activity on AMN3, HEp2, and GB cells along cell lines tested with less effect on Vero and HeLa while have minor incident on REF and RD cells ($P<0.01$).



(R2)

Figure1. Chemical structure of R1 3-amino-2-methylquinazolin-4(3H)-one(L).

The inhibitory effect was related to the concentration used of the compound (Figure 2). Initial R1 concentration 15.63 µg/mL have reduced living cells percentage significantly ($P < 0.01$) down to 19% in GB cells and ($P<0.05$) 66, 74% in HEp2 and Vero cells respectively.

Whereas this concentration did not reduces the viability of REF and RD cells only to 90 and 88%, meanwhile it did not affect HeLa cells viability. Elevating the concentration in double folds produces gradual growth inhibition in HeLa, AMN3, Vero, and HEp2 cell lines. On the contrary REF and RD affected insignificantly ($P<0.01$) by R1 elevated folds concentration and remained in the least inhibition levels.

This R1 increment produces insignificant ($P<0.01$) GB inhibition, these cells was notably reduced by initial concentration of R1 and this reduction continued in the same level through the experiment.

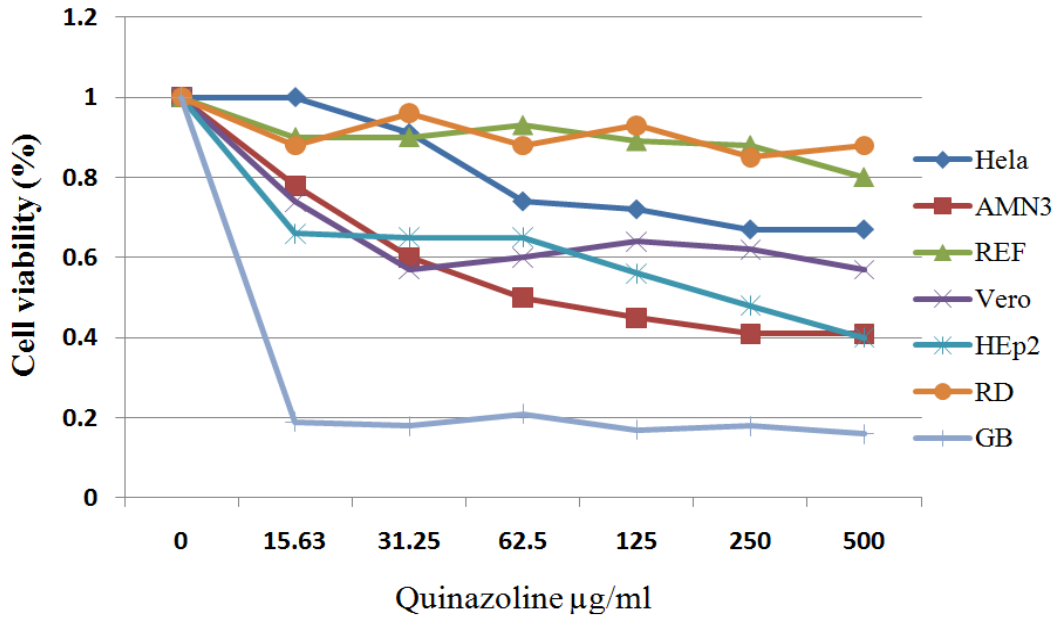


Figure 2. Effect of 3-amino-2-methylquinazolin-4(3H)-one (L) on the growth inhibition of different cancer cells. Hela, AMN3, REF, Vero, HEp2 and RD cells were incubated with the indicated concentrations of the compound for 72 hours. The cells growth was evaluated by MTT assay. The cell viability expressed as percentage of the compound-treated viable cells divided by the viable cells of the untreated control. The data are the means of triplicates.

These results demonstrated that 3-amino-2-methylquinazolin-4(3H)-one (L) inhibits the growth of most cancer cells of different tissue type and origins.

Cell lines under experiment possesses different inhibition concentration fifty (IC50) and different maximum viability reduction percentage (Table 1). While it possesses no IC50 toward REF and RD cells even at highest concentration (500 µg/mL), its IC50 for Vero, AMN3, and HEp2, was 31.25, 62.5, and 125 µg/mL respectively. The maximum viability reduction percentage was variable according to the cell type and it was achieved with the highest concentration of R1 (500 µg/mL). To investigate if the growth inhibition property imposed by R1 was due to the induction of apoptosis in cancer cells tested, we conducted 4,6-Diamidino-2-phenylindole (DAPI) staining assay. Cell's nucleus stained with DAPI showed heavily condensed DNA indicating the fragmentation of the cell's DNA upon treatment of the cells with the inhibition concentration 50 of R1 (figure 3). However REF cells showed almost an intact nucleus

Table 1: Inhibition concentration 50 of cancer cells treated with R1 and the maximum viability reduction percentage achieved with R1 treatment, (*) concentration of initial inhibitory

Cell Line	Inhibition concentration 50 (µg/mL)	Maximum viability reduction %
GB	*15.63	84
AMN3	62.5	59
HEp2	125	60
Vero	31.25	43
Hela	250	33
REF	*250	20
RD	*250	15

To a certain point this assay provide evidence that RD cells undergo through apoptosis when challenged with R1 at maximum concentration used (500 µg/mL). A pronounced RD nucleus DNA condensation was evident under fluorescent microscope. To investigate furthermore the apoptosis induction in the R1 treated cells we conducted DNA fragmentation assay in agarose gel electrophoreses. This assay give extra weight to our gesture that R1 is able to induce apoptosis in most of the treated cell lines. The fragmented DNA of treated cells appears as blemished smear over the line of electrophoresis run orientation (Figure 4). This DNA appearance was less pronounced in REF cells when treated with 500µg/mL of R1. The RD cells exhibited visible or noticeable DNA fragmentation in this assay; they have been treated with 500µg/mL for 48 hr.

In order to follow the mechanism that induce apoptosis in the tested cells, three types of intracellular antioxidant enzymes activity was assessed in the treated cells. It's well known that cancer cells maintain their antioxidant enzymes activity in certain level to keep reactive oxygen species (ROS) in an active form to allow it's over expressed signal transduction pathways (23, 24). Moderate levels of intracellular ROS is thought to be essential to maintain appropriate redox balance and to stimulate cellular proliferation (25).

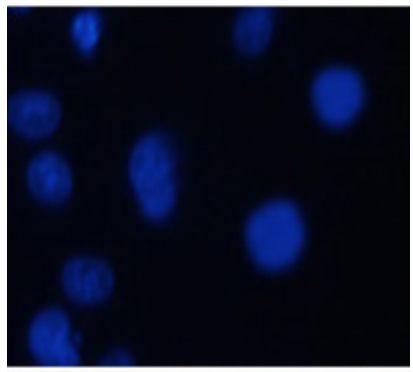
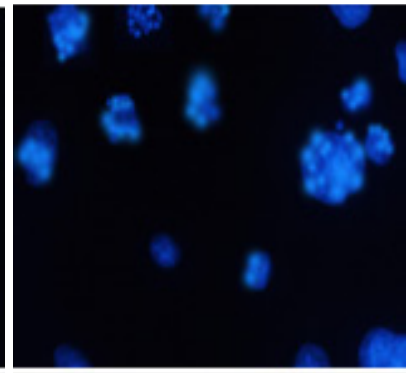
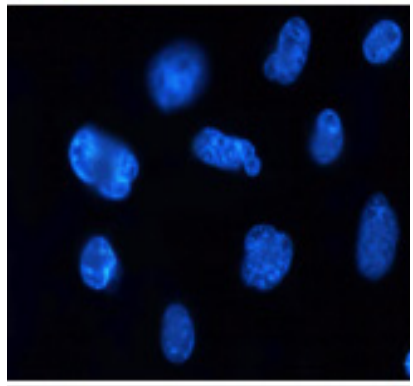
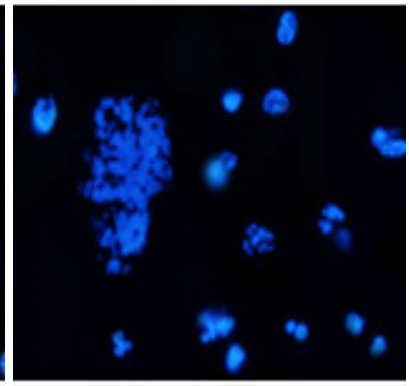
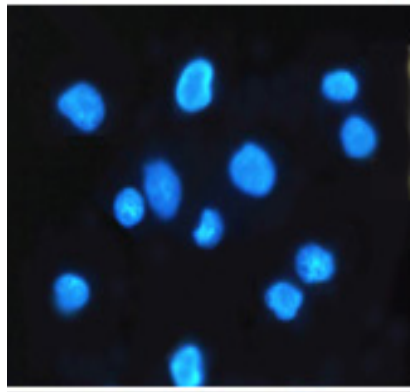
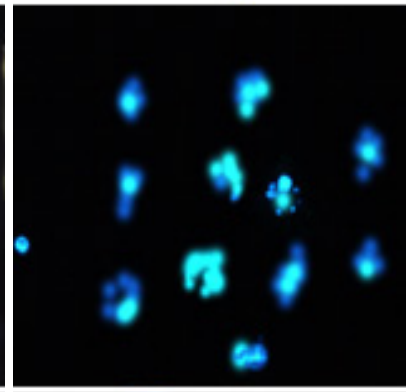
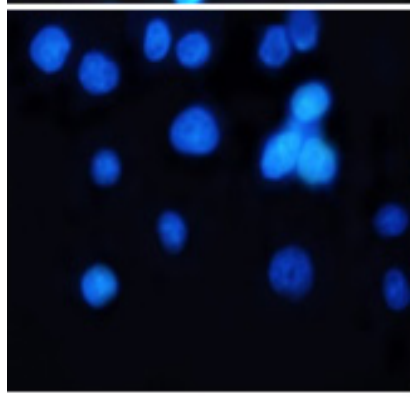
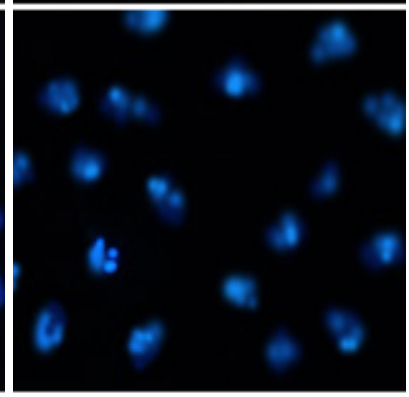
Cell Line	Control	R1 Treated	apoptotic Cell %
Hela			68
Vero			76
AMN3			82
HEP2			71

Figure 3: induction of apoptosis in R1 treated cell lines. After treatment with IC50 final concentration of R1 (500 µg/mL for cells don't have IC50) or vehicle control (DMSO) for 48 h, cells were stained with DAPI and observed under fluorescence microscope (Olympus, Japan). Apoptotic cell percent counted in 25-30 microscopic fields. C= control, T= treated. 400 X

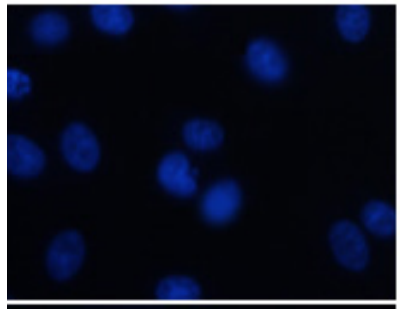
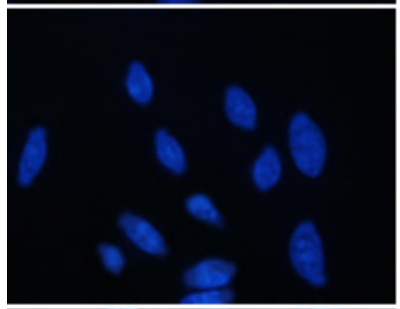
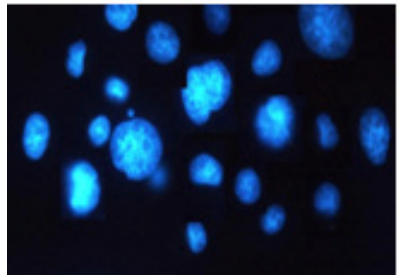
Cell Line	Control	R1 Treated	apoptotic Cell %
RD			35
GB			93
REF			4

Figure 3: Continuo.

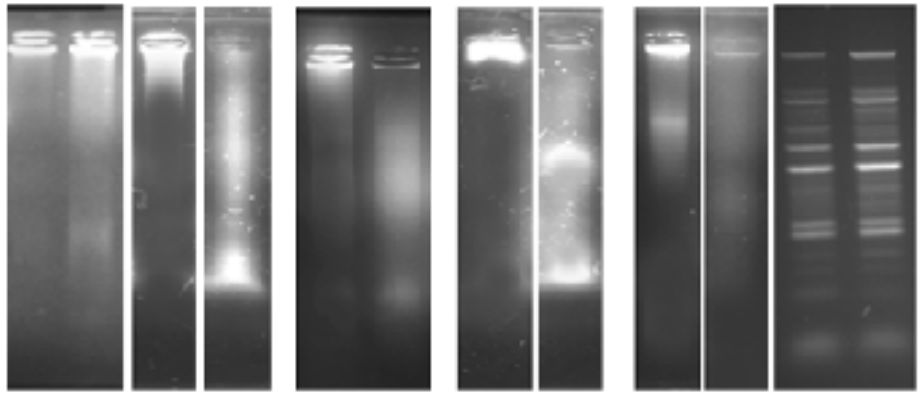


Figure 4 : DNA fragmentation assay . After each cell line treatment with its specific R1 inhibition concentration 50 for 48 h, cell's DNA were extracted and loaded on 0.7 % agarose gel. After electrophoresis, DNA was photographed by gel documentation system (Scie-plas, UK), C= control, T= treated.

The compound under investigation seems to succeed in modulating the enzyme super oxide dismutase (SOD) activity in HEp2, AMN3, Vero, and HeLa cells (Table 2). The SOD activity was significantly (P<0.0001) increased in cells treated with their IC50 of R1. Glutathione-S-transferase in all cell line treated with R1 was significantly (P<0.0001) reduced in its levels (Table 3). The concentration of intracellular reduced form of glutathione (GSH) was significantly (P<0.0001) decreased in cells treated with the IC50 of R1 (Table 4).

The tested components of the intracellular reactive oxygen species controlling system resemble one of the most important weapons of cancer cells to maintain it contentious proliferation status.

Table 2: Intracellular activity of super oxide dismutase (SOD) in cancer cells treated with inhibition concentration 50 of R1 or with vehicle control (DMSO) for 72 hr at 37˚C. Activity determination conducted using water soluble tetrazolium salt[WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)5-(2,4-disulfophenyl)-2-H-tetrazolium] and standard SOD solution.

Cell Type	Enzyme activity U/ml	Probability
HEp2	**130**	**0.0001**
Control	**27**	
AMN3	**7.9**	**0.0001**
Control	**0.27**	
Vero	**23**	**0.0001**
Control	**0.41**	
Hela	**76**	**0.0001**
Control	**0.75**	

Table 3: Intracellular activities of glutathione-S-transferase (GST) in cancer cells treated with inhibition concentration 50 of R1 or with vehicle control (DMSO) for 72 hr at 37˚C. Activity determination conducted utilizing 1-chloro-2-dinitrobenzen as substrate and standard GST solution

Cell Type	Enzyme activity mM	Probability
HEp2	**0.01**	**0.0001**
Control	**0.8**	

AMN3	**0.02**	**0.0001**
Control	**1**	
Vero	**0.07**	**0.0001**
Control	**3.21**	
Hela	**0.1**	**0.0001**
Control	**11**	

Table 4: Intracellular concentrations of glutathione (GHS) activity in cancer cells treated with inhibition concentration 50 of R1 or with vehicle control (DMSO) for 72 hr at 37˚C. intracellular GHS activity determination conducted utilizing its oxidation by 5,5-dithiobis(2-nitrobenzoic acid

Cell Type	Enzyme Concentration mM	Propability
HEp2	**0.01**	**0.0001**
Control	**17.1**	
AMN3	**0.01**	**0.0001**
Control	**9**	
Vero	**0.01**	**0.0001**
Control	**17**	
Hela	**0.01**	**0.0001**
Control	**1.2**	

Discussion:

The search for synthetic quinazoline derivative compounds that would exert cytotoxic effect against cancer cell specifically is ongoing effort (2). The effective biochemical mechanisms for such compounds depend on the differences between the metabolism activities of cancer and normal cells (26).

In this research we identified the anticancer activity of a novel synthetic aquinazoline compound (R1). It was locally synthesized in the Department of Applied Science, University of Technology, Iraq and it antibacterial and antifungal properties were previously characterized (7). This compound was able to inhibit cancer cell lines proliferation of different type and origin, its IC50 differed considerably from one type of cancer to another. Its severally inhibit the viability of Glyoblastoma (GB) and green African kidney transformed (Vero) cells. The toxic activity was moderate when tested against Human epidermoied larynx carcinoma cells (HEp2) and cervical cancer cells (Hela).

Minimal toxicity was noticed toward Rabdomyosarcoma cells (RD) as well as toward normal primary rat embryo fibroblast cells (REF). Such a result directs the attention to the differences in the mechanisms that R1 interfere with to induce toxicity in cancers. Derivatives of quinozoline compound are known to have more than one toxic effect toward cancer cells, one of such toxic effects could be enough to induce apoptosis when tested in vitro (27, 28). This compound (R1) may hold multiple mechanisms of action to induce cell toxicity in the same cancer cell, and may also have different mechanisms relevant to the specific type of cancer (29, 30).

Although we did not tested R1 activity as kinase inhibitor, quinazoline derivatives are particularly of great significance when targeting kinases (31, 32, 33). It's well documented that these compounds also have other drug function in addition to their antioxidant activity such as antiarrhythmic (34) and antihypertensive agents (35).

Conclusively DAPI staining assay and DNA fragmentation assay indicate obvious apoptotic characteristics in all cancer cells tested, even in RD cells which was less affected by R1. It's fortunate to imply that although R1 incline toxicity to REF cells but it did not indicate numerous apoptosis event in REF cell line as observed by DAPI staining and DNA fragmentation assays. This result is very precious; it's of great significance to have such a compound, which its toxicity relay on physiological differences between cancer and normal cells (33, 36).

Many reports referred to the derivatives of quinazoloine compounds as an antioxidant system modulator or can impose antioxidant activity if they were synthetic or isolated from natural sources (37, 38). The antioxidant as well as radical scavenging activity of quinazoline compounds are well documented, new chemically synthesized quinazoline compounds derivatives profound a potential reactive oxygen species (ROS) scavenging activity when the measurement of this activity were performed using 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) assay method (39, 40, 41, 42, 43).

We represent here an evident that R1 is a modulator of cancer cell antioxidant controlling system. Additionally our R1 compound may have ORS scavenging activity, many researchers indentified that quinazoline derivative compounds have potent reactive oxygen species scavenging activity they even propos that these compounds can be invested in cancer chemo-preventive therapy (44).

Since different types of quinazoline compounds were capable of perform radicals scavenging activity in vitro, the situation in our case could be relatively comparable; R1 may possess this activity although we did not masseur it chemically with DPPH. K. Manasa et al., (2010) indicated that synthesized quinazoline compounds have antioxidant properties as well as radicals scavenger activity correlated with its anticancer activity as determined by MTT assy. (45).

The capacity of R1 to modulate antioxidant status in the cancer cell lines under investigation represented by the increment of intracellular SOD activity in Human epidermoied larynx carcinoma cells (HEp2), transformed green African monkey kidney cells (Vero), whit mice mammary adenocarcenoma cells (AMN3), and cervical cancer cell line (Hela). Growing evidence indicate that cancer cells are under increased internal oxidative stress compared to normal cells due in part to mitochondrial dysfunction (46, 47).

Palma et al., (2010) studded the expression profiles of antioxidant superoxide dismutase-1 and -2 (SOD-1), (SOD-2) genes they found to be significantly hypo-expressed in cancerous tissues of patients undergoing lung resection for non-small cell lung cancer than in unaffected tissues (48). The chemically synthesized quinazoline derivatives found to be capable of increasing the activity of super oxide dismutase (SOD) and catalase enzymes (CAT) in rats as well as reduced glutathione (GSH) and glutathione peroxidase activity during prolonged treatment.

Furthermore researchers put forth the assumption of an antioxidant action of quinazolines by inhibition of the enzyme xanthine oxidase responsible for the formation of free radicals in particular the anion superoxide °O2 and reported that the quinoline derivative, aminoxyl protects from the oxidizing effect of the free radicals by preventing the production of the bio-membranes lipid peroxidation (49).Cellular GSH can regulate cell growth and apoptosis (50). Therefore; we analyzed the intracellular changes of GSH levels in the cell lines under investigation.

Apparently R1 was capable of depleting cells under investigation of their GSH. It has been reported that the intracellular GHS content has a decisive effect on anticancer drug induced apoptosis, indicating that apoptotic effect are inversely comparative to GSH content (51) the same is her our results demonstrate that treatment of cancer cells with

R1 depleted GSH level in these cells, and that would reduce cancer cells under investigation capacity to avoid the increment in super oxide radicals (O2-) consequentially the induction of apoptosis (52) Changes in the rate of cancer cell proliferation are accompanied by changes in their intracellular GSH levels and consequently these could be reflected in their antioxidant machineries (53).

Two decades ago the hypothesis which recognizes oxidative stress as survival stimulator of cancer cells was proposed (54) (55). Later it was observed that the Akt pathway is critical for cancer cell survival and the oxidative stress eventually led to the activation of the Akt (protein kinase B) pathway, which enhance cancer cells survival under chronic oxidative stress (56).

Other investigator suggests that inhibition of glutathione (GSH) with quinazoline derivative compounds may combine with Akt inhibitor to induce cytotoxicity via metabolic oxidative stress in human head and neck cancer (HNSCC) cells (57).

Since sublethal levels of reactive oxygen species (ROS) may promotes cancer cell proliferation and increase its resistant to apoptosis, the modulation of oxidative stress have great influence on cancer cell biology, and imbalance cancer cell needs to sublethal levels of oxidative stress may induce its apoptosis. Alteration of ROS levels in cancer cells through the alteration of its antioxidant defense enzyme levels would be a useful approach to treat cancer (58, 59). Conclusively R1 may be capable to perform such a behavior; it's of great significance to understand its full anticancer mechanism.

Dedicated to the memory of Dr. Zaid abdul munim Al-Hilli.
You are living in our hearts, we well continuo your mission.

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مقدرة مركب 3-amino-2-methylquinazolin-4(3H)-one(L) على حث الموت المبرمج وتحوير فعالية الأنزيمات المضادة للأكسدة في عدد من الخلايا السرطانية

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الخلاصة:

تمتلك المركبات المشتقة من حلقة الكوينازولين فعالية بايولوجة مميزة ، جرى التحري عن تثبيط النمو و السمية الخلوية وحث الموت المبرمج وقدرة تحوير فعالية الأنزيمات المضادة الأكسدة لأحد المركبات الجديدة المشتقة من هذه الحلقة والذي صنع في مختبرات قسم العلوم التطبيقية-الجامعة التكنولوجية وهو المركب3-amino-2-methylquinazolin-4(3H)-one(L) وذلك في عدد من الخطوط الخلايا السرطانية . والذي أظهر مقدرة عالية على قتل الخلايا السرطانية عن طريق حث الموت المبرمج من خلال تحوير فعالية الأنزيمات المضادة للأكسدةSOD و GST و GSHفي تلك الخلايا . يظهر هذا المركب قدرة دوائية مثيرة للأهتمام تتطلب المزيد من البحث.