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Synthesis, Characterization and Cytotoxic Activity of Some Pyrimidine Derivatives

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Abstract

Synthesis of some pyrimidine derivatives which plays an important role in the medicinal chemistry because it possesses promising cytotoxic activity. The synthesized compounds were characterized by UV-Visible and FT-IR spectral data. Some of the new compounds were evaluated for their potential cytotoxicity against two different human cancer cell lines HepG2 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma) in vitro, using Neutral Red and MTT assays. The synthesized compounds were active against two cell lines under study and a toxic effect was clear with a significant difference at the level of probability ($p< 0.0001$) and this effect was contrasted among different concentrations for each synthesized compound.

Keywords: Pyrimidine derivatives, Cytotoxic activity, Human cancer cell lines.

Introduction:

Pyrimidine derivatives are well known for their pharmacological activities. Various drugs containing pyrimidine nucleus were synthesized and used as anticancer agents like 5-Fluorouracil (5-FU), Tegafur and Thioguanine[1] (Fig.(1)).

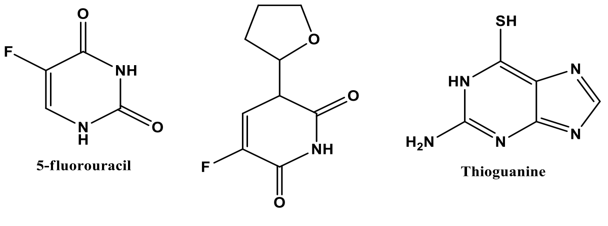

Fig. (1) Pyrimidine derivatives as anticancer agents.

An interest in pyrimidine derivatives as anticancer agents has led to the preparation and anticancer activity evaluation of hundreds of such molecules. For example, 2-cyanopyrimidines [2], hydrazino pyrimidine-5-carbonitriles [3], 1,3-dialkylated-pyrimidin-2,4-diones[4] and 4-anilino-2-(2-pyridyl). Pyrimidines were evaluated as a new class of potent anticancer agents [5]. Pyrimidines and their derivatives have been found to possess a broad spectrum of biological activities such as antimicrobial, anti-inflammatory, analgesic, antiviral and anticancer activities [6-13]. Quantitative methods for in vitro cytotoxicity have been described and recommended in the literature, although these methods present available cannot be easily automated. A range of assays based on different aspects of cellular activity can be applied for the assessment of biocompatibility. In the present study, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red (NR) assays for quantitative evaluation which have been adapted for human inactivated allografts are presented [14]. The first group of assays measures the ability of viable cells to reduce a water-soluble yellow dye, MTT, to a water-insoluble purple formazan product. This is converted by intracellular dehydrogenase to colored formazans. A second group of assays monitors' cell membrane integrity, and is based on the spectro-photometric determination of NR (3-amino-2-methyl-phenazine hydrochloride) taken up by viable cells and stored in their lysosomes.

In view of these facts, we aimed to investigate these uracil derivatives for their anti-cancer activities. Four compounds were subjected to 2 human tumor cell lines and all of these compounds are active against two cell lines.

Instrumental Method:

Synthetic methods for the preparation of pyrimidine derivatives are summarized in (Scheme (1)). Melting points were measured

by using (Gallen Kamp) melting point (Table (1)). Infrared spectra were recorded on F.T.IR-8300 Fourier transforms infrared spectrophotometer SHIMADZU as potassium bromide disc in the (600-4000) cm^{-1} spectral range (Table (2)). The electronic spectra of the compounds were obtained using (SHIMADZU UV-Vis. 160A) ultraviolet spectrophotometer (Table (2)). The reactions were monitored by TLC and purification of the compounds were carried out by recrystallization method using suitable solvent. The cytotoxic effect of the synthesized compounds was evaluated by Neutral Red and MTT assays.

Synthesis of 5,6-diaminopyrimidine-2,4-diol hydrochloride (a): [15]

Three-necked flask equipped with a reflux condenser and an efficient stirrer was placed. To 203 ml. of absolute ethanol (99.99%), (8g, 0.34 g. atom) of sodium, (18.5 ml, 0.17 mole) of ethyl cyanoacetate , and (10.4 g, 0.17 mole) of urea were added. The mixture was heated under reflux for 4 hours. (203ml) of hot (80°) water was added; the stirred mixture was heated at 80° for 15 minutes and then neutralized to litmus with glacial acetic acid. Additional glacial acetic acid (15.2 ml.) was added, followed by cautious addition of a solution of (13.1 g, 0.19 mole) of sodium nitrite which was dissolved in (14.1ml) of water. The nitroso compound was removed by filtration and washed twice with a small amount of ice water. The moist material was transferred back to the flask, and (87.3 ml) of warm water (50°) was added. Then an additional (6.1g) of sodium hydrosulfite was added. The dense diaminouracil bisulfite was filtered from the cooled solution, washed well with water, and partially dried. Then concentrated hydrochloric acid was added until the consistency of the resulting mixture was such as to permit mechanical stirring (20 to 40 ml. of acid). The slurry was heated on a steam bath with stirring for 1 hour. Tan diaminouracil hydrochloride was filtered on a sintered glass funnel, washed well with acetone.

Synthesis of 5,6-diamino-2-mercapto pyrimidine-4-ol hydrochloride (b): [15]

Three-necked flask equipped with a reflux condenser and an efficient stirrer was placed. To 203ml. of absolute ethanol (99.99%), (8g, 0.34 g. atom) of sodium, (18.5 ml, 0.17 mole) of ethyl cyanoacetate, and (13.29 g, 0.17 mole) of thiourea were added. The mixture was heated under reflux for 4 hours. (203ml) of hot (80°) water was added; the stirred mixture was heated at 80° for 15 minutes and then neutralized to litmus with glacial acetic acid. Additional glacial acetic acid (15.2 ml.) was added, followed by cautious addition of a solution of (13.1 g, 0.19 mole) of sodium nitrite which was dissolved in (14.1ml) of water. The nitroso compound was removed by filtration and washed twice with a small amount of ice water. The moist material was transferred back to the flask, and (87.3 ml) of warm water (50°) was added. Then an additional (6.1g) of sodium hydrosulfite was added. The dense diaminouracil bisulfite was filtered from the cooled solution, washed well with water, and partially dried. Then concentrated hydrochloric acid was added until the consistency of the resulting mixture was such as to permit mechanical stirring (20 to 40 ml. of acid). The slurry was heated on a steam bath with stirring for 1 hour. Tan diaminouracil hydrochloride was filtered on a sintered glass funnel, washed well with acetone.

Synthesis of 2-mercapto-6-methylpyrimidin -4(3H)-one (c): [16]

0.1 mole, 3.9 g of sodium hydroxide in (2.4 ml) water was added to the mixture of the (0.04 mole, 3 g) of thiourea and (0.04 mole, 5.0ml) of ethylacetoacetate in (4 ml) of ethanol in round bottomed flask and the mixture was refluxed for (2 hours), then hot solution was added (8 ml) of concentrated hydrochloric acid in (4 ml) of water to the product. The product was filtered and washed with cold distilled water.

Synthesis of 2-hydrazinyl-6-methylpyrimidin-4(3H)-one (d): [17]

A mixture of 0.02 mole, 3.63 g of 2-mercapto-6-methylpyrimidin-4(3H)-one and (12.7 ml) of hydrazine hydrate (99%) was refluxed in around bottomed flask for (3 hours). The product was filtered and washed with cold distilled water.

Synthesis of 2-(2-(4-(dimethylamino) benzylidene)hydrazinyl)6-methylpyrimidin-4(3H)-one (e):

A mixture of 0.002 mole, 0.28g of 2-hydrazinyl-6-methylpyrimidin-4(3H)-one and (0.002 mole, 0.29g) of 4-N, N-dimethylamino benzaldehyde in around bottomed flask with (5ml) ethanol, 1-2 drops of glacial acetic acid were added, and the mixture was refluxed for 5 hours, ice bath was used to separate the product, and the mixture was then filtered and precipitate was isolated.

Statistical Analysis:

The values of the investigated parameters were given in terms of mean ± standard error, and differences between means were assessed by analysis of variance (ANOVA) and Duncan test, using SAS computer program version 7.5 [18] Differences in results were considered significant at probability value equal or less than 0.0001.

Scheme (1).

Table (1)
Physical Data of the Prepared Compounds.

Name of compound.	*Structural Formula*	*M.wtg. mol^{-1}*	*M.P.*	*Yield %*	*Color*
5,6-diaminopyrimidine-2,4- diol hydrochloride (a)	HO N NH_2 .HCl N NH_2 OH	178.58	298-300C°	59.4%	Light tan
5,6-diamino-2-mercaptopy rimidine-4-ol hydrochlori de (b)	HS N NH_2 .HCl N NH_2 OH	194.64	300-303C°	30.3%	Light Yellow
2-mercapto-6-methyl pyrimidin-4(3H)-one (c)	H_3C N SH NH O	142.02	330C° (sublime)	64.2%	Yellowish-white
2-hydrazinyl-6-methyl pyrimidin-4(3H)-one (d)	H_3C N $NHNH_2$ NH O	140.07	212-214C°	42.8%	white
2-(2-(4-(dimethylamino) benzylidene)hydrazinyl)-6-methylpyrimidin -4(3H)-one (e)	Me N NH-N=CH- -$N(CH_3)_2$ NH O	271.14	≥300C°	55.5%	orange

Table (2)
Spectral Data of the Prepared Compound.

Compounds	*IR spectral data ν(cm^{-1})*	*UV-Visible λmax (nm)*
5,6-diaminopyrimidine-2,4- diol hydrochloride (a)	3406.1-3300(NH_2), 1712.7(C=O), 1668.3(C=N), 1618.2(C=C), 1195.8(C-N)	253nm
5,6-diamino-2-mercaptopy rimidine-4-ol hydrochlori de (b)	3390-3400(NH_2), 2300.9(S-H), 1631.7(C=N), 1548.7(C=C), 1225(C=S), 1176.5(N-C), 763.8(C-S)	271nm
2-mercapto-6-methyl pyrimidin-4(3H)-one (c)	3112.9(N-H), 3010arm 2887.2alph(C-H), 2580.6-2370.4(S-H), 1631.7(C=O), 1556.4(C=N), 1240.1-1197.7(C=S), 740(C-S)	292nm, 271nm
2-hydrazinyl-6-methyl pyrimidin-4(3H)-one (d)	3211.3-3250(N-H), 2925.8alph 3010arm(C-H), 1643.2(C=O), 1593.1(C=N), 1157.2(C-N)	404nm, 251nm
2-(2-(4-(dimethylamino) benzylidene)hydrazinyl)-6-methylpyrimidin -4(3H)-one (e)	3209.3-3290(N-H), 3010arm 2927.7 alph(C-H), 1641.3(C=O), 1600broad(C=N), 1164.9(C-N)	264nm, 355nm

Method of cytotoxicity assay

A-Neutral red assay:

Single cell suspension was prepared by treating 25 cm^3 tissue culture flask with 2 ml trypsin solution incubated for 2 min at 37°C in an incubator supplemented with (5%) CO_2 after detachment of the cells from the flask surface single cell suspension by gently taping of the flask followed by the addition of 20 ml of growth medium supplemented with 10% fetal calf serum then the viability test of the cells was made by using trypan blue dye which stains the dead cells. Cells suspension was well mixed followed by transferring 200 µl/well to the 96 well flat bottom micro titer plate using automatic micropipette containing ($1x10^5$ cell/well). Plates were incubated at 37°C in an incubator supplemented with (5%) CO_2 until 60-70% confluence of the internal surface area of the well for HepG2 cell line, the cells were then exposed to different concentration (600,300,150,75and 37.5 µg/ml) of new synthesized compounds, each compound was added to the cells in triplicate form of each concentration, only cells incubated with culture media represented the negative control, then the 96-well cell culture plate incubated at 37°C in an incubator supplemented with (5%) CO_2 for 48 hrs. After elapsing the incubation period, 50 µl/well of neutral red dye freshly prepared were added to each well and incubated again for 2 hrs, viable cells will uptake the dye and the dead not, the plates washed by PBS to remove the excess dye, then 100µl/well of eluent solution were added to each well to draw out the dye from the viable cells. Optical density of each well was measured by using *ELISA* reader at a transmitting wave length on 492nm [19].

MTT based cytotoxicity assay:

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] cytotoxicity assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. The number of surviving cells is directly proportional to the level of the formazan created. Cell proliferation assay was carried out by MTT cell proliferation assay kit (Roche applied sciences, Germany). Equal number of (MCF7) cells were seeded in each well of 96-well micro plate and incubated at 37C°, in presence of 5% CO_2. The cells were treated with synthesized compounds at various concentrations (100µg/ml, 50µg/ml, 25µg/ml, 12.5µg/ml, 6.25µg/ml and 3.125µg/ml) for 24 hrs. In vehicle control culture wells, a maximum of 0.5% DMSO was added. After 24 hrs. treatment, 5 µl of MTT reagent(R&D systems USA) along with 45µl of phenol red and FBS free DMEM (sigma Life Science, USA) was added to each well and incubated for 4 hrs. at 37C° in presence of 5% CO_2. Thereafter 50 µl of solublization buffer (R&D systems, USA) was added to each well to solubilize the coloured formazan crystals produced by the reduction of MTT. The optical density was measured at 570 nm using micro plate reader (Hidex Chamelon plate reader). The results (mean O.D.± SD) obtained from quadruplicate wells were used in calculation to determine the cytotoxicity (50% inhibitory concentration, IC50) of the test compounds.

Results and Discussion

Some new pyrimidine derivatives were synthesized in good yields (Scheme (1)). The antitumor activity results indicated that all the tested compounds are active against two cell lines (HepG2 and MCF7). The results of treated of cancer cell lines under study showed a toxic effect is clear with a significant differences at the level of probability ($p \leq 0.0001$) and this effect was found among different concentrations of the compounds prepared.

Cytotoxic effect of synthesized compounds on HepG2 cell line:

Results indicated in (Table (3)) showed that diamino uracil hydrochloride (a) has a cytotoxic effect on HepG2 cell line with a significant differences ($P \leq 0.0001$) started at the lower concentration and continued to the higher concentrations where the growth inhibition of HepG2 cell line was increased gradually with the increased treatment with Diamino uracil hydrochloride concentration, when compared with the negative control (the same cell line without any treatment), Maximum inhibitory effect of Diamino uracil hydrochloride was reached 61.3% when the

cell culture of HepG2 was treated with 600µg/ml of 5,6-diaminouracil-2,4-diol hydrochloride while the inhibitory effect decreased to 9% after treatment with 37.5% µg/ml of diaminouracil hydrochloride.

Cytotoxic effect of synthesized compounds on MCF7 cell line:

(Table (4)) showed that all compounds exhibited growth inhibition activity on the tested tumor panel breast cancer cell line (MCF7) between 100-3.12µg/ml in comparison to the negative control. And the synthesized compounds having IC50 values in the range of 20.9–23.2µg/mL can be classified as possessing mild cytotoxic activity against MCF7 cell line**.** The compounds **b** and **c** (with nitrogen heterocyclic ring in the molecule) showed moderate anticancer activity against MCF7 cell line while compound **d** showed high selectivity for MCF7 cell lines. Compound **a** exhibited high anticancer activity against HEPG2 and MCF7 cell lines.

Table (3)

Cytotoxicity Effect of Synthesized Compounds (at Different Conc.)on HepG2 Tumor Cell Line after, Incubation for 48 Hours Measured at 492nm.

compounds	*Concentration µg/ml*	*Absorbance of compounds*	*Inhibition Rate% ±SE*	*$IC_{50\%}$*
5,6-Diaminouracil-2,4diol hydrochloride (a)	37.5	1	9.00±0.57 e	432.6141
	75	0.968	12.00±0.00 d	
	150	0.788	28.30±0.34 c	
	300	0.599	45.50±0.28 b	
	600	0.425	61.3±0.17 a	
5,6-diamino-2-mercapto pyrimidine-4-ol hydrochloride (b)	37.5	0.978	11.00±0.57c	447.9962
	75	0.967	12.00±1.15 c	
	150	0.967	12.00±0.00 c	
	300	0.703	36.00±0.57b	
	600	0.368	66.5±0.28 a	
2-mercapto-6-methyl pyrimidin-4(3H)-one (c)	37.5	1.099	0.09±0.00d	531.6179
	75	1.097	0.27±0.00d	
	150	1.056	4.00±0.57 c	
	300	1.037	5.7±0.11b	
	600	0.371	66.2±0.11 a	
2-(2-(4-(dimethyl amine) benzylidene) hydrazinyl)-6-methyl pyrimidine-4(3H)-one (e)	37.5	0.997	9.30±0.17c	451.5608
	75	1.075	2.20±0.20e	
	150	1.03	6.30±0.00d	
	300	0.765	30.40±0.40b	
	600	0.422	61.60±0.11 a	

***** ($P \leq 0.0001$), different letters= significant differences between mean.***

Table (4)
Cytotoxicity Effect of Synthesized Compounds (at Different Conc.)on MCF7 Tumor Cell Line after, Incubation for 24 Hours Measured at 570nm.

Compounds	*Concentration μg/ml*	*Absorbance of compounds*	*Viable cell% ± SE*	*$IC_{50\%}$*
5,6-diaminouracil-2,4-diol hydrochloride(a)	3.125	0.646	96.12±0.22 a	21.9
	6.25	0.540	80.35±0.30 b	
	12.5	0.463	68.89±0.34 c	
	25	0.318	47.31±0.22 d	
	50	0.190	28.32±0.26 e	
	100	0.084	12.54±0.30 f	
5,6-diamino-2-mercapto pyrimidine-4-ol hydrochloride (b)	3.125	0.645	95.97±0.30 a	23.2
	6.25	0.558	83.03±0.30 b	
	12.5	0.467	69.53±0.34 c	
	25	0.335	49.84±0.30 d	
	50	0.202	30.10±0.52 e	
	100	0.090	13.38±0.39 f	
2-mercapto-6-methyl pyrimidin-4(3H)-one (c)	3.125	0.661	98.36±0.17 a	22.6
	6.25	0.559	83.18±0.17 b	
	12.5	0.478	71.17±0.21 c	
	25	0.342	50.98±0.48 d	
	50	0.175	26.13±0.26 e	
	100	0.074	11.00±0.25 f	
2-(2-(4-(dimethyl amine) benzylidene) hydrazinyl)-6-methyl pyrimidine-4(3H)-one (e)	3.125	0.653	97.16±0.17 a	20.9
	6.25	0.566	84.27±0.17 b	
	12.5	0.447	66.56±0.21 c	
	25	0.320	47.71±0.48 d	
	50	0.169	25.14±0.26 e	
	100	0.055	8.27±0.25 f	

Structural-activity relationship (SAR)

From the obtained results (Table (3,4)), we can conclude that the anticancer activity is due to:

(i) The presence of nitrogen heterocyclic rings.

(iii) The presence of 2-thiouracil moiety is essential for inhibition activity.[20]

Conclusion

Due to the presence of sulphur and nitrogen in the heterocyclic compounds skeleton, they show diverse biological activities. Pyrimidines are the important heterocyclic compounds which show promising pharmacological activities i.e.

anticancer agent. The present results suggests that 4 compound (a, b, c and e) induced cytotoxicity on HepG2 and MCF7 cell lines.

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الخلاصة

تضمن هذا البحث تحضير عدد من (مشتقات البريميدنات) التي تلعب دوراً هاماً في مجالات الكيمياء الطبية وذلك لانها تمتلك فعالية حياتية (فعالية سُمية أتجاه الخلايا)، و تم تشخيص المركبات المحضرة بواسطة الطرق الطيفية (طيف الاشعة تحت الحمراء F.T.IR) و الاشعة فوق البنفسجية (UV-VISIBLE) و قياس درجة الانصهار لهذه المركبات، وخضعت هذه المركبات الى نوعين من خطوط الخلايا السرطانية البشرية (سرطان الكبد (HepG2) و سرطان الثدي (MCF7)) خارج الجسم الحي وذلك بأستخدام فحوصات مختلفة (متعادل الصبغة الحمراء (neutral red) و (MTT))، و وجدَ أن هذه المركبات ذات فعالية اتجاه كلا الخطين السرطانيين وبفروق معنوية عند مستوى احتمالية ($P \leq 0.0001$) وكان هذا التأثير متباين بين التراكيز المختلفة لكل مركب من المركبات المحضرة .