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Original article

Antimicrobial, antitumor and 5α-reductase inhibitor activities of some hydrazonoyl substituted pyrimidinones

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ABSTRACT

A series of 2-[*N*-aryl-2-oxo-2-(4-chlorophenyl)ethanehydrazonoyl]-6-methyl-4(3*H*)-pyrimidinones **5** were prepared by coupling the diazonium salt of aniline derivatives with 2-(4-chlorobenzoylmethylene)-6-methyl-4(3*H*)-pyrimidinone **4** in sodium hydroxide solution. The structures of these newly synthesized compounds were confirmed by IR, NMR, mass spectrometry and elemental analyses and the tautomeric structure of these compounds was discussed. All the newly synthesized compounds were screened for their antibacterial and antifungal activities, some of which exhibited moderate activity. Also, the above compounds were evaluated for their antitumor activity against a panel of 60 human tumor cell lines by the National Cancer Institute (NCI), USA. Compounds **5b**, **5d** and **5i** showed good cytotoxic activities against the tested cell lines. In addition, the newly synthesized compounds were screened for their 5α-reductase inhibitor activity and all the tested compounds showed activities in descending order as follows **5b**, **5c**, **5g**, **5j**, **5d**, **5h**, **5f**, **5e** and **5i**.

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1. Introduction

The pyrimidinone skeleton exists in many natural and synthetic biologically active materials and its derivatives are applied in various pharmaceutical and biochemical fields [1,2]. It is of great interest that specifically functionalized pyrimidinones may possess specific biological properties. For example, it was recently found that some of pyrimidinones carrying an arylidene moiety have potential as antitumor agents [3], some of these analogues also showed, besides broad-spectrum antitumor activity, a distinctive pattern of selectivity toward individual cell lines, such as that of leukemia. In addition, hydrazones are known as very important class of organic compounds, some of which show significant biological activities, such as analgesic, anticancer, immunomodulating, antitumor, anti-inflammatory, antibacterial, and antifungal activities [4–9]. In continuation to our previous work [10], we wish to report herein synthesis of a new series of hydrazones namely, 2-{[*N*-aryl-2-oxo-2-(4-chlorophenyl)] ethanehydrazonoyl}]-6-methyl-pyrimidin-4(3*H*)-ones **5** and study their tautomeric structures and their biological activities like antibacterial, antifungal, antitumor and 5α-reductase inhibitor activities. We design the compounds in this study based on the synthesis of new pyrimidinone derivatives with certain co-planarity characters so that they can bind and interact with the grooves in the DNA and RNA-transcriptase interfering with the cancer gene coding and preventing cancer cell replication. Also, we aimed to replace the triazine moiety in the clinical worldwide used anstrazole®, a 5α-reductase inhibitor, by its bio-isoster pyrimidine and try to assemble its electronic and inter-atomic distance in the synthesized compounds **5a**–**j**.

2. Results and discussion

The required compound **4** was prepared from 6-methyl-2-thiouracil as described by Hurst et al. [11]. Reaction of compound **4** with diazotized anilines in ethanolic sodium hydroxide solution at 0–5 °C afforded the corresponding arylhydrazono derivatives **5a**–**j** (Scheme 1). The mass spectra of products **5a**–**j** revealed the molecular ion peaks at the expected *m*/*z* values, with relative intensities varying from 37 to 80%. Compound **5** can exist in one or more of five tautomeric structures **A**–**E** (Chart 1). Infrared spectral data (see Experimental) appear to be more consistent with the hydrazone structure (**A** or **B**) rather than the keto-azo form **C**, enol-azo tautomeric form **D** or the CH-azo form **E** (Chart 1). For example, all compounds exhibit two carbonyl bands in the regions

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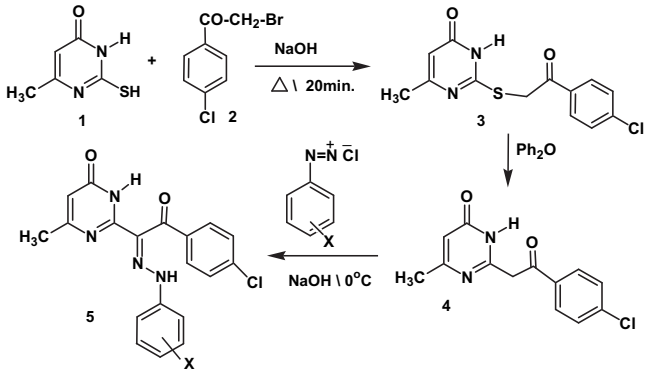

Scheme 1. Synthesis of compounds **5a**–**j**.

1690–1660 and 1659–1617 cm^{-1} corresponding to the stretching vibrations of the pyrimidinone and the benzoyl carbonyl groups, respectively. The low value of the wave number assigned for the latter CO stretching band in compounds **5a**–**j** seems to result from strong chelation with NH and conjugation with the C=N double bond as required by hydrazone form **A** or **B** [12]. This finding excludes the enol-azo form **D**.

To elucidate the actual tautomeric form of the studied compounds **5a**–**j**, the electronic absorption spectra were measured. The data are summarized in Table 1. As shown, each of compounds **5a**–**j** (in dioxane) exhibits two characteristic absorption bands in the regions 403–389 and 311–285 nm. Such an absorption pattern is similar to that of some typical hydrazone chromophores [10a,13,14]. The spectrum of **5d** (unsubstituted phenyl ring), as a representative example of the two series studied (a–c: electron donating substituents X; e–j: electron-withdrawing substituents X) was recorded in solvents of different polarities. The spectra showed little, if any, difference in shift (Table 1). The small shifts in λ_{max} of **5d** in different solvents are due to solute–solvent interactions. In agreement with this conclusion, is the observation that the spectra of aryl hydrazones derived from the reaction of quinones with *N*-alkyl-*N*-phenylhydrazines, are largely independent of the solvent polarity, unlike those of *o*- and *p*-hydroxyazo compounds [12]. This finding, while it excludes the azo tautomeric forms **C**, **D** and **E**, indicates that each of compounds **5a**–**j** exists in one tautomeric form, namely **A** or **B** (Chart 1).

Table 1
UV spectra of coupling compounds **5a**–**j**.

Compd. no.	λ_{max} (log ε)
5a	393 (4.52), 305 (4.00)
5b	401 (4.21), 307 (3.81)
5c	399 (4.01), 306 (3.65)
5d[a]	392 (3.99), 304 (3.80)
5e	393 (4.57), 299 (3.85)
5f	389 (4.22), 298 (3.74)
5g	403 (3.01), 311 (4.23)
5h	390 (4.30), 305 (4.00)
5i	390 (4.12), 305 (4.05)
5j	394 (4.15), 285 (4.20)

[a] Solvent: λ_{max} (log ε): acetic acid 396 (4.11), 300 (4.25); chloroform 394 (4.50), 300 (4.60); ethanol 392 (4.27), 302 (4.44); cyclohexane 394 (4.00), 304 (3.98).

Finally, to distinguish between the two forms **A** and **B**, ^{13}C NMR of compounds **5a**–**d** and **5g**–**j** were recorded. The data showed signals for the carbonyl carbon resonance at $\delta = 160$–164 ppm. This chemical shift suggests that N-(3) near to C=O is sp^3-hybridized (pyrrole type as in compound **6**) and different from the sp^2-hybridized carbon C-4, which appears at 170–175 ppm (compound **7**) (Chart 2) [15–19]. Based on the above finding, we conclude that the isolated products are found in each case in one tautomeric form namely, **A** rather than **B**.

3. Pharmacology

3.1. Antimicrobial and antifungal activities

The novel compounds **5** were screened for their antibacterial and antifungal properties using agar well diffusion method [20].

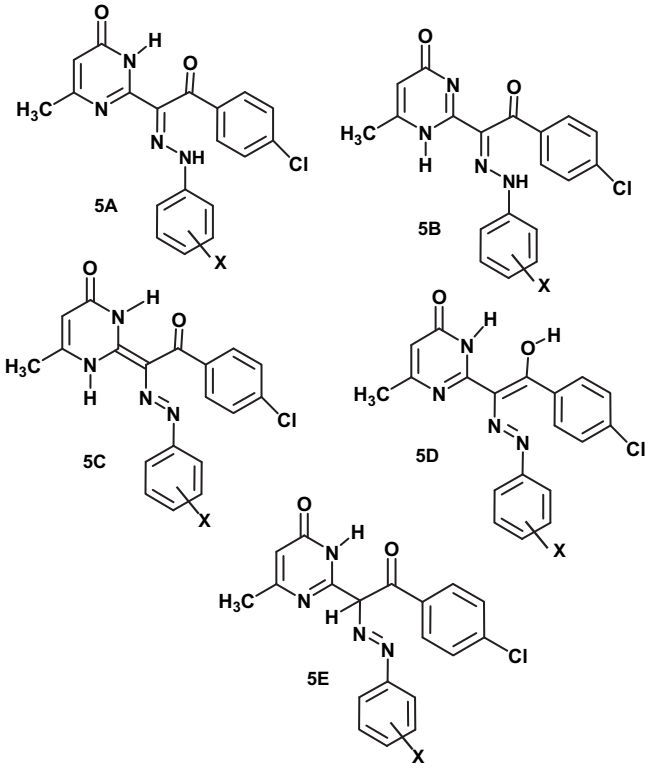

Chart 1. Tautomeric structures of compound **5**.

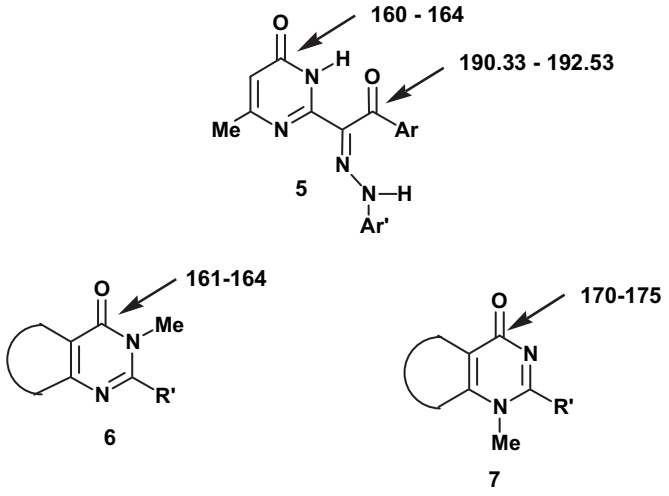

Chart 2. The ^{13}C NMR chemical shift.

Table 2
Antimicrobial activity of compounds **5**.

Compd. no.	Micro-organism/IZD (cm)[a]							
	AF	PI	SR	CA	SA	PA	BS	EC
5a	0	0	0	0	0	0	0	0
5b	0	0	0	0	0	0	0	++
5c	0	0	0	0	0	0	0	0
5d	0	0	0	0	0	0	0	++
5e	0	0	0	0	0	0	0	0
5f	0	0	0	0	0	0	0	+
5g	0	0	0	0	++	0	+	+
5h	0	0	0	0	++	0	0	+
5i	0	0	0	0	0	0	0	0
5j	0	0	0	0	0	0	0	0
CA[b]					++	+++	+++	++
TE[c]	+++	+++	+++	++				

[a] 50 mL of solution in DMSO whose concentration 5, 2.5, 1.0 μg/mL was tested.
[b] Chloramphenicol as standard antibacterial agent.
[c] Terbinafin as standard antifungal agent. +++, inhibition value 1.5–1.1 cm; ++, inhibition value 0.6–1.0 cm; +, inhibition value 0.1–0.5 cm beyond control; 0, no inhibition detected.

The antibacterial activity of the testing compounds was evaluated against four Gram-Positive and Gram-Negative bacterial species; *Staphylococcus aureus* **SA**, *Pseudomonas aeruginosa* **PA**, *Bacillus subtilis* **BS**, and *Escherichia coli* **EC**. *Chloramphenicol* was used as a reference to evaluate the potency of the testing compounds under the same conditions. Antifungal activity was screened against four fungal species; *Aspergillus fumigatus* **AF**, *Penicillium italicum* **PI**, *Syncephalastrum racemosum* **SR** and *Candida albicans* **CA**, using Terbinafin as the reference antifungal agent. All the compounds were dissolved in DMSO to concentrations of 5, 2.5, and 1.0 μg/mL, using inhibition zone diameter (IZD, cm) as a measure for the antimicrobial activity. The antibacterial screening revealed that compounds **5f**, **5g**, and **5h** showed moderate activity against **EC**, however, compounds **5b** and **5d** are highly active against **EC**, and compounds **5g** and **5h** are active against **SA**. Antifungal results indicated that all compounds showed no activity against chosen fungal species. All the data are shown in Table 2.

3.2. Anticancer assay for preliminary in vitro testing

The human tumor cell lines of the cancer screening panel were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. For a typical screening experiment, cells were inoculated in 96 well microtiter plates in 100 μL assay volume, at plating densities ranging from 5000 to 40,000 cell/well. After cell inoculation, the microtiter plates were incubated at 37 °C, under an atmosphere of 5:95 CO_2:air (v/v) at 100% relative humidity, for 24 h prior to addition of drugs under assessment. Following drug addition, the plates were incubated for an additional 48 h, under the same conditions. Sulfarohodamine B (SRB) solution (100 μL, 0–4% w/v in 1% aq acetic acid) is added to each well and plates were incubated for 10 min at room temperature [21,22].

3.2.1. In vitro assay

Evaluation of anticancer activity was performed on all compounds by NCI, USA. The recorded data demonstrates that among all tested compounds possessing a pyrimidinone moiety; three compounds produced the highest growth inhibition percentage. These three compounds were tested against 60 human tumor cell lines, from 9 cancer types. Initially, a single high dose was used (1.0 M) in the full NCI 60-cell panel. Only compounds which satisfied predetermined threshold inhibition criteria were progressed to the 5-dose screen, which was designed to efficiently capture compounds with anti-proliferate activity. This screen was

Table 3
Developmental therapeutics program one-dose mean graph.

Panel/cell line	Growth percent	Growth percent	Growth percent
	5b	**5d**	**5i**
Leukemia			
CCRF-CEM	−15.08	66.03	nd
HL-60(TB)	108.59	50.74	115.14
K-562	91.74	52.73	87.61
MOLT-4	98.62	39.84	84.16
RPMI-8226	85.08	96.57	91.91
SR	84.35	nd	62.16
Non-small cell lung cancer			
A549/ATCC	100.03	92.54	94.61
EKVX	91 .46	103.39	105.04
HOP-62	94.24	104.07	116.72
HOP-92	90.99	88.71	97.73
NCI-H226	107.41	108.67	111.00
NCI-H23	111 .33	105.18	86.10
NCI-H322M	106.17	107.24	100.21
NCI-H460	105.30	108.76	103.13
NCI-H522	nd	nd	84.76
Colon cancer			
COLO 205	116.23	105.71	121.73
HCC-2998	115.84	109.10	111.21
HCT-116	86.94	86.88	90.39
HCT-15	110.86	112.06	96.72
HT29	110.66	105.27	101.59
KM12	114.61	101.95	100.14
SW-620	93.70	94.56	98.45
CNS cancer			
SF-268	107.28	117.24	95.91
SF-295	90.05	75.72	95.38
SF-539	85.29	92.33	90.87
SNB-19	103.64	95.47	95.91
SNB-75	115.66	128.46	91.94
U251	103.37	106.82	96.78
Melanoma			
LOX IMVI	92.62	98.95	90.57
MALME-3M	126.09	113.86	110.47
M14	82.73	97.08	88.06
MDA-MB-435	123.17	107.78	95.80
SK-MEL-2	95.33	102.42	99.06
SK-MEL-28	117.97	110.14	106.93
SK-MEL-5	82.48	92.44	88.40
UACC-257	104.52	102.52	99.94
UACC-62	90.80	91.99	80.29
Ovarian cancer			
IGROV1	7.59	17.65	94.20
OVCAR-3	95.76	97.53	104.16
OVCAR-4	116.35	110.81	99.20
OVCAR-5	121.85	117.08	91.69
OVCAR-8	100.70	92.85	92.95
NCI/ADR-RES	102.57	105.88	93.99
SK-OV-3	115.86	98.31	95.27
Renal cancer			
786-0	82.91	82.24	103.17
A498	107.77	83.96	97.59
ACHN	99.08	110.63	90.63
CAKI-1	112.71	103.86	95.00
RXF393	40.73	28.48	94.35
SN12C	99.97	93.24	95.54
TK-10	136.08	65.49	120.15
UO-31	nd	116.06	75.87
Prostate cancer			
PC-3	95.01	92.58	90.50
DU-145	102.09	104.88	95.47
Breast cancer			
MCF7	91.01	90.11	89.39
MDA-MB-231/ATCC	95.90	101.67	85.96
HS 578T	99.88	83.59	90.12
BT-549	117.79	97.44	99.42
T-47D	88.32	77.02	75.56
MDA-MB-468	nd	nd	94.65

nd = not done.

Table 4
Evaluation of ED_{50}, LD_{50}, and 5α-reductase inhibitor activities relative to anastrozole.

Compd. No.	ED_{50}[a] ($\mu g\ kg^{-1}$)	LD_{50}[b] ($\mu g\ kg^{-1}$)	LD_{90}[c] ($\mu g\ kg^{-1}$)	Potency relative to anastrozole
5b	0.173	2663	4356.87	6.300578
5c	0.174	2555	3986.54	6.264368
5d	0.211	2111.22	3245.92	5.165877
5e	0.611	2345.37	3123.76	1.783961
5f	0.5911	2133.45	2980.32	1.84402
5g	0.188	2553.48	3300.12	5.797872
5h	0.478	2345.23	3290.98	2.280335
5i	0.698	1870.34	2250.87	1.561605
5j	0.198	2341.33	3217.17	5.505051
Anastrozole	1.09	2.415	4.314	1.00

[a] ED_{50}: Dose caused 50% of pharmacological response in the test.
[b] LD_{50}: Dose killed 50% of the tested animals.
[c] LD_{90}: Dose killed 90% of the tested animals.

based on careful analysis of historical DTP screening data, and the data set was reported as a mean graph giving the growth percent for each compound. The growth percent data is presented in Table 3. The activity of the compounds is measured according to a value of 100 means no growth inhibition. A value of 40 would mean 60% growth inhibition. A value of 0 means no net growth over the course of the experiment. A value of −40 would mean 40% lethality. A value of −100 means all cells are dead, this means that compound **5b** exhibits its highest activity against leukemia CCRF-CEM, ovarian IGROV1 cell lines and moderate activity against renal RXF393 cell line. Compound **5d** exhibits the highest activity against ovarian IGROV1 and renal RXF393 cell lines, and moderate activity against leukemia CCRF-CEM, HL-60(TB), K-562, MOLT-4 cell lines renal TK-10 cell lines. Compound **5i** exhibits moderate activity against leukemia SR, and the less activity against renal UO-31, and breast T-47D cell lines which is presented in Table 3.

3.3. Pharmacological screening

Circulating testosterone and dihydrotestosterone hormone levels or tissue concentrations were measured after administration of 5α-reductase inhibitor radioimmuno assays.

All synthesized compounds were tested for their 5α-reductase inhibitor activity in vivo; the ED_{50}, and LD_{50} data were determined and are given in Table 4. All the tested compounds showed 5α-reductase inhibitor activities with good LD_{50}, the order of activity in descending order is **5b**, **5c**, **5g**, **5j**, **5d**, **5h**, **5f**, **5e** and **5i**. All compounds were highly enough to provide good therapeutic windows and soft profile margin (Table 4).

From the data in Table 4, we concluded that:

- The influence of the substituent group toward decreasing the activity was as follow: methyl, nitro, ethyl ester, hydrogen, chloro and acetyl substituents.
- The methyl substituent on the diazo entity highly increases the activity (the para substituent provides more effect than the meta substituent).
- The chloride atom at position-4 increases the activity more than that at position-3 in the benzene ring.
- The decrease in the degree of intramolecular hydrogen bonding greatly increases the 5α-reductase inhibitor activity.
- Replacing the triazine moiety in the clinical used 5α-reductase inhibitor anstrazole® by its bioisoster pyrimidine highly increases the activity.
- The hydrazonoyl group is essential for the anticancer activity, while the substituents do not play a great role, hence +M substituents slightly increase the activity with respect to −M ones.
- The hydrazonoyl group with nitro substituents contributes to add antimicrobial activities to the tested compounds.

It is worth to mention that all the tested compounds have high safety margin and broad therapeutic windows.

4. Conclusion

In this context, we synthesized a new series of hydrazones and investigated their biological activities. For the activity against fungi and Bacteria, four compounds exhibited moderate activity and three have low activity against some kinds of bacteria. For what concern the antitumor activity, three compounds namely, **5b**, **5d** and **5i** showed significant inhibition of tumor cells at various cell lines. Regarding the 5α-reductase inhibitor, all the tested compounds except compound **5a** are highly enough to provide good therapeutic windows and soft profile margin activities. Also, the relation between the structure of the tested compounds and their biological activities was discussed and showed that replacing the triazine moiety in the clinical used 5α-reductase inhibitor anstrazole® by its bioisoster pyrimidine highly increases the activity. The hydrazonoyl group is essential for the anticancer activity, while the substituents do not play a great role. Moreover, the hydrazonoyl group with nitro substituents contributes to add antimicrobial activities to the tested compounds.

5. Experimental protocols

5.1. Chemistry

Melting points were determined on a Gallenkamp apparatus and are uncorrected. IR spectra were recorded in potassium bromide using Perkin Elmer FTIR 1650 and Pye-Unicam SP300 infrared spectrophotometers. ^1H and ^{13}C NMR spectra were recorded on a varian mercury VXR-300 spectrophotometer (300 MHz for ^1H and 75 MHz for ^{13}C) and the chemical shifts were related to that of the solvent DMSO-d_6. Mass spectra were recorded on a GCMS-Q 1000 EX Shimadzu and GCMS 5988-A HP spectrometers, the ionizing voltage was 70 eV. Electronic absorption spectra were recorded on Perkin–Elmer Lambada 40 spectrophotometer. Elemental analyses were carried out at the Microanalytical Laboratory of Cairo University, Giza, Egypt. 2-(4-chlorobenzoylmethylthio)-1,2-dihydro-6-methyl-4(3*H*)-pyrimidinones **3** was prepared as previously described [11].

5.2. 2-[N-aryl-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (*5*)

To a stirred solution of 2-(4-chlorobenzoylmethylene)-6-methyl-4(3*H*)-pyrimidinone **4** (0.65 g, 5 mmole) in ethanol (25 mL) was added sodium hydroxide (0.2 g, 5 mmole) and the mixture was cooled in an ice bath to 0–5 °C. To the resulting solution, while being stirred, was added dropwise over a period of 20 min, a solution of the appropriate arenediazonium chloride, prepared as usual by diazotizing the respective aniline (5 mmole) in hydrochloric acid (6 M, 3 mL) with sodium nitrite (1 M, 5 mL). The whole mixture was then left in a refrigerator overnight. The precipitated solid was collected, washed with water and finally crystallized from ethanol to give the respective hydrazone **5**.

5.2.1. 2-[N-(4-Methoxyphenyl)-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (*5a*)

Orange crystals, Yield 1.63 g, 82%, m.p. 206–208 °C. ^1H NMR (DMSO-d_6): δ 2.33 (s, 3H, 6-CH_3), 3.79 (s, 3H, OCH_3), 6.24 (s, 1H, 5-CH), 6.78 (d, $J = 8$ Hz, 2H, ArH), 6.84–7.22 (m, 4H, ArH), 7.69

(d, $J = 8$ Hz, 2H, ArH), 12.85 (s, 1H, NH), 16.28 (s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 21.94, 58.21, 117.37, 120.21, 126.58, 129.0, 129.20, 130.40, 131.31, 132.84, 135.98, 137.99, 143.55, 148.04, 162.11, 190.49. IR (KBr) ν 3423, 3172, 1681, 1658 cm^{-1}. MS m/z (%) 396 (M^+, 7), 152 (100), 111 (42). Anal. Calcd for $C_{20}H_{17}ClN_4O_3$ (396.84): C, 60.53; H, 4.32; N, 14.12. Found: C, 60.19; H, 4.12; N, 13.91%.

*5.2.2. 2-[N-(4-Methylphenyl)-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5b**)*

Yellow crystals, Yield 1.62 g, 85%, m.p 190 °C (ethanol). ^{1}H NMR (DMSO-d6): δ 2.27 (s, 3H, Ar-CH_3), 2.37 (s, 3H, 6-CH_3), 6.23 (s, 1H, CH), 7.32 (d, $J = 8$ Hz, 2H, ArH), 7.58 (d, $J = 8$ Hz, 2H, ArH), 7.78 (d, $J = 8$ Hz, 2H, ArH), 7.93 (d, $J = 8$ Hz, 2H, ArH), 12.45 (s, 1H, NH), 14.64 (s, 1H, NH).^{13}C NMR (DMSO-d_6): δ 20.51, 21.98, 116.80, 120.60, 126.51, 128.29, 129.21, 129.90, 131.32, 132.11, 135.61, 137.75, 143.65, 147.58, 163.14, 191.48. IR (KBr) ν 3424, 3129, 1690, 1659 cm^{-1}. MS m/z (%) 382 ($M^+ + 2$, 35), 381 ($M^+ + 1$, 48), 380 (M^+, 51), 379 (100), 111 (53). Anal. Calcd for $C_{20}H_{17}ClN_4O_2$ (380.84): C, 63.08; H, 4.50; N, 14.71. Found: C, 63.00; H, 4.70; N, 14.93%.

*5.2.3. 2-[N-(3-Methylphenyl)-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5c**)*

Dark orange crystals, yield 1.48 g, 78%, m.p. 234–236 °C. ^{1}H NMR(DMSO-d_6): δ 2.35 (s, 3H, Ar-CH_3), 2.41 (s, 3H, 6-CH_3), 6.31 (s, 1H, 5-CH), 7.06–7.31 (m, 4H, ArH), 7.58 (d, $J = 8$ Hz, 2H, ArH), 7.78 (d, $J = 8$ Hz, 2H, ArH), 12.42 (s, 1H, NH), 15.32 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 20.97, 22.66, 116.28, 120.94, 127.81, 127.64, 129.18, 130.46, 131.0, 132.43, 134.25, 135.28, 136.43, 138.11, 142.02, 147.09, 160.36, 191.07. IR (KBr) ν 3424, 3105, 1683, 1617 cm^{-1}. MS m/z (%) 382 ($M^+ + 2$, 42), 381 ($M^+ + 1$, 38), 380 (M^+, 54), 111 (43), 91 (100). Anal. Calcd for $C_{20}H_{17}ClN_4O_2$ (380.84): C, 63.08; H, 4.50; N, 14.71. Found: C, 63.20; H, 4.65; N, 14.81%.

*5.2.4. 2-[N-Phenyl-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5d**)*

Orange solid, yield 1.52 g, 83%, m.p. 198 °C. ^{1}H NMR (DMSO-d_6): δ 2.38 (s, 3H, 6-CH_3), 6.29 (s, 1H, 5-CH), 7.23 (d, $J = 8$ Hz, 2H, ArH), 7.38–7.79 (m, 5H, ArH), 7.82 (d, $J = 8$ Hz, 2H, ArH), 12.43 (s, 1H, NH), 14.32 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 21.35, 118.02, 121.21, 126.50, 128.38, 129.37, 132.01, 133.60, 134.51, 138.37, 142.25, 147.18, 148.56, 163.01, 190.78. IR (KBr) ν 3421, 3090, 1670, 1631 cm^{-1}. MS m/z (%) 368 (M^+2, 36), 367 ($M^+ + 1$, 43), 366 (M^+, 62), 111 (63). Anal. Calcd for $C_{19}H_{15}ClN_4O_2$ (366.81): C, 62.22; H, 4. 12 ; N, 15.27. Found: C, 62.30; H, 4.35; N, 15.34%.

*5.2.5. 2-[N-(4-Chlorophenyl)-2-oxo-2-(4-chlorophenyl)-ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5e**)*

Orange crystals, yield 1.70 g, 85%, m.p. 145 °C. ^{1}H NMR ($CDCl_3$): δ 2.36 (s, 3H, 6-CH_3), 6.30 (s, 1H, 5-CH), 7.22 (d, $J = 9$ Hz, 2H, ArH), 7.52 (d, $J = 9$ Hz, 2H, ArH), 7.63 (d, $J = 8$ Hz, 2H, ArH), 7.80 (d, $J = 8$ Hz, 2H, ArH), 11.04 (s, 1H, NH), 12.84 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 22.63, 117.22, 127.71, 128.42, 129.17, 129.30, 130.29, 131.62, 133.26, 136.50, 136.56, 139.13, 140.93, 162.00. 190.95. IR (KBr) ν 3419, 3186, 1667, 1620 cm^{-1}. MS m/z (%) 403 ($M^+ + 2$, 20), 402 ($M^+ + 1$, 32), 401 (M^++, 78), 136 (100), 111 (95). Anal. Calcd for $C_{19}H_{14}Cl_2N_4O_2$ (401.26): C, 56.87; H, 3.52; N, 13.96. Found C, 56.52; H, 3.29; N, 13.70%.

*5.2.6. 2-[N-(3-Chlorophenyl)-2-oxo-2-(4-chlorophenyl) ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5f**)*

Orange solid, yield 1.5 g, 75%, m.p. 222 °C. ^{1}H NMR ($CDCl_3$): δ 2.35 (s, 3H, 6-CH_3), 6.34 (s, 1H, 5-CH), 7.14–7.54 (m, 4H, ArH), 7.58 (d, $J = 8$ Hz, 2H, ArH), 7.81 (d, $J = 8$*Hz*, 2H, ArH), 12.33 (s,1H, NH), 15.32 (S, 1H, NH). MS m/z (%) 403 ($M^+ + 2$, 17), 402 ($M^+ + 1$, 29), 401 (M^+, 69), 111 (78). Anal. Calcd for $C_{19}H_{14}Cl_2N_4O_2$ (401.26): C, 56.87; H, 3.52; N, 13.96. Found: C, 57.05; H, 3.82; N, 13.71%.

*5.2.7. 2-[N-(3-Nitrophenyl)-2-oxo-2-(4-chlorophenyl) ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5g**)*

Yellow crystals, yield (1.63 g, 79%), m.p. 280 °C. ^{1}H NMR ($CDCl_3$): δ 2.36 (s, 3H, 6-CH_3), 6.35 (s, 1H, 5-CH), 7.53–7.81, 8.02–8.33 (m, 4H, ArH), 7.82 (d, $J = 8$ Hz, 2H, ArH), 7.87 (d, $J = 8$ Hz, 2H, ArH), 12.37 (s, 1H, NH), 13.10 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 22.75, 117.82, 121.46, 127.84, 128.28, 129.07, 129.19, 130.31, 130.71, 131.61, 133.28, 136.17, 138.03, 143.61, 148.56, 164.0, 190.33. IR (KBr) ν 3423, 3172, 1681, 1658 cm^{-1}. MS m/z (%) 411 (M^+, 64), 410 (100), 111 (30). Anal. Calcd for $C_{19}H_{14}ClN_5O_4$ (411.81): C, 55.42; H, 3.43; N, 17.01. Found: C, 55.66; H, 3.51; N, 17.36%.

*5.2.8. 2-[N-(4-Nitrophenyl)-2-oxo-2-(4-chlorophenyl) ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5h**)*

Orange crystals, yield 1.69 g, 82%, m.p. 130–132 °C. ^{1}H NMR ($CDCl_3$): δ 2.06 (s, 3H, CH_3), 6.25 (s, 1H, 5-CH), 7.62 (d, $J = 9$ Hz, 2H, ArH), 7.63 (d, $J = 9$ Hz, 2H, ArH), 7.81 (d, $J = 9$ Hz, 2H, ArH), 7.93 (d, $J = 9$ Hz, 2H, ArH), 12.83 (s, 1H, NH), 12.82 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 22.66, 118.15, 120.44, 127.74, 128.38, 129.48, 130.33, 131.96, 133.78, 136.0, 139.47, 143.05, 146.24, 162.15, 189.27. IR (KBr) ν 3400, 3308, 1673, 1666 cm^{-1}. MS m/z (%) 413 ($M^+ + 2$, 1), 412 ($M^+ + 1$, 2), 411 (M^+, 2), 152 (100), 111 (44). Anal. Calcd for $C_{19}H_{14}ClN_5O_4$ (411.81): C, 55.42; H, 3.43; N, 17.01. Found: C, 55.27; H, 3.60; N, 17.35%.

*5.2.9. 2-[N-(4-Acetylyphenyl)-2-oxo-2-(4-chlorophenyl) ethanehydrazonoyl]-6-methyl-4(3H)-pyrimidinone (**5i**)*

Redish brown solid, Yield (1.49 g, 73%), m.p. 182 °C. ^{1}H NMR ($CDCl_3$): δ 2.06 (s, 3H, 6-CH_3), 2.36 (s, 3H, $COCH_3$), 6.34 (s, 1H, 5-CH), 7.28 (d, $J = 9$ Hz, 2H, ArH), 7.62 (d, $J = 9$ Hz, 2H, ArH), 7.86 (d, $J = 9$ Hz, 2H, ArH), 7.95 (d, $J = 8$ Hz, 2H, ArH), 11.18 (s, 1H, NH), 12.86 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 22.36, 26.24, 115.32, 118.56, 123.23, 126.80, 128.45, 132.62, 137.46, 141.23, 148.44, 150.21, 154.05, 159.43, 161.66, 164.0, 192.53. IR (KBr) ν 3429, 3062, 1708, 1658, 1627 cm^{-1}. MS m/z (%) 410 ($M^+ + 2$, 12), 409 ($M^+ + 1$, 30), 408 (M^+, 37), 111 (81). Anal. Calcd for $C_{21}H_{17}ClN_4O_3$ (408.85): C, 61.69; H, 4.19; N, 13.70. Found: C, 61.90; H, 4.00; N, 14.01%.

*5.2.10. 2-[N-(Ethoxycarbonylphenyl)-2-oxo-2-(4-chlorophenyl)-ethanehydraz-onoyl]-6-methyl-4(3H)-pyrimidinone (**5j**)*

Red solid, Yield (1.64 g, 75%), m.p. 238 °C. ^{1}H NMR ($CDCl_3$): δ 1.29 (t, $J = 7$ Hz, 3H, CH_3), 2.06 (s, 3H, 6-CH_3), 4.25 (q, $J = 7$ Hz, 2H, CH_2), 6.26 (s, 1H, 5-CH), 7.31 (d, $J = 9$ Hz, 2H, ArH), 7.64 (d, $J = 8$ Hz, 2H, ArH), 7.80 (d, $J = 9$ Hz, 2H, ArH), 7.87 (d, $J = 8$ Hz, 2H, ArH), 11.20 (s, 1H, NH), 12.95 (s, 1H, NH); ^{13}C NMR (DMSO-d_6): δ 15.21, 20.93, 59.21, 116.99, 119.82, 121.63, 128.38, 131.26, 132.89, 133.05, 135.09, 139.0, 149.56, 152.68, 154.0, 158.21, 161.83, 190.56. IR (KBr) ν 3413, 3120, 1728, 1689,1631 cm^{-1}. MS m/z (%) 440 ($M^+ + 2$, 18), 439 ($M^+ + 1$, 36), 438 (M^+, 51), 437 (100), 111 (40). Anal. Calcd for $C_{22}H_{19}ClN_4O_4$ (438.87): C, 60.21; H, 4.36; N, 12.77. Found: C, 60.43; H, 4.72; N, 12.89%.

5.3. Biological assay

5.3.1. Treatment of animals

Animals were obtained from the animal house colony of the company. All animals were allowed free access to water and were kept on a constant standard diet. Twenty three groups, each of 12 male Sprague–Dawley rats in the postnatal third days, were treated subcutaneously with the 5α-reductase inhibitor (tested compound or reference standard). The tested compounds were dissolved in 5%. Tween 80 in water. The solvent was used for both standard and negative control group, beginning on the postnatal third day until the age of seven weeks.

Twenty-one groups were used to test the activities, of which one was used as the positive control for anastrozole and another served as the negative control group. After scarifying blood was withdrawn for testosterone and dihydrotestosterone (DHT) determination [23]. Moreover, intraprostatic concentrations of testosterone and DHT were determined [24].

The biological experiments were performed according to the official standards.

5.3.2. Radioimmuno assay for testosterone and dihydrotestosterone

Serum testosterone and dihydrotestosterone were measured by radioimmuno assay in serum extracts using specific antisera without prior chromatography. Serum samples of 0.5 mL were extracted with 2 mL of freshly purified peroxide-free diethyl ether by shaking for 60 s on a Vortex mixer. The aqueous phase was frozen at −70 °C, the ether phase containing steroids was transferred to a conical test tube and evaporated in BSA/phosphate buffer (pH = 7.4) containing (1,2,6,7-3H)-testosterone or (1,2,6,7-3H)-dihydrotestosterone and then specific antisera were added and incubated over a period of 24 h at 4 °C under non-equilibrium conditions. Bound hormone and free hormone were separated by adsorption on dextran-coated charcoal. The activity of each sample was determined in a Beckman-counter (USA) using a commercially available scintillation cocktail (Mini-RIA, Zinsser, Spain).

As for other steroid hormones, commercially available KIA-kits, e.g., Biermann GmbH, Germany, can be used.

The hormone level in the sample was calculated from a standard curve by means of a computer program (KIA-Calc, LKB, Canada), using appropriate control sera. Steroid levels of rats treated with different doses of 5α-reductase inhibitors were compared with vehicle-treated controls (Table 4). The relative potency was calculated by dividing the ED_{50} (dose that causes 50% of pharmacological response in the test) of anastrozole by that of a tested compound.

5.4. Determination of acute toxicity

LD_{50} and LD_{90} were determined by using male albino rats and injecting them with different increasing doses of agents. Doses that killed 50% and 90% of the tested animals, respectively, were calculated according to Austen and Brocklehurst [25] (Table 4).

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