Turk J Chem
28 (2004) , 559 – 571.

Synthesis of Novel 4-Alkylidene- and 4-Alkylamino-5-oxo-4,5-Dihydro-[1,2,4]triazole Derivatives and Investigation of Their Antitumor Activities

Neslihan DEMİRBAŞ*, Reyhan UĞURLUOĞLU
Karadeniz Technical University, Department of Chemistry, 61080 Trabzon-TURKEY
e-mail: neslihan@ktu.edu.tr

Received 21.05.2003

A series of novel 3-alkyl-4-cyclohexylmethylenamino-5-oxo-4,5-dihydro-[1,2,4]triazoles **(6a-e)** and 3-alkyl-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazoles **(7b-e)** were synthesized from the reaction of corresponding 3-alkyl-4-amino-5-oxo-4,5-dihydro-[1,2,4]triazoles (**1**) with cyclohexancarboxaldehyde and capronaldehyde. The acetylation of compounds **6e** and **7b** resulted in the formation of 1-acetyl-4-cyclohexylmethylenamino-5-oxo-3-(*p*-tolyl)-4,5-dihydro-[1,2,4]triazole **(10)** and 3-benzyl-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazole **(11)**. 3-Alkyl-4-cyclohexylmethylamino-5-oxo-4,5-dihydro-[1,2,4]triazoles (**8a-e**) and 3-alkyl-4-hexylamino-5-oxo-4,5-dihydro-[1,2,4]triazoles **(9b-e)** were obtained from the selective reduction of compounds **6a-e** and **7b-e** with $NaBH_4$. The in vitro antitumor activity of some selected compounds was screened by the National Cancer Institute (USA) against several human tumor cell lines, and compounds **8c, 9d** and **11c** were found to be active.

Key Words: Imine bond, antitumor activity, capron aldehyde, cyclohexane carboxaldehyde, 5-oxo-[1,2,4]triazole.

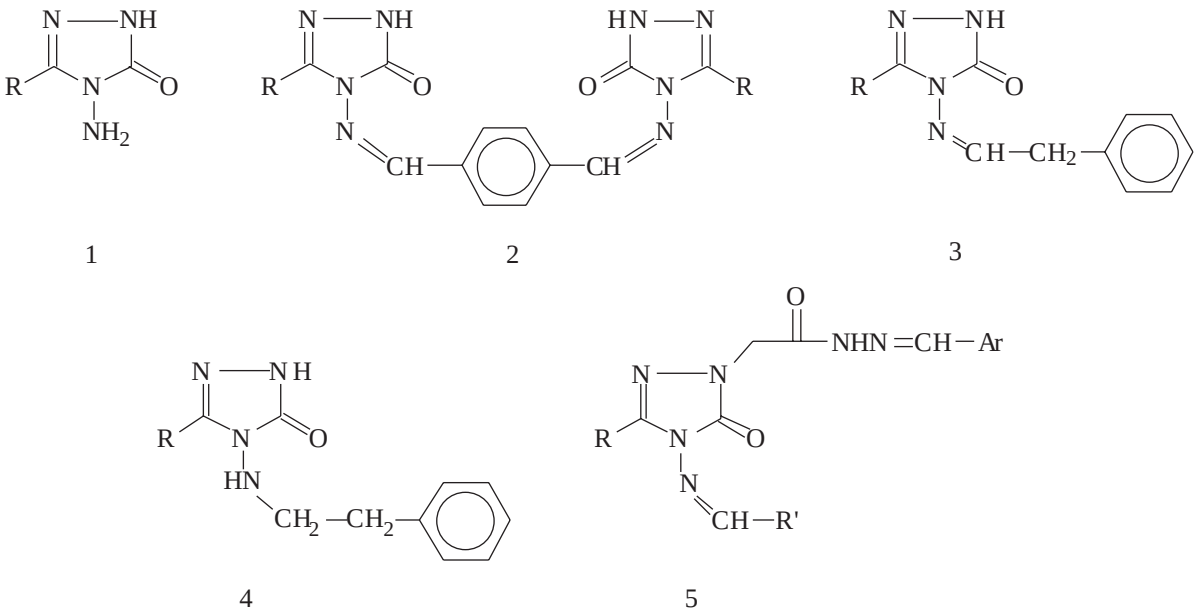
Introduction

In recent years, considerable attention has been devoted to the generation of new antitumor drugs for the treatment of cancer. Thus, several compounds having a wide range of different structures have been synthesized. Among these, there are structurally simple azole derivatives besides the complex molecules incorporating an azole moiety[1−11]. However, cancer is still a major health problem due to the insufficiency of conventional methods.

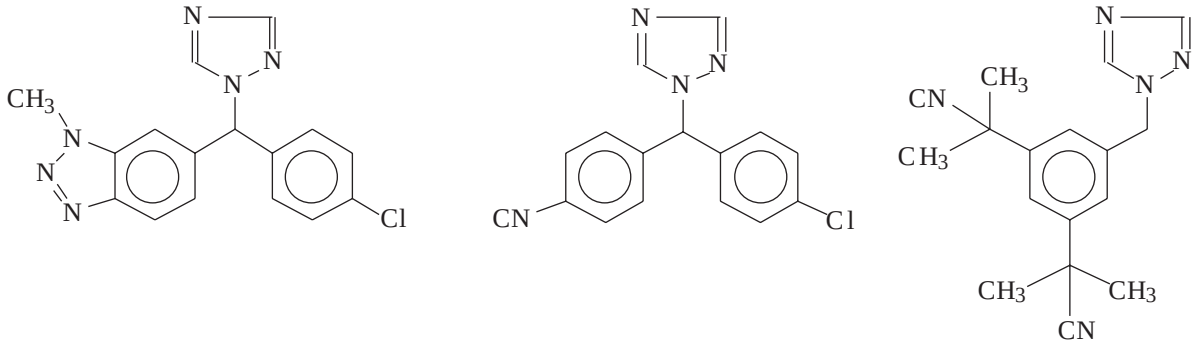
The chemistry of 3-alkyl-4-amino(or substitutedamino)-5-oxo-4,5-dihydro-[1,2,4]triazoles (**1**) has been studied in detail. For example, the alkylation, acylation, nitration and bromination of compounds **1** were performed in our laboratories along with their conversion into Schiff bases[4,12,13]. It has also been reported

*Corresponding author

that the conversion of the amino group at position 4 of the 5-oxo-[1,2,4]-triazole ring into the arylidenamino group may cause antitumor activity[14]. Several 4-alkylidenamino-5-oxo-[1,2,4]triazole derivatives have been synthesized using type **1** compounds and reported to be antitumor active agents[15]. It has been reported that the compounds having the highest activity contain aromatic groups such as phenyl and *p*-tolyl at position 3 and electron withdrawing groups such as *p*-nitrophenylmethylenamino, *o*-chlorophenylmethylenamino and phenylmethylenamino at position 4 of the 5-oxo-[1,2,4]triazole ring[15]. Moreover, some N, N'-bis(3-alkyl-5-oxo-4,5-dihydro-[1,2,4]triazol-4-yl)-1,4-xylenediimines (**2**), 3-alkyl-4-(2-phenylethylenamino)- (**3**) and 3-alkyl-4-(2-phenylethylamino)-5-oxo-4,5-dihydro-[1,2,4]triazoles (**4**) were synthesized from the reaction of compounds **1** with terephtalaldehyde and phenyl acetaldehyde, respectively (Scheme 1). Compounds **2-4** were found to possess activity against breast cancer, nonsmall cell lung cancer and brain tumors (CNC cancer)[4,12]. Furthermore, different type Schiff base derivatives (**5**) of compounds **1** synthesized in our laboratory were found to be active against only breast cancer[16]since breast cancers are generally estrogen dependent and aromatase enzyme catalyzes the conversion of androgens into estrogens in breast cancer tissues[17,18]. Hence, inhibitors of this enzyme are potential therapeutics for the treatment of estrogen dependent breast cancer[5]. It has been reported that compounds having triazole moieties such as vorozole, letrozole and anastrozole (Scheme 2) appear to be very effective aromatase inhibitors and may be effective in the treatment of breast cancer[19−21]. Among type **5** compounds the highest activity was observed in the compound containing a phenyl group at position 3 of the 5-oxo-[1,2,4]triazole ring. It is known that [1,2,4]triazole moieties interact strongly with the heme iron and aromatic substituents in the active site of aromatase[22]. It is speculated that the reason for the type **5** compound including a phenyl group at position 3 being the most effective against breast cancer cell line is a phenyl group that seems to fit to the active site of aromatase.



Scheme 1.



Vorozole Letrozole Anastrozole

Scheme 2. Known antitumor drugs incorporating the [1,2,4]triazole ring.

In view of these facts, our aim was to obtain 3-alkyl-4-alkylidenamino- and 3-alkyl(aryl)-4-alkylamino-5-oxo-4,5-dihydro-[1,2,4]triazole compounds to screen them for their potential antitumor activity.

Experimental

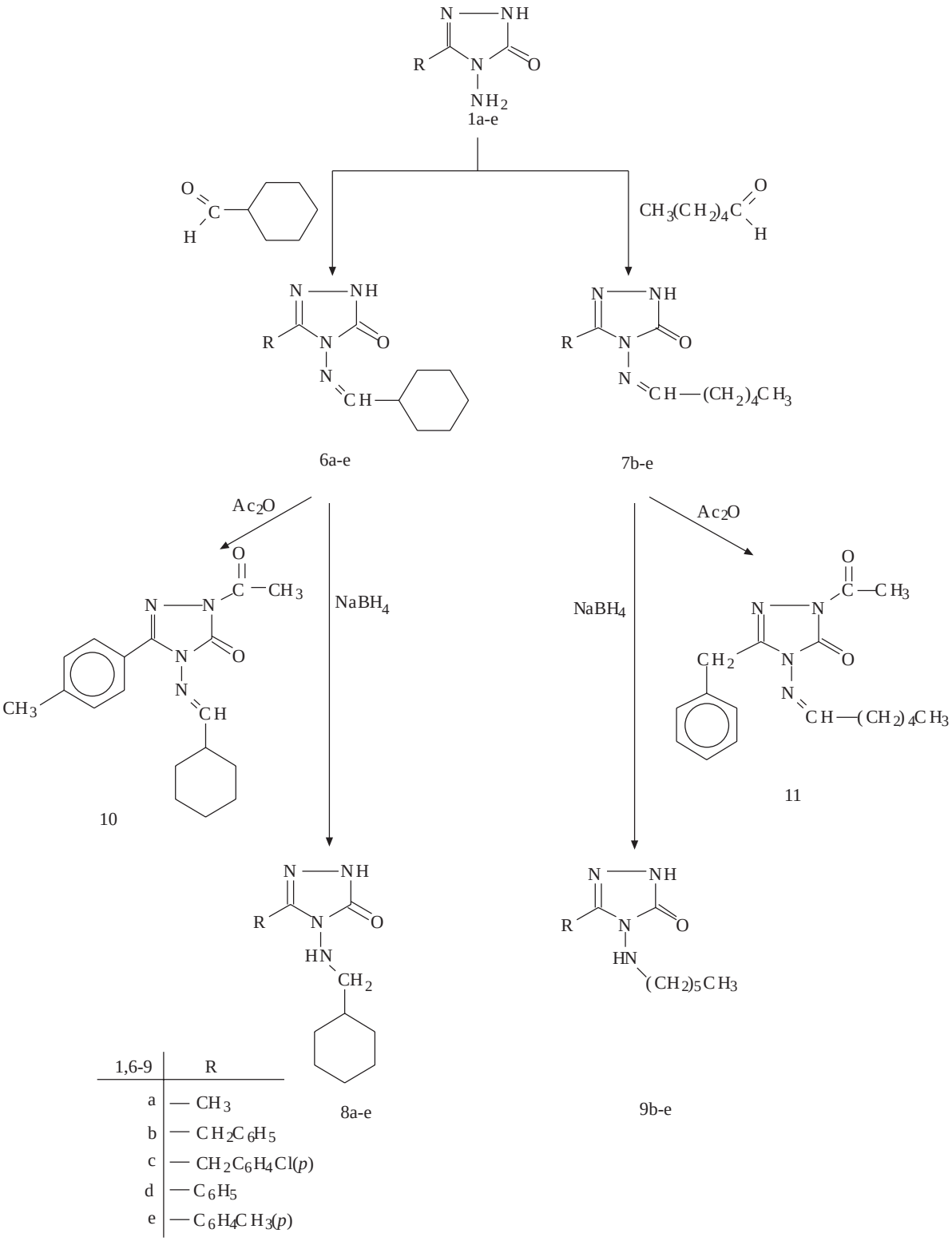
Melting points were determined on a Gallenkamp melting point apparatus and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Varian-Mercury 200 MHz spectrometer. The IR spectra were measured as potassium bromide pellets using a Perkin-Elmer 1600 series FTIR spectrometer. Combustion analysis was performed on a Carlo Erba 1106 elemental analyzer. All the chemicals were obtained from Fluka Chemie AG Buchs (Switzerland). Compounds **1** were synthesized by a published method[26].

General method for the synthesis of compounds 6

The corresponding compound **1** (0.01 mol) was heated in an oil bath with cyclohexancarboxaldehyde (0.01 mol) at 120-130 °C for 2 h. After cooling the mixture to room temperature a solid appeared. This was recrystallized from an appropriate solvent to afford the desired compound.

4-Cyclohexylmethylenamino-3-methyl-5-oxo-4,5-dihydro-[1,2,4]triazole (6a): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethyl acetate (yield: 58%) to afford the desired compound. M.p. 205-206 °C. Analysis (% Calc/found): for $C_{10}H_{16}ON_4$ C: 57.67/57.13, H: 7.74/7.08, N: 26.90/27.50; IR (KBr) cm^{-1}: 3261 (ν_{NH}), 1717 ($\nu_{C=O}$), 1599 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d$_6$) δ 1.25 (bs, 2CH$_2$) 1.50-2.10 (m, 3CH$_2$), 2.10-2.45 (m, CH), 2.20 (s, CH$_3$), 8.76 (d, N=CH, J=4.8Hz), 11.26 (s, NH); ^{13}C NMR (DMSO-d$_6$) δ 163.68 (N=CH), 153.59 (triazole C-5), 145.23 (triazole C-3), 40.99 (CH), 29.13 (2CH$_2$), 25.51 (CH$_2$),25.11 (2CH$_2$), 11.18 (CH$_3$).

3-Benzyl-4-cyclohexylmethylenamino-5-oxo-4,5-dihydro-[1,2,4]triazole (6b): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethyl acetate (yield: 50%) to afford the desired compound. M.p. 121-122 °C. Analysis (% Calc/found): for $C_{16}H_{20}ON_4$ C: 67.58/66.95, H: 7.09/7.08, N: 19.71/18.87; IR (KBr) cm^{-1}: 3179 (ν_{NH}), 1705 ($\nu_{C=O}$), 1590 ($\nu_{C=N}$); ^{1}H NMR (CDCI$_3$) δ 1.30 (bs, 2CH$_2$), 1.60-1.90 (m, 3CH$_2$), 2.25-2.45 (m, CH), 4.00 (s, CH$_2$), 7.30-7.40 (m, 5H, ar-H), 8.85 (d, N=CH, J=4.8Hz), 10.75 (s, NH); ^{13}C NMR (CDCI$_3$) δ 164.48 (N=CH), 152.26 (triazole



Scheme 3. Synthetic pathway for the preparation of compounds **6-11**.

C-5), 147.24 (triazole C-3), [ar C: 135.11, 129.01 (2C), 128.40 (2C), 126.89], 41.61 (CH), 31.69 (CH_2) 29.32 ($2CH_2$), 25.83 (CH_2), 25.31 ($2CH_2$).

3-(*p*-Chlorobenzyl)-4-cyclohexylmethylenamino-5-oxo-4,5-dihydro-[1,2,4]triazole (6c): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethyl acetate (yield: 50%) to afford the desired compound. M.p. 155-156 °C. Analysis (% Calc/found): for $C_{16}H_{19}ON_4CI$ C: 60.27/60.10, H: 6.01/5.97, N: 17.57/17.69; IR (KBr) cm^{-1}: 3154 (ν_{NH}), 1711 ($\nu_{C=O}$), 1592 ($\nu_{C=N}$);^{1}H NMR (DMSO-d_6) δ 1.25 (bs, $2CH_2$), 1.65-1.80 (m, $3CH_2$), 2.20-2.40 (m, CH), 4.00 (s, CH_2), 7.20-7.50 (m, 4H, ar-H), 8.85 (d, N=CH, J=4.8Hz), 11.90 (s, NH); ^{13}C NMR (DMSO-d_6) δ 166.43 (N=CH), 154.84 (triazole C-5), 145.20 (triazole C-3), [ar C: 129.91, 128.20 (2C), 127.30 (2C), 126.20], 40.75 (CH), 28.86 ($3CH_2$), 25.10 (CH_2), 24.76 ($2CH_2$).

4-Cyclohexylmethylenamino-5-oxo-3-phenyl-4,5-dihydro-[1,2,4]triazole (6d): Following the general procedure reported above, a white solid was obtained. It was recrystallized from acetone-water (1:3) (yield: 52%) to afford the desired product. M.p. 126 °C. Analysis (% Calc/found): for $C_{15}H_{18}ON_4$ C: 66.66/67.21, H: 6.71/6.58, N: 20.73/20.89; IR (KBr) cm^{-1}: 3160 (ν_{NH}), 1690 ($\nu_{C=O}$), 1584 ($\nu_{C=N}$);^{1}H NMR (DMSO-d6) δ 1.20 (bs, $2CH_2$), 1.55-1.80 (m, $3CH_2$), 2.20-2.45 (m, CH), 7.35-7.45 (m, 3H, ar-H), 7.75-7.45 (m, 2H, ar-H), 8.75 (d, N=CH, J=4.8Hz), 12.20 (s, NH); ^{13}C NMR (DMSO-d6) δ 166.59 (N=CH), 151.54 (triazole C-5), 144.39 (triazole C-3), [ar C: 130.09, 128.55 (2C), 127.86 (2C), 126.83], 40.94 (CH), 29.06 ($2CH_2$) 25.56 (CH_2), 24.96 ($2CH_2$).

4-Cyclohexylmethylenamino-5-oxo-3-(*p*-tolyl)-4,5-dihydro-[1,2,4]triazole (6e): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethyl acetate (yield: 50%) to afford the desired compound (yield: 60%). M.p. 200-201 °C. Analysis (% Calc/found): for $C_{16}H_{20}ON_4$ C: 67.58/66.80, H: 7.07/7.37, N: 19.71/19.21; IR (KBr) cm^{-1}: 3160 (ν_{NH}), 1698 ($\nu_{C=O}$), 1582 ($\nu_{C=N}$);^{1}H NMR (DMSO-d6) δ 2.25 (s, CH_3), 1.30 (bs, $2CH_2$), 1.60-1.95 (m, $3CH_2$), 2.30-2.60 (m, CH+ CH_3), 7.72 (d, 2H, ar-H, J=10 Hz), 7.30 (d, 2H, ar-H, J=10 Hz), 8.62 (d, N=CH, J=4.6Hz), 12.20 (s, NH); ^{13}C NMR (DMSO-d6) δ 166.19 (N=CH), 151.35 (triazole C-5), 144.28 (triazole C-3), [ar C: 139.65, 128.90 (2C), 127.58 (2C), 123.87], 40.75 (CH), 28.87 ($2CH_2$) 25.38 (CH_2), 24.79 ($2CH_2$), 20.86 (CH_3).

General method for the synthesis of compounds 7

A solution of corresponding compound **1** (0.01 mol) in 30 mL of glacial acetic acid was refluxed with capronaldehyde for 4 h. After cooling to room temperature, it was poured into 100 mL of water. On cooling in deepfreeze, a solid product occurred. This was recrystallized from an appropriate solvent to afford the desired compound.

3-Benzyl-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazole (7b): Following the general procedure reported above, a white solid was obtained. It was recrystallized from petroleum ether (yield: 55%) to afford the desired compound. M.p. 94-95 °C. Analysis (% Calc/found): for $C_{15}H_{20}ON_4$ C: 66.15/66.76, H: 7.40/7.35, N: 20.57/20.95; IR (KBr) cm^{-1}: 3168 (ν_{NH}), 1702 ($\nu_{C=O}$), 1589 ($\nu_{C=N}$); ^{1}H NMR ($CDCI_3$) δ 0.90 (t, CH_3, J=6.8 Hz), 1.20-1.45 (m, $2CH_2$), 1.45-1.70 (m, CH_2), 2.25-2.40 (m, CH_2), 4.00 (CH_2), 7.20-7.40 (m, 5H, ar-H), 8.95 (t, N=CH, J=5.5 Hz), 10.54 (s, NH); ^{13}C NMR ($CDCI_3$) δ 161.61 (N=CH), 152.28 (triazole C-5), 147.19 (triazole C-3), [ar C: 135.07 129.01 (2C), 128.77 (2C), 126.95], 33.44 (CH_2), 31.69 (CH_2), 31.20 (CH_2), 25.29 (CH_2), 22.36 (CH_2), 13.89 (CH_3).

3-(*p*-Chlorobenzyl)-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazole (7c): Following the general procedure reported above, a white solid was obtained. It was recrystallized from petroleum ether (yield: 58%) to afford the desired compound. M.p. 110-111 °C. Analysis (% Calc/found): for $C_{15}H_{10}ON_4CI$ C: 58.72/58.75, H: 6.25/6.83, N: 18.26/19.94; IR (KBr) cm^{-1}: 3173 (ν_{NH}), 1702 ($\nu_{C=O}$), 1584 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.85 (bs, CH_3), 1.15-1.38 (m, $2CH_2$), 1.38-1.58 (m, CH_2), 2.25-2.30 (m, CH_2), 3.95 (s, CH_2), 7.20-7.40 (m, 4H, ar-H), 8.90 (bs, N=CH), 11.87 (s, NH); ^{13}C NMR (DMSO-d_6) δ 160.88 (N=CH), 151.50 (triazole C-5), 145.35 (triazole C-3), [ar C: 134.62, 131.10, 130.56 (2C), 128.22 (2C)], 32.60 (CH_2), 30.54 (CH_2), 30.31 (CH_2), 24.70 (CH_2), 21.79 (CH_2), 13.72 (CH_3).

4-Hexylidenamino-5-oxo-3-phenyl-4,5-dihydro-[1,2,4]triazole (7d): Following the general procedure reported above, a white solid was obtained. It was recrystallized from petroleum ether (yield: 65%) to afford the desired compound. M.p. 106-107 °C. Analysis (% Calc/found): for $C_{14}H_{18}ON_4$ C: 65.09/65.16, H: 7.02/7.08, N: 21.69/21.55; IR (KBr) cm^{-1}: 3164 (ν_{NH}), 1694 ($\nu_{C=O}$), 1628 ($\nu_{C=N}$);); ^{1}H NMR (DMSO-d_6) δ 0.90 (bs, CH_3), 1.20-1.45 (m, $2CH_2$), 1.45-1.68 (m, CH_2), 2.35-2.45 (m, CH_2), 7.42-7.58 (m, 3H, ar-H), 7.78-7.95 (m, 2H, ar-H), 8.85 (t, N=CH, J=5.4 Hz), 12.27 (s, NH); ^{13}C NMR (DMSO-d_6) δ 163.13 (N=CH), 150.33 (triazole C-5), 143.07 (triazole C-3), [ar C: 128.84, 127.26 (2C), 126.58 (2C), 125.59], 31.49 (CH_2), 29.54 (CH_2), 23.64 (CH_2), 20.72 (CH_2), 12.70 (CH_3).

4-Hexylidenamino-5-oxo-3-(*p*-tolyl)-4,5-dihydro-[1,2,4]triazole (7e): Following the general procedure reported above, a white solid was obtained. It was recrystallized from petroleum ether (yield: 53%) to afford the desired compound. M.p. 99-100 °C. Analysis (% Calc/found): for $C_{15}H_{20}ON_4$ C: 66.15/66.62, H: 7.40/7.23, N: 20.57/21.17; IR (KBr) cm^{-1}: 3165 (ν_{NH}), 1700 ($\nu_{C=O}$), 1624 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.90 (bs, CH_3), 1.20-1.50 (m, $2CH_2$), 1.50-1.70 (m, CH_2), 2.30-2.45 (m, CH_2), 2.40 (S, CH_3), 7.30 (d, 2H, ar-H, J=11.11 Hz), 7.70 (d, 2H, ar-H, J=11.11 Hz), 8.85 (t, N=CH, J=5.4 Hz), 12.20 (s, NH); ^{13}C NMR (DMSO-d_6) δ 164.17 (N=CH), 151.38 (triazole C-5), 144.50 (triazole C-3), [ar C: 139.66, 128.88, 127.54, 123.66], 32.52 (CH_2), 30.59 (CH_2), 24.70 (CH_2), 21.76 (CH_2), 20.85 (CH_3), 13.76 (CH_3).

General method for synthesis of compounds 8

A solution of corresponding compound **6** (0.01) in 40 mL of diglime was treated with a solution of $NaBH_4$ (0.03 mol) in 30 mL of diglime. The mixture was refluxed for 8 h and then poured into 500 mL of water. On cooling in a deepfreeze, a solid appeared. This was recrystallized from an appropriate solvent to afford the desired compound.

4-Cyclohexylmethylamino-3-methyl-5-oxo-4,5-dihydro-[1,2,4]triazole (8a): Following the general procedure reported above, a white solid was obtained. It was recrystallized from benzene-n-hexan (1:2) (yield: 59%) to afford the desired compound. M.p. 149-150 °C. Analysis (% Calc/found): for $C_{10}H_{18}ON_4$ C: 57.11/57.20, H: 8.63/8.34, N: 26.65/27.33; (KBr) cm^{-1}: 3257 and 3161 (ν_{2NH}), 1711 ($\nu_{C=O}$), 1595 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.84-1.05 (m, CH_2), 1.17-1.30 (m, $2CH_2$), 1.60-1.85 (m, $2CH_2$+CH), 2.07 (s, CH_3), 2.75 (bs, CH_2), 5.82 (bs, NNH), 11.34 (s, NH); ^{13}C NMR (DMSO-d_6) δ 153.99 (triazole C-5), 145.48 (triazole C-3), 56.05 (NCH_2), 35.94 (CH), 30.90 ($2CH_2$), 24.30 ($2CH_2$), 25.56 ($2CH_2$), 10.90 (CH_3).

3-Benzyl-4-cyclohexylmethylamino-5-oxo-4,5-dihydro-[1,2,4]triazole (8b): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethanol-water (1:3)

(yield: 62%) to afford the desired compound. M.p. 132-133 °C. Analysis (% Calc/found): for $C_{16}H_{22}ON_4$ C: 67.10/67.15, H: 7.74/7.35, N: 19.57/19.39; (KBr) cm^{-1}: 3258 and 3165 (ν_{2NH}), 1695 ($\nu_{C=O}$), 1578 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.77-0.88 (m, CH_2), 1.09-1.30 (m, $2CH_2$), 1.60-1.75 (m, $2CH_2$+CH), 2.60 (bs, CH_2), 3.82 (s, CH_2), 5.80 (t, NNH, J=5.8 Hz), 7.25-7.45 (m, 5H, ar-H),11.52 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.27 (triazole C-5), 145.48 (triazole C-3), [ar C: 136.19 (C), 128.74 (2CH), 128.49 (2CH), 126.70 (CH)], 55.82 (NCH_2), 35.89 (CH), 30.89 ($3CH_2$), 26.24 (CH_2), 25.53 ($2CH_2$).

3-(*p*-Chlorobenzyl)-4-cyclohexylmethylamino-5-oxo-4,5-dihydro-[1,2,4]triazole (8c): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethanol-water (1:3) (yield: 65%) to afford the desired compound. M.p. 131-132 °C. Analysis (% Calc/found): for $C_{16}H_{21}ON_4CI$ C: 59.90/59.83, H: 6.18/6.50, N: 17.46/17.59; (KBr) cm^{-1}: 3242 and 3161 (ν_{2NH}), 1698 ($\nu_{C=O}$), 1579 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.80-1.00 (m, CH_2), 1.15-1.20 (m, $2CH_2$), 1.60-1.90 (m, $2CH_2$+ CH), 2.65 (bs, CH_2), 3.82 (s, CH_2), 5.82 (bs, NNH), 7.20-7.50 (m, 4H, ar-H),11.54 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.03 (triazole C-5), 147.18 (triazole C-3), [ar C: 135.16 (C), 131.43 (C), 130.65 (2CH), 128.42 (2CH)], 55.80 (NCH_2), 35.75 (CH), 30.77 ($2CH_2$), 26.11 (CH_2), 25.50 ($2CH_2$).

4-Cyclohexylmethylamino-5-oxo-3-phenyl-4,5-dihydro-[1,2,4]triazole (8d): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethanol-water (1:3) (yield: 67%) to afford the desired compound. M.p. 165-166 °C. Analysis (% Calc/found): for $C_{15}H_{20}ON_4$ C: 66.15/65.40, H: 7.40/7.52, N: 20.57/21.12; (KBr) cm^{-1}: 3258, 3165 (ν_{2NH}), 1695 ($\nu_{C=O}$), 1578 ($\nu_{C=N}$);^{1}H NMR (DMSO-d_6) δ 0.70-0.95 (m, CH_2), 0.90-1.15 (m, $2CH_2$), 1.45-1.70 (m, $2CH_2$+CH), 2.80 (t, CH_2, J=5.4), 6.05 (t, NNH, J=5.2 Hz), 7.45-7.55 (m, 3H, ar-H), 7.90-7.95 (m, 2H, ar-H), 11.95 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.25 (triazole C-5), 145.41 (triazole C-3), [ar C: 129.04 (C), 128.93 (2CH), 127.31 (2CH), 124.34 (C),], 55.80 (NCH_2), 35.75 (CH), 30.77 ($2CH_2$), 26.11 (CH_2), 25.50 ($2CH_2$).

4-Cyclohexylmethylamino-5-oxo-3-(*p*-tolyl)-4,5-dihydro-[1,2,4]triazole (8e): Following the general procedure reported above, a white solid was obtained. It was recrystallized from ethanol-water (1:3) (yield: 64%) to afford the desired compound. M.p. 195-196 °C. Analysis (% Calc/found): for $C_{16}H_{22}ON_4$ C: 67.10/67.87, H: 7.74/8.48, N: 19.57/20.36; (KBr) cm^{-1}: 3241 and 3154 (ν_{2NH}), 1700 ($\nu_{C=O}$), 1521 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 1.00-1.10 (m, $2CH_2$), 1.25-1.70 (m, $2CH_2$+ CH), 2.35 (s, CH_3), 2.80 (bs, CH_2), 6.00 (bs, NNH), 7.25 (d, 2H, ar-H, J=8.2 Hz), 7.85 (d, 2H, ar-H, J=8.2 Hz), 11.88 (s, NH); ^{13}C NMR (DMSO-d_6) δ ^{13}C NMR (DMSO-d_6) δ 154.25 (triazole C-5), 145.41 (triazole C-3), [ar C: 129.04 (C), 128.93 (2CH), 127.31 (2CH), 124.34 (C)], 55.85 (NCH_2), 35.79 (CH), 31.81 ($2CH_2$), 26.13 (CH_2), 25.52 ($2CH_2$), 21.05 (CH_3).

General method for the synthesis of compounds 9

A solution of corresponding compound **7** (0.01) in 40 mL of diglime was treated with a solution of $NaBH_4$ (0.03 mol) in 30 mL of diglime. The mixture was refluxed for 8 h and then poured into 500 mL of water. On cooling in a deepfreeze, a solid appeared. This was recrystallized from an appropriate solvent to afford the desired compound.

3-Benzyl-4-hexylamino-5-oxo-4,5-dihydro-[1,2,4]triazole (9b): Following the general procedure reported above, a white solid was obtained. It was recrystallized from acetone-water (1:3) (yield: 50%)

to afford the desired compound. M.p. 96-97 °C. Analysis (% Calc/found): for $C_{15}H_{22}ON_4$ C: 65.66/65.43, H: 8.08/8.17, N: 20.42/20.36; (KBr) cm^{-1}: 3257 and 3190 (ν_{2NH}), 1697 ($\nu_{C=O}$), 1581 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.84 (s, CH_3), 1.20-1.40 (m, $4CH_2$), 2.70 (bs, CH_2), 3.82 (s, CH_2), 5.85 (bs, NNH), 7.25-7.35 (m, 5H, ar-H), 11.53 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.05 (triazole C-5), 147.62 (triazole C-3), [ar C: 136.23 (C), 128.78 (2CH), 128.48 (2CH), 126.72 (CH)], 49.11 (NCH_2), 31.24 (CH_2), 30.86 (CH_2), 27.18 (CH_2), 26.26 (CH_2), 26.16 (CH_2), 14.04 (CH_3).

3-(*p*-Chlorobenzyl)-4-hexylamino-5-oxo-4,5-dihydro-[1,2,4]triazole (9c): Following the general procedure reported above, a white solid was obtained. It was recrystallized from acetone-water (1:3) (yield: 53%) to afford the desired compound. M.p. 72-73 °C. Analysis (% Calc/found): for $C_{15}H_{21}ON_4CI$ C: 58.34/57.97, H: 6.85/6.60, N: 18.14/18.89; (KBr) cm^{-1}: 3242 and 3161 (ν_{2NH}), 1704 ($\nu_{C=O}$), 1521 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.85 (bs, CH_3), 1.18-1.35 (m, $4CH_2$), 2.72 (bs, CH_2), 3.85 (s, CH_2), 5.87 (bs, NNH), 7.25-7.40 (m, 4H, ar-H), 11.58 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.05 (triazole C-5), 147.30 (triazole C-3), [ar C: 135.19 (C), 131.49 (CH), 130.68 (2CH), 128.52 (2CH)], 49.11 (NCH_2), 31.23 (CH_2), 30.29 (CH_2), 27.22 (CH_2), 26.54 (CH_2), 22.20 (CH_2), 14.14 (CH_3).

4-Hexylamino-5-oxo-3-phenyl-4,5-dihydro-[1,2,4]triazole (9d): Following the general procedure reported above, a white solid was obtained. It was recrystallized from acetone-water (1:3) (yield: 69%) to afford the desired compound. M.p. 118-119 °C. Analysis (% Calc/found): for $C_{14}H_{20}ON_4$ C: 64.59/64.76, H: 7.74/7.68, N: 21.52/21.05; (KBr) cm^{-1}: 3242 and 3161 (ν_{2NH}), 1704 ($\nu_{C=O}$), 1521 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.82 (bs, CH_3), 1.10-1.30 (m, $4CH_2$), 2.97 (bs, CH_2), 6.12 (bs, NNH), 7.48 (bs, 3H, ar-H), 7.98 (bs, 2H, ar-H), 11.97 (s, NH); ^{13}C NMR (DMSO-d_6) δ 154.34 (triazole C-5), 147.31 (triazole C-3), [ar C: 129.87 (CH), 128.40 (C), 127.31 (2CH), 127.17 (C)], 49.11 (NCH_2), 31.19 (CH_2), 27.06 (CH_2), 26.26 (CH_2), 22.18 (CH_2), 13.98 (CH_3).

4-Hexylamino-5-oxo-3-(*p*-tolyl)-4,5-dihydro-[1,2,4]triazole (9e): Following the general procedure reported above, a white solid was obtained. It was recrystallized from acetone-water (1:3) (yield: 49%) to afford the desired compound. M.p. 141-142 °C. Analysis (% Calc/found): for $C_{15}H_{22}ON_4$ C: 65.66/66.12, H: 8.08/8.56, N: 20.42/19.53; (KBr) cm^{-1}: 3244 and 3152 (ν_{2NH}), 1701 ($\nu_{C=O}$), 1508 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.80 (bs, CH_3), 1.10-1.35 (m, $5CH_2$), 2.35 (s, CH_3), 2.90-3.05 (m, CH_2), 6.10 (bs, NNH), 7.28 (d, 2H, ar-H, J=7.6Hz), 7.89 (d, 2H, ar-H, J=7.6), 11.91 (bs, NH); ^{13}C NMR (DMSO-d_6) δ 154.36 (triazole C-5), 145.31 (triazole C-3), [ar C: 1329.50 (C), 128.96 (2CH), 127.21 (2CH), 124.42 (C)], 49.20 (NCH_2), 31.24 (CH_2), 27.13 (CH_2), 22.22 (CH_2), 21.07 (CH_3), 13.96 (CH_3).

Synthesis of 1-acetyl-4-cyclohexylmethylenamino-3-(*p*-tolyl)-5-oxo-4,5-dihydro-[1,2,4]triazole (10)

Compound **6e** (0.01 mol) was refluxed with 10 mL of acetic anhydride for 2 h. The mixture was cooled to room temperature and, after 40 mL of ethanol was added, it was refluxed for an additional 30 min. After evaporation at 35-40 °C under reduced pressure, a solid appeared. This was recrystallized from benzene-petroleum ether (1:2) to afford the desired compound. (yield: 61%). M.p. 106-107 °C. Analysis (% Calc/found): for $C_{18}H_{24}O_2N_4$ C: 65.83/65.48, H: 7.34/7.54, N: 17.06/17.66; (KBr) cm^{-1}: 1783 and 1749 ($\nu_{2C=O}$), 1602 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 1.10-1.50 (m, $2CH_2$+ CH_3), 1.60-1.90 (m, $2CH_2$), 2.36 (s,

CH_2), 2.50-2.60 (m, CH_3+CH), 7.76 (d, 2H, ar-H, J=8.0 Hz), 7.32 (d, 2H, ar-H, J=8.0), 8.66 (t, N=CH, J=4.8 Hz); ^{13}C NMR (DMSO-d_6) δ 168.56 (N=CH), 165.55 (C=O), 147.75 (triazole C-5), 145.19 (triazole C-3), [ar C: 140.69 (C), 128.56 (2CH), 127.77 (2CH), 121.96 (C)], 28.26 ($2CH_2$+CH), 24.93 (CH_2), 24.33 ($2CH_2$), 23.00 (CH_3), 20.50(CH_3).

Synthesis of 1-acetyl-3-benzyl-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazole (11)

Compound **7b** (0.01 mol) was refluxed with 10 mL of acetic anhydride for 2 h. The mixture was cooled to room temperature and 40 mL of ethanol added. It was then refluxed for an additional 30 min. After evaporation at 35-40 °C under reduced pressure, a solid appeared. This was recrystallized from benzene-petroleum ether (1:2) to afford the desired compound. (yield: 50%). M.p. 43-44 °C. Analysis (% Calc/found): for $C_{17}H_{24}O_2N_4$ C: 64.53/64.60, H: 7.65/7.98, N: 17.71/17.43; (KBr) cm^{-1}: 1780 and 1733 ($\nu_{2C=O}$), 1602 ($\nu_{C=N}$); ^{1}H NMR (DMSO-d_6) δ 0.86 (s, CH_3), 1.26 (s, CH_3), 1.43-1.60 (m, $2CH_2$), 2.28-2.45 (m, CH_2), 2.50-2.60 (m, $2CH_2$), 4.01 (s, CH_2) 7.18 (m, 5H, ar-H), 8.76 (bs, N=CH); ^{13}C NMR (DMSO-d_6) δ 163.46 (N=CH), 160.58 (C=O), 147.76 (triazole C-5+ triazole C-3), [ar C: 134.38 (C), 128.75 (2CH), 128.67 (2CH), 126.76 (CH)], 32.57 (CH_2), 30.81 (CH_2), 30.41 (CH_2), 24.46 (CH_2), 23.35 (CH_3), 21.71(CH_2), 13.52 (CH_3).

Pharmacology

The screening experiments were performed by the Developmental Therapeutic Program of the National Cancer Institute (NCI), Bethesda, Maryland, USA. Seventeen compounds were selected by the NCI for screening of 3 human tumor cell lines, breast cancer (MCF7), nonsmall cell lung cancer (NCI-H460) and brain tumors (SF-268). The screening results are summarized in Table 1. Results for each test agent are reported as the percentage of growth of the treated cells when compared to the untreated control cells. Compounds (**6c** and **7d** and **9c)** that reduce the growth of any one of the cell lines to approximately 32% or less are passed on for evaluation in the full panel of 46 cell lines derived from human solid tumors (brain, breast, colon, leukemia, lung, melanoma, ovarian and renal) over a 5-log dose range. Each selected compound was tested at a minimum of 5 concentrations at 10-fold dilutions. A 48 h continuous drug exposure protocol was used and a sulforhodamin B (SRB) protein assay was used to estimate cell viability or growth[27]. The screening results of compounds **8c**, **9d** and **11c** towards several tumor cell lines are presented in Table 2 as GI_{50} values.

Results and Discussion

In order to synthesize 3-alkyl-4-alkylamino-5-oxo-4,5-dihydro-[1,2,4]triazoles, 2 methods have been developed[23,24]. One of these involves the reaction of ester ethoxycarbonylhydrazones with alkyl- or arylhidrazines[23], but there are only a few known aryl or alkyl hidrazynes and they are unstable in the reaction temperature. In the other method reported recently[24], 4-alkylamino compounds were obtained by the reduction of 3-alkyl-4-alkylidenamino-4,5-dihydro-[1,2,4]-triazoles obtained from the reaction of type **1** compounds with some halogenobenzaldehydes and *p*-tolualdehyde.

Table 1. Antitumor screening data for the selected compounds.

Comp. no.	Number assigned by NCI	Growth percentage of tumor cell			Activity
		MCF7	HCI-H460	SF-268	
6a	S722893	89	95	102	-
6b	S723214	112	106	115	-
6c	S721920	0	0	0	+
6d	S722894	101	79	98	
7b	S722899	108	100	103	-
7d	S722895	10	79	88	+
7a	S721925	108	96	105	-
8b	S721927	96	96	128	-
8c	S721929	110	102	139	-
8d	S721926	111	97	125	-
8e	S721928	108	95	129	-
9b	S721921	81	96	126	-
9c	S721923	44	31	42	+
9d	S721922	93	87	114	-
9e	S721924	91	95	99	-
10	S722889	91	101	102	-
11	S722890	80	103	96	-

In the present study, to improve the reported method[24], and to investigate the effect of the substituent at position 4 of the [1,2,4]triazole ring on antitumor activity, 4-alkylidenamino-5-oxo-4,5-dihydro-[1,2,4]triazole compounds were obtained. Thus, the synthesis of new 3-alkyl-4-cyclohexylmethylenamino- (**6a-e**) and 3-alkyl-4-hexylidenamino-5-oxo-4,5-dihydro-[1,2,4]-triazoles (**7b-e**) was achived in reasonably good yields. The IR, ^{1}H NMR and ^{13}C NMR spectra and elemental analysis of the new compounds are consistent with the suggested structures. In the ^{1}H NMR spectra of compounds **6a-e** the signals belonging to the cyclohexyl group were observed at 1.20-2.60 ppm as separate peaks, while the signal representing the $-NH_2$ group of compounds **1** and recorded at 5.30 ppm was absent. The ^{1}H NMR spectra of compounds **7b-e** showed peaks due to the hexylidene group at 0.85-2.45 ppm. In the ^{1}H NMR spectra of compounds **6a-e** and **7b-e** the signal derived from the -N=CH group was recorded at 8.75-8.95 ppm. The carbon-13 peak of the same group was seen at 160.88-166.59 ppm in the ^{13}C NMR spectra of these compounds. The ^{13}C signals of $4CH_2$+CH_3 belonging to the hexylidene group on compounds **7b-e** were observed at 30.54-12.70 ppm, while the signals belonging to the cyclohexyl group on compounds **6a-e** were seen at 41.61-24.96 ppm.

3-Alkyl-4-cyclohexylmethylamino- (**8a-e**) and 3-alkyl-4-hexylamino-5-oxo-4,5-dihydro-[1,2,4]-triazoles (**9b-e**) were obtained from the reduction of the exocyclic imine bond of type **6** and **7** compounds using a $NaBH_4$ as reducing agent. It was reported that the reduction of the 5-oxo-4,5-dihydro-[1,2,4]triazole ring is also possible[25]. Since $NaBH_4$ is a selective reducing agent, it did not reduce the 5-oxo-[1,2,4]triazole ring in the present study. The fact that both elemental analysis and spectroscopic data are consistent with the suggested structures for compounds **8a-e** and **9b-e** shows that the reduction took place only at the exocyclic imine bond of compounds **6a-e** and **7b-e** using $NaBH_4$ in diglime (diethylene glycol dimethyl ether). When $NaCNBH_3$ was used, no reduction was observed to take place in compounds **6a-e** and **7b-e** and the compounds remained unchanged.

Table 2. The screening results of compounds **6c**, **7d**, **9c** towards several tumor cell line)[a] (μM).

Panel/Cell line	**6c** GI_{50}	**7d** GI_{50}	**9c** GI_{50}
Leukemia			
CCRF-CEM	36.7	b	52.1
HL-60 (TB)	32.6	34.5	46.2
K-562	54.0	82.9	39.9
MOLT-4	35.1	69.8	48.5
SR	24.1	62.0	33.8
Non-small cell lung cancer			
A549/ATCC	39.7	b	30.8
HOP-62	b	18.9	57.6
HOP-92	28.6	b	85.5
NCI-H226	25.1	b	25.2
NCI-H322M	50.0	b	30.6
NCI-H460	44.5	b	35.1
NCI-H522	36.7	b	24.0
Colon Cancer			
COLO 205	84.9	b	40.4
HCT-116	b	b	30.0
HCT-15	60.9	b	42.2
HT29	65.4	60.7	b
ACHN	b	b	66.9
CAKI-1	46.8	b	48.4
RXF 393	18.4	b	19.5
SN12C	29.3	b	22.4
UO-31	44.7	b	31.4
Breast Cancer			
MCF7	22.8	89.4	25.1
NCI/ADR-RES	83.4	b	80.2
MDA-MB-231/ATCC	b	b	49.6
HS 578T	b	67.1	45.7
MDA-MB-435	69.3	b	28.6
T-47D	39.9	30.7	74.6
KM12	31.3	b	26.5
SW-620	56.6	b	b
CNC cancer			
SF-268	40.2	b	29.9
SF-295	b	b	53.1
SNB-19	b	b	46.0
SNB-75	b	b	b
Melanoma			
LOX IMIV	25.0	b	19.6
MALME-3M	b	b	31.8
M14	b	b	44.5
SK-MEL-2	28.9	b	16.7
SK-MEL-5	39.4	44.2	25.9
UACC-257	76.5	b	49.6
UACC-62	89.2	b	24.4
Ovarian Cancer			
IGROVI	63.7	b	34.0
OVCAR-3	50.6	b	55.1
OVCAR-4	21.1	b	b
OVCAR-5	b	b	b
OVCAR-8	89.2	b	46.7
Renal Cancer			
A498	21.5	30.2	28.2

[a)] GI_{50}: Molar concentration for 50% growth inhibition
[b)] The values GI_{50}, TGI or $LC_{50} \geq 100$ μM

The IR, ^{1}HNMR and ^{13}C NMR data of compounds **8a-e** and **9b-e** are very informative. In the IR spectra of compounds **8a-e** and **9b-e,** the presence of a carbonyl peak at about 1700 cm^{-1} shows that this group on the 5-oxo-[1,2,4]triazole ring was not reduced by $NaBH_4$. In the ^{1}H NMR spectra of these compounds, exocyclic -NH and -NH$\underline{CH_2}$ signals were observed at 5.80-6.10 ppm (exchangeable with D_2O) and 2.70-2.97 ppm, respectively, while the -N=CH signal was absent. The ^{13}C peaks of the -NHCH_2 group of compounds **8a-e** were recorded at 55.80-56.05 ppm, while the signals belonging to the same group of compounds **9b-e** were observed at lower field, 49.11-49.20 ppm. The endocyclic NH peaks of compounds **8a-e** and **9b-e** appeared at 11.52-11.95 ppm.

The 1-acetylated derivatives (**10** and **11**) were afforded when compounds **6e** and **7b** were treated with acetic anhydride. The IR spectra of compounds **10** and **11** displayed additional carbonyl absorption at 1780-1783 cm^{-1}originating from the acetyl group. In addition, the ^{1}H NMR spectra of acylated products (**10** and **11)** contain an additional signal belonging to the methyl protons of the acetyl group at 0.86-1.10 ppm. In the ^{13}C NMR spectra of compounds **10** and **11**, the carbonyl function of the acetyl group was observed at 160.58 -165.55 ppm, while the signal belonging to the carbonyl group on the 5-oxo-[1,2,4]triazole ring appeared at 147.75 ppm. The signal observed at 23.00-23.35 ppm in the ^{13}C NMR spectra of compounds **10** and **11** was attributed to the methyl carbon of the acetyl group.

The screening experiments were performed by the Developmental Therapeutic Program of the NCI. These results demonstrate that the acetyl group at position 1 of the 5-oxo-[1,2,4]triazole ring is not essential for antitumor activity. Compound **6c** exhibited activity towards leukemia, non-small cell lung cancer (except HOP-62), colon cancer (except HCT-116 and ACHN), breast cancer (except MDA-MB-435 and HS 578T), brain tumor (SF-268), melanoma (except MALME-3M, M14), ovarian cancer (except OVCAR 5) and renal cancer to inhibit 50% of the growth of tumor cells with GI_{50} values less than 100 μM. Moreover, compound **6c** showed marginal activity against HOP-62, HCT-116, ACHN, MDA-MB-435, HS 578T, SF-268, MALME-3M, M14 and OVCAR 5 with GI_{50} values equal to or greater than 100 μM. Compound **9c** displayed moderate activities towards all test cell lines with GI_{50} values less than 100 μM except for HT29, SW-620, SNB-75, OVCAR-4 and OVCAR-5. Compound **7d** was more selective towards the test cell lines; moderate activities were observed against the leukemia cell line (except CCRF-CEM), HOP-62, HT29, MCF7, HS 578T, T-47DSK-MEL-5 and A498 with GI_{50} values less than 100 μM. Although there is a *p*-chlorobenzyl or phenyl group at position the 3 of 4-alkyliden(or alkyl)amino-5-oxo-[1,2,4]triazole ring of compounds **6c**, **7d** and **9c** the results obtained are not suitable for an evaluation of structure-activity relationships. However, this stimulated us to investigate structural modifications in the [1,2,4]triazole ring to obtain potential antitumor activity.

Acknowledgments

This work was supported by the Research Fund of Karadeniz Technical University. The authors thank the National Cancer Institute, USA, for the antitumor screening results, and Dr. S. Güner (Karadeniz Technical University, Department of Chemistry) and Dr. R.Ş. Aslan (Karadeniz Technical University, Department of Foreign Languages and Literature) for their critical reading of the manuscript.

References

1. S. Yano, H. Kazuno, N. Suzuki, T. Emura, K. Wierzba, J. Yamashita, Y. Tada, Y. Yamada and M. Fukushima, T. Asao, **Bioorg. Med. Chem. 12,** 3431-41 (2004).
2. Y. Sadzuka, T. Sugiyama, H. Suzuki, H. Sawanishi and K. Miyamato, **Toxicology Lett. 150**, 341-40 (2004).
3. Y. Tsuzuki, K. Tomita, Y. Sato, S. Kashimoto and K. Chiba, **Bioorg. Med. Chem. 14,** 3189-93 (2004).
4. N. Demirbas, R. Uğurluoğlu, A. Demirbas, **Bioorg. Med. Chem. 10,** 3717-23 (2002).
5. P. Marchand, M. Le Borgne, M. Palzer, G. Le Baut and R. W. Hartmann, **Bioorg. Med. Chem. Lett. 13,** 1553-55 (2003).
6. C. Foulon, C. Danel, C. Vaccher, S. Yous, J.-P. Bonte and J.-F. Gossens, **J. Chromatography A, 1035** 131-36 (2004).
7. J.-Y. Chou, S.-Y. Lai, S.-L. Pan, G.-M. Jow, J.-W. Chern and J.-H. Guh, **Biochem. Pharmacol. 66** 115-24 (2003).
8. B.S. Holla, K.N. Poorjary, B.S. Rao and M.K. Shivananda, **Eur. J. Med. Chem. 37** 511-17 (2002).
9. B.S. Holla, B.K. Sarojini, B.S. Rao, P.M. Akberali, N.S. Kumari and V. Shetty, **Il Farmaco, 56** 565-70 (2001).
10. E. Pomarnacka and A. Kornicka, **Il Farmaco, 56** 571-77 (2001).
11. A.A. Ikizler, E. Uzunali and A. Demirbaş, **Ind. J. Pharm. 5**, 289-92 (2000).
12. H. Yüksek, A. Demirbaş, A. Ikizler, C.B. Johansson, C. Çelik and A.A. Ikizler, **Arzneim Forsh. Drug Res. 47**, 405-09 (1997).
13. A. Demirbas, N. Demirbas and A.A. İkizler, **Ind. J. Het. Chem. 9**, 87-94 (1999).
14. A.A. İkizler, A. İkizler, H. Yüksek and M. Serdar, **M. Model. Meas. Cont. C, 57**, 25-29 (1997).
15. A.A. İkizler, A. İkizler, M. Serdar and N. Yıldırım, **Acta Pol. Pharm. Drug-Res., 54**, 360-63 (1997).
16. N. Demirbas, S. Alpay Karaoğlu, A. Demirbas and K. Sancak, **Eur. J. Med. Chem.** (in press).
17. T. Nakata, S. Takashima, Y. Shiotsu, C. Murakata, H. Ishida, S. Akinaga, P.K. Li, H. Sasano, T. Suzuki and T. Saeki, **Steroid Biochem. Mol. Biol., 86**, 455-60 (2003).
18. N. Zilembo, E. Bajetta, E. Bichisao, A. Martinetti, I. La Torre, P. Bidoli, R. Longarini, T. Portale, E. Seregni and E. Bombardieri, **Biomed. Pharmacother.**, (in press).
19. P.E. Goss and K. Strasser-Weippl, **Best Pract. Res. Clin. End. Met., 18,** 113-30 (2004).
20. J.R. Santen, **Steroids**, **68**, 559-67 (2003).
21. M. Clemons, R.E. Coleman and S. Verma, **Cancer Treat. Rev**., **30**, 325-32 (2004).
22. S. Chen, Y.C. Kao and A. Laughton, **J. Steroid Biochem.**, **61**, 107-15 (1997).
23. E. Ayça, A.A. Ikizler and M. Serdar, **Chem. Acta Turc.** 9, 381-88 (1981).
24. B. Kahveci and A.A. İkizler, **Turk. J. Chem. 24**, 343-51 (2001).
25. J. Daunis, Y. Guindo, R. Jacquier and P. Vialefont, **B. Soc. Chim. Fr.** q, 3296-99 (1971).
26. A.A. İkizler and R. Ün, **Chem. Acta Turc. 7**, 119-22 (1979).
27. G.D. Gray and E. Wickstrom, **Biotechniques, 21,** 780-83 (1996).