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To cite this article: C Saturnino et al 2018 IOP Conf. Ser.: Mater. Sci. Eng. 459 012023

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Abstract. Carbazoles are aromatic heterocyclic compounds derived from the fusion of a benzene ring with an indole nucleus in 2,3 position. Today, many carbazole derivatives are widely studied as anticancer, anti-fungal, antioxidants, photoconductor, anti-bacterial, antimalarial, anti-Alzheimer, anti-tuberculosis, anti-HIV agents and for the treatment of obesity. Some of them, differently substituted on carbazole nucleus, have been synthetized and their biological activity have been evaluated. The purpose of this work is the study of new species obtained binding, a series of carbazole derivatives in which the carbazolic nitrogen has been functionalized with different alkyl-thiol chains, with gold nanoparticles, synthesized in organic solvent. The formation of self-assembled monolayers of these ligands is obtained on the spherical surface of gold nanoparticles, with a stabilizing effect against aggregation. These systems might present a double innovative function, matching an antineoplastic activity typical of carbazoles, with the photothermal effects of gold nanoparticles.

1. Introduction
Pharmaceutical research is focused on the design of new antineoplastic drugs [1] with higher selectivity on tumoral cells, which are able to overcome any resistance of the diseased cells and that cause bland side effects [2]. Carbazoles were largely studied for all their properties [2-4], [27-30], which can implement by changing functionalized groups on the nitrogen atom or inserting suitable substituents on carbazole core, with the purpose of obtaining new and unique properties as antioxidant or antimicrobial [5-7]. Carbazoles derivatives has become important for their efficient topoisomerase, tubulin, telomerase, kinase and integrase inhibition activity [8, 27, 31]. These compounds induce
antiproliferative activity and significant apoptotic response approaching cancer cells selectively when compared to the normal ones [9-11]. A new way of administering therapeutic agents, as carbazoles, involves the use of nanocarriers [12]. Recently, a lot of delivery carrier based on nanometric size, as nanotubes [13], nanorods [14], dendrimers [15] and lysosomes [16] have been designed; among these methods gold nanoparticles (Au-NPs) are good carrier of different payloads into target cells [17]. Gold nanoparticles (Au-NPs) take advantage of their unique chemical and physical properties to carry and release drugs, they are able to carry different size of drug molecules from little one to large biomolecules as DNA, RNA or proteins. A promising aspect in the treatment of cancer disease is the photothermal damage to cells caused by the presence of gold nanoparticles [20]. Au-NPs photothermal therapy is a technique for selective damage of cancer cells that is based on the use of gold nanospheres irradiated with 20 ns laser pulses (λ= 532 nm) to generate local heating [21-22]. A condition for an effective therapy provides for an efficient release of the drug, which can be released thanks to external stimuli such as light [18], or from the inside through variations in the pH levels. This paper describes a method to synthesize Au-NPs functionalized with thio-carbazole derivatives, as potential antiproliferative agents against breast and uterine cancer cell lines-without affecting non-tumoral cell lines viability.

2. Results and Discussion

2.1. Synthesis and Biological evaluation of N-thioalkylcarbazole derivatives (1a-1c, 2a, 3a).

The N-thioalkylcarbazole derivatives were synthesized by the reaction of 9-(bromo-alkyl)-9H-carbazoles (P1a-P1c, P1a-P3a) with thiourea using i-PrOH as solvent under reflux for 12 hours, in a nitrogen atmosphere (Figure 1).

After removal of the i-PrOH, the residue was treated with 6N NaOH under reflux for 5 h, and then HCl 3N has been added at rt. The obtained product was purified by PTLC in a mixture n-hexane/ethyl acetate (97/3=v/v) to collect the pure compounds. The intermediates 9-(bromo-alkyl)-9H-carbazoles (P1a-P1c, P1a-P3a) were prepared according to the general synthetic methods [23]. In fact, the proper carbazole derivatives (a-c) were stirred at rt with dry DMF until became clear. Then, NaH 60% oil dispersion and, successively, the appropriate terminal dibromoalkane [(=CH2)n, n=7-9] were added at 0 °C. The mixture was stirred for 5 hours at rt, then water was added and the resulting mixture was extracted with EtOAc. The obtained residue was purified by silica gel column chromatography (Et2O/hexane, 2/3 as eluent) to give the pure compounds.

The new synthesized carbazole derivatives (1a-1c, 2a, 3a) have been examined for their antiproliferative activity against two human breast cancer cell lines, namely MCF-7 and MDA-MB-231 and two human uterine cancer cell lines, cervix epithelium HeLa and endometrial Ishikawa. Only 1c, demonstrated a significant anti-proliferative activity on uterine cancer cells and, to a lesser extent, on breast cancer cell lines. Moreover, the assayed compounds did not show effects on the proliferation of non-tumoral MCF-10A and 3T3-L1 cells (Figure 2). Next, the molecular mechanism of compound
1c has been studied, proving that the observed anti-tumoral effects on HeLa cells depend on the induction of apoptosis [8].

![Figure 2](image-url) **Figure 2.** Antiproliferative activity of compound 1c (1-10-20-40 µM) on tumoral cell lines (MCF-7, MDA-MB-231, HeLa, Ishikawa) and non tumoral cells (MCF-10A, 3T3-L1).

Compound 1c induces apoptosis through the intrinsic pathway, also known as mitochondrial pathway, producing a clear increase of caspases 3/7 activity, which are cleaved by the initiator caspase-9 and that, in turn may activate other pro-apoptotic proteins and cleaves Parp-1, a protein involved in repairing DNA damages.

2.2 Synthesis of Au-NPs functionalized with N-thioalkylcarbazole derivatives.
The Au-NPs are synthesized by modifying the procedure described by Brust [24] (**Figure 3**), using the appropriate amount of binders.

![Figure 3](image-url) **Figure 3.** General procedure of synthesis of Au-NP@N-thio-alkylcarbazoles.

The required amount depends on molecular weight of chosen thio-carbazole, but, in general, the [Au]/[thiol] ratio is 5/1. In a typical synthesis 6 mL of a solution of TOAB in toluene (50 mM) were added under stirring to 2.3 mL of an aqueous solution of 30 mM HAuCl₄ to extract Au(III) from the...
aqueous phase; then 1.91 mL of an aqueous solution of NaBH₄ 0.5 M was added to the heterogeneous mixture, under vigorous stirring to reduce the Au(III) to Au (0). In a short time, a dark red organic phase is obtained, which is kept under stirring at room temperature for ~ 1h. After this period of time the thio-alkylcarbazole derivative 1a are added in 1/5 molar ratio to the organic phase and stirred for 24 hours. After this time the Au-NPs were purified, by very dry the solution by a rotary evaporator under vacuum; then the precipitate is dissolved in a minimum volume of dichloromethane and reprecipitated by addition of 10mL of ethanol. The suspension is treated with ultrasound and then it is centrifugated in order to isolate the pure Au-NPs@N-thioalkylcarbazole. All the other Au-NPs are synthesized following the same procedure with the chosen N-thioalkylcarbazole derivatives (1a, 1c, 2a, 3a).

2.3 Chemical-Physical Characterization of AuNP@N-thioalkylcarbazole.

The UV-Vis spectra of a toluene solution of Au-NP@carbazoles show a surface plasmonic resonance (SPR) band at 536 nm, which is characteristic of about 10 nm-sized nanoparticles. The particle size was also measured using a Dynamic Light Scattering (DLS) and the samples were prepared with TOAB or without it, to control and compare the growing of nanoparticles size. As shown in Table 1 the difference between the hydrodynamic radius, the nanoparticles synthesized with the use of TOAB present a smaller aggregation therefore form particles with lower hydrodynamic radius compared to the same nanoparticles synthesized without the TOAB.

<table>
<thead>
<tr>
<th>Name Sample</th>
<th>R_{hyd}/nm</th>
<th>% polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNP@1a (TOAB)</td>
<td>60</td>
<td>25%</td>
</tr>
<tr>
<td>AuNP@1a</td>
<td>100</td>
<td>10%</td>
</tr>
<tr>
<td>AuNP@2a (TOAB)</td>
<td>70</td>
<td>15%</td>
</tr>
<tr>
<td>AuNP@2a</td>
<td>140</td>
<td>20%</td>
</tr>
<tr>
<td>AuNP@3a (TOAB)</td>
<td>35</td>
<td>15%</td>
</tr>
<tr>
<td>AuNP@3a</td>
<td>35</td>
<td>10%</td>
</tr>
</tbody>
</table>

Freshly prepared colloids have been then casted onto stubs for SEM imaging, and particle analysis indicates that the diameter of all Au-NPs can be described approximately as Gaussian distribution (Figure 4). SEM images show different size of nanoparticles after toluene evaporation and DLS measurements of Au-NPs toluene solutions reveal a two peaks distribution ($R_{hyd}/nm \approx 50$ and $150$), at different hydrodynamic radius, which demonstrates that nanoparticles aggregation is occurring in solution maybe induced by $\pi-\pi$ stacking interaction.

![Figure 4](image-url) SEM images of nanoparticles of AuNP@1a (left side), and relative statistical distributions of the Au-NPs diameters size in the selected area (right side).
3. Conclusions
Cancer is one of the principal deathly diseases in developed countries, probably due to lifestyle choices, such as smoking, lack of physical activity and unbalanced diets [25]. Compound 1c induces apoptosis through the mitochondrial pathway, producing a clear increase of caspases which is involved in repairing DNA damages. New thiol-functionalized carbazoles, in which the carbazole nitrogen has been functionalized with alkyl-thiol chains, have been prepared [22], and used to stably functionalize Au-NPs. They are soluble in organic solvents, and are stable over weeks on the air. Au-NP@carbazoles show typical band in UV-Vis spectra. Morphological images of these nanoparticles were carried out in order to determine the gold dimension, while DLS studies were run directly in solutions. The cytotoxicity evaluation of the carbazole-capped Au-NP is actually in progress. These systems might present a double innovative function, combining an antitumor activity common of carbazoles, with the photothermal effects of gold nanoparticles.

4. Experimental Details
All reagents, excluding carbazole binders, and all solvents has been bought from Sigma-Aldrich and they are used without any other purification. Carbazole derivatives has been synthesized as already reported [25, 31, 32, 33]. Au-NPs are prepared by changing the reported literature method of Brust and co-workers [24].

The UV-vis absorption spectra were recorded using an HP spectrophotometer mod. 8451, capable of operating between 190 and 1100 nm. The set-up of the Dynamic Light Scattering (DLS) experiments is constituted of a He-Ne laser source (λ=632.8 nm and power of 35 mW), linearly polarized, focused onto the sample, and of a single photon counting detector (avalanche photodiode) for the scattered light. The detection technique is based on a self-beating detection mode, the scattering angle is fixed at 90° and temperature at T=24°C. A Malvern 4700 correlator is used to obtain the scattered intensity autocorrelation function. The hydrodynamic radius of particles RH is obtained from the normalized scattered electric field autocorrelation function by using a second-order cumulant expansion. The size distribution of the particles in solution was obtained through the Laplace inversion method. SEM morphology and SEM-EDS of the investigated samples were obtained using a FEI Quanta FEG 450 microscope.

Cell cultures, cell viability assay, tunel assay, western blot analysis, immunocytochemistry and caspase assay were performed as already described in Iacopetta et al. and Tundis et al.

Acknowledgments
Authors wish to thank: MIUR and CNR for financial support, Prof. C. Milone and Dr. E. Piperopoulos (Dept. of Engineering UniME, Italy) for SEM characterizations, Dr. N. Micali (CNR-IPCF Messina, Italy) for DLS measurements.

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