9-Fluorenon-4-carboxamides: synthesis, conformational analysis, anti-HSV-2, and immunomodulatory evaluation. Note II

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Dedicated to Professor Vincenzo Tortorella in the occasion of his "Fuori Ruolo" status

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Abstract

Title 9-fluorenon-4-carboxamides has been prepared as asymmetrically-substituted analogues of the antiviral tilorone and their antiherpetic and cytokine-inducing properties have been explored. The best pharmacological profiles were shown by carboxamides **1**, **4**, **9**, and **10** endowed with anti-HSV-2, IFNα, IFNγ and TNFα-inducing properties assayed on peripheral blood mononuclear cells (PBMC). The conformational analysis of compounds 1-10 has been performed by molecular modelling techniques and a geometrical descriptor was evaluated.

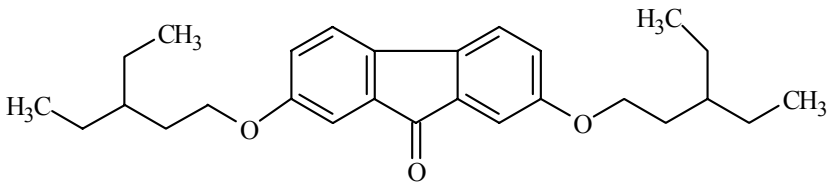
Keywords: 9-Fluorenon-4-carboxamides, tilorone analogues, anti-HSV-2 activity, cytokine-inducing properties, conformational analysis

Introduction

In pursuing our research on DNA-intercalating 9-fluorenone congeners[1,2] designed as potential immunomodulatory, chemotherapeutic agents, analogous to the antiviral drug tilorone, a series of 9-fluorenon-4-carboxamides have been synthesised and assayed for cytotoxicity, anti-HSV-2 and IFN-, TNF-inducent activities.

Tilorone, (2,7-bis[2-(diethylamino)ethoxy]-9H-fluoren-9-one), was discovered in 1970 as the first low molecular-weight IFN-inducing agent, effective *in vivo* against several DNA and RNA viruses in connection with its DNA intercalating property.[3-5]

In fact, the mechanism of action of tilorone is related to DNA-intercalation induced by the two main components of the drug: the chromophore and the side chains.[6]
Intercalation alters DNA chemo-physical properties stimulating cytokines expression[7].



Tilorone

Tilorone was marketed in USA and used for many years as a potent oral and intranasal active antiviral agent.[8] Clinical use, however, was stopped because of ascertained side effects such as mucopolysaccharidosis. In particular, tilorone proved to be a potent inducer of lysosomal storage of sulphated glycosaminoglycans (GAGs). Such an effect was then associated with the presence of the symmetrical basic side chains in 2 and 7 positions, responsible for the formation of the complexes with GAGs, resistant to lysosomal enzymes attack.[9-11] Different, non-symmetrically substituted, monobasic tilorone analogues have subsequently been reported, able to maintain intercalating and immunomodulatory properties but, lacking parent drug adverse effects, unable to disturb GAG degradation.[12,13]

The 9-fluorenon-4-carboxamides could belong to this class of non-symmetrically-substituted tilorone derivatives.

Nowadays many treatment protocols for viral and tumoral diseases rely on the combined administration of chemotherapeutics and immunomodulators able to counteract immunological disorders, consequent to the pathology.[7]

The pivotal role of mononuclear phagocytes in effective antiviral immunity is well established. Defined effector mechanisms include the release of cytokines such as IFN-γ, tumor necrosis factor-α (TNF-α) and other types of interferons such as IFN-α, with wide-ranging antiviral effects. These cytokines, regulating the inflammatory and immune responses, may induce an antiviral state in the cells (i.e. via IFNs) or destroy virus-infected cells (i.e. via TNF-α).

It is well known that endogenous IFNs are a large family of multifunctional secreted proteins involved in antiviral defence, cell growth regulation and immune activation.[14,15]

Recombinant IFN dominates the cytokines market, but its clinical use may cause unfavourable immune responses with the formation of neutralising antibodies that reduce the effectiveness of a particular therapy.

Therefore, enhancing the release of endogenous IFN by oral administration of low-molecular-weight compounds has been ardently desired.[16] Various synthetic compounds possessing IFN-inducing activity have been reported [17,18] and, among them, tilorone adopted by us as the parent drug of our recent research.[1,2]

In addition, many studies have demonstrated that TNF-α also exhibits potent antiviral activity *in vitro* which may be mediated, at least in part, through the action of IFN-α/β. Thus, synergy with IFN-γ and IFN-α/β dramatically enhances the antiviral effects of TNF-α, which includes the inhibition of virus replication and the induction of an antiviral state in uninfected neighbouring cells.[19]

Herpes simplex viruses establish lifelong, latent infections in the peripheral nervous system, that enables them to escape from the immune system and to persist in the host.[20]

Although genital infections due to HSV-1 occur, the vast majority of genital herpes infections are caused by HSV-2.[21] In addition, in immunocompromised patients, transplant recipients and individuals with acquired immune deficiency syndrome (AIDS), HSV-2 may reactivate from its latent state and cause severe recurrent and chronic diseases.[7]

The antivirals used nowadays (acyclovir and its congeners) can produce significant side effects and often have limited oral bioavailability. Moreover, virus strains resistant to most of these drugs are emerging.[22-24]

In the current treatment protocols for viral infections based on the combined use of preparations of immunomodulator cytokines with chemotherapeutics, the capability of a synthetic agent to modulate also cytokines production by peripheral blood mononuclear cells (PBMC) could constitute a new strategy in antiviral therapy.

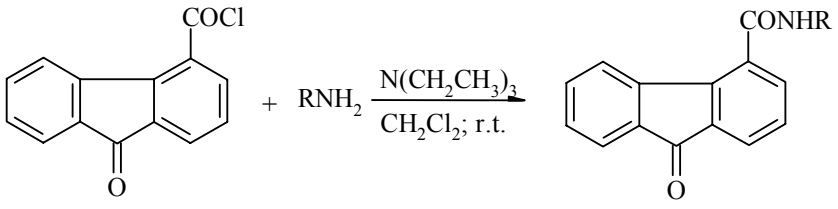
On the basis of these considerations we designed and investigated compounds **1-10**. Their citotoxicity on Wish cell lines, their potential anti-HSV-2 activity, based or not on their capacity to trigger PBMC in releasing IFN-α, IFN-γ, TNF-α, have been explored.

Aiming of investigating the action mechanism at molecular level, computerized conformational analysis, in comparison with parent tilorone, has been also performed.

Results and Discussion

Chemistry

Scheme 1 shows the synthetic pathway used for the preparation of 4-carboxamides **1-10** (Table 1) starting from commercial 9-fluorenon-4-carbonyl chloride by condensation with selected primary amines, in presence of distilled triethylamine, in dichloromethane for 24h. We have used amines endowed with different basic chains bearing OH groups or nitrogen and oxygen atoms in the aim to help DNA-intercalation or to stabilize it. Moreover an improvement of hydrosolubility was greatly aimed in view of biological *in vitro* assays.

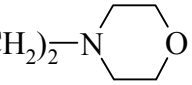
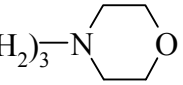


1 - 10

Scheme 1

The yields range between 73 and 91%.
Elemental and ^{1}H-NMR analyses have confirmed the assigned structures.

Table 1. 9-fluorenon-4-carboxamides

Comp.	R	Comp.	R
1	—$(CH_2)_2OH$	**6**	—$(CH_2)_2N(CH_3)_2$
2	—$(CH_2)_3OH$	**7**	—$(CH_2)_3N(CH_3)_2$
3	—$CH_2CHOHCH_3$	**8**	—$(CH_2)_2N(CH_2CH_3)_2$
4	—$(CH_2)_4OH$	**9**	—$(CH_2)_2$— 
5	—$(CH_2)_2O(CH_2)_2OH$	**10**	—$(CH_2)_3$— 

Conformational search study

The conformational search was carried out by molecular mechanics techniques coupled to the analysis of the averaged solvent accessible surface area (ASASA) computed on the fluorenone moiety. This descriptor was considered as able to take into account the putative mechanism of action of these compounds that could intercalate the duplex DNA by the fluorenone moiety.

On the other hand, ASASA allows the estimation of the degree of exposition of the planar tricyclic ring to the aqueous solvent, providing an indirect measure of pharmacokinetic properties of our compounds in which the fluorenone lipophilic moiety is linked to hydrophilic side chains.

In detail, after building the 3D structures of each compound, we proceeded with the exploration of the internal degrees of freedom by Monte Carlo (MC) randomization of any rotatable bond. 5000 conformations were generated and energy minimized with the AMBER* force field using the notation of all the atoms and the implicit model of solvation GB/SA water[25] as implemented in Macro Model ver. 7.2.[26] The convergence in the conformational search was evaluated for each molecule using the averaged number of duplicate conformers (AND). Usually an AND value higher than 2 indicates a good conformational space exploration.

For all conformers, the solvent accessible surface area, according to the water property suggested by the Macro Model program, was performed, considering the interaction with a 1.4 Å radius probe. The fluorenone moiety atoms were selected and the averaged value (ASASA) was computed for each compound. With respect to our previous work[1] on 9-fluoren derivatives, compounds **1**-**10** bring their side chain in 4 and are deprived of glycosidic units. As a consequence, the planar aromatic moiety differs by one atom avoiding the opportunity to make a direct comparison of the ASASA descriptor between the two series of compounds.

In Table 2 the MC results are summarized.

Table 2. The Summary of the Monte Carlo conformational search conditions and results

Compound	RB [a]	MC ite [b]	NCONF [c]	AND [d]	ASASA [e]	Min [f]	Max [g]
1	5	5000	89	54.0	430.1	408.5	438.8
2	6	5000	258	18.9	428.1	396.1	439.5
3	5	5000	196	6.3	429.1	398.5	438.7
4	7	5000	776	24.4	418.4	383.2	439.9
5	8	5000	1881	2.6	406.1	376.1	439.6
6	5	5000	201	24.0	411.5	391.7	439.3
7	7	5000	530	9.2	413.1	377.1	439.8
8	6	5000	1204	4.0	392.1	371.0	439.5
9	8	5000	746	6.4	393.7	371.0	439.9
10	9	5000	1588	3.0	399.1	364.5	439.9

[a] Number of rotatable bonds. [b] Monte Carlo iterations. [c] Number of conformers within 12.5 kcal/mol. [d] Averaged number of duplicates with RMS (root mean square) deviation computed on the atomic coordinates lower than 0.25 Å and with energy difference lower than 1 kcal/mol. [e] Average; [f] Minimum and [g] Maximum Solvent Accessible Surface Areas.

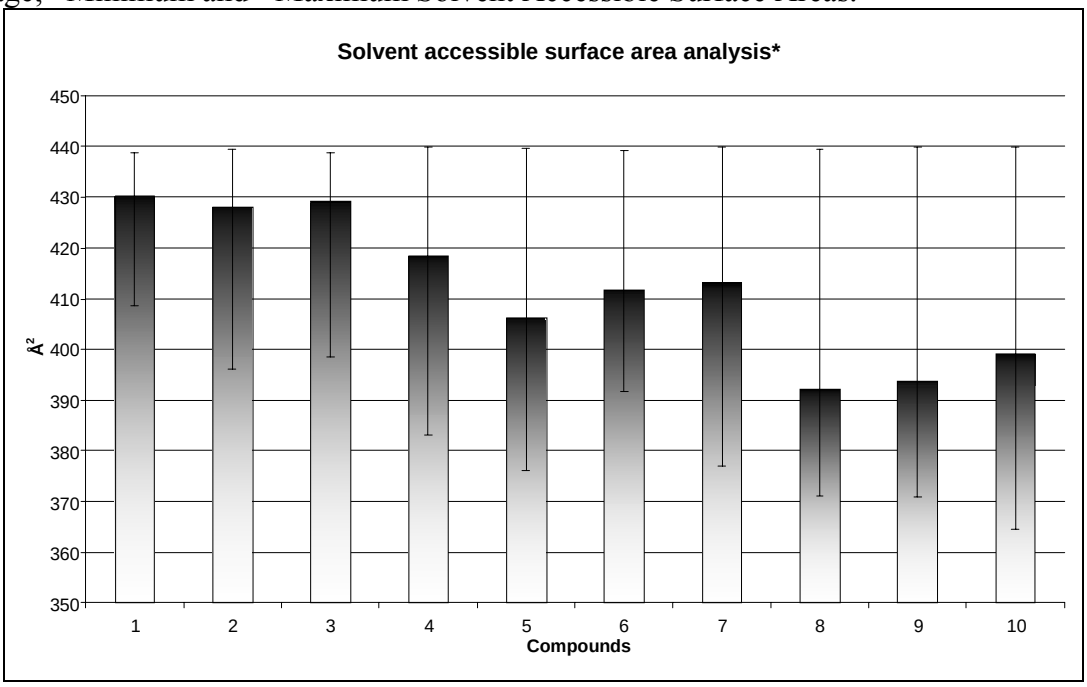


Figure 1. Fluorenone ASASA results for compounds **1-10**. *Bar plots refer to the averaged solvent accessible surface area in Å² for the fluorenone moiety. Error bars regard the maximum and minimum ASASA values in the conformational ensemble generated in the Monte Carlo search.

As indicated in Table 2 and Figure 1, we found three different kinds of fluorenone moiety solvent accessibility. Compounds **1-3** showed the highest ASASA values close to 430 Å², **4-7** values between 420 and 408 Å², **8-10** with ASASA values lower than 400 Å². In the extended conformation all compounds are able to expose the fluorenone moiety at the same level as shown by the maximum ASASA value close to 440 Å².

In Figure 2 the comparison of the energy global minimum conformers of all compounds is demonstrated.

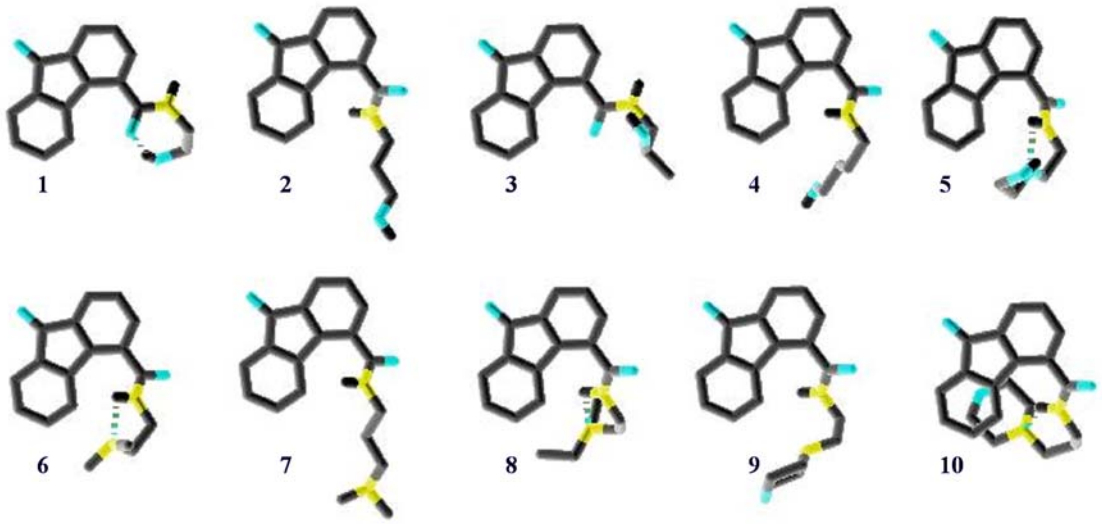


Figure 2. Energy global minimum conformers of compounds **1-10** (polytube models) obtained in the Monte Carlo conformational search. Hydrogen bonds are represented with the dashed lines.

The side chain of compounds **1-10** contains hydrogen bond (HB) acceptor and donor atoms. The conformation of the such side chains in 4 is influenced by the HB network that can be established. HB interactions of global minimum energy conformers give an idea of such an influence, but cannot be considered as an exhaustive analysis due to the large amount of conformers found in the MC search (Table 2). However some considerations can be made on observing the structures of Figure 2. Hydrogen bond interactions are carried for global minimum conformers of compounds **1**, **5**, **6**, **8** and **10** only, even if the other five compounds contain similar HB acceptors and donors. The reason for such a different behaviour can be attributed to the nature of the cyclic conformation bridged by the HB. In particular, where the number of atom terms is between 5 and 7 units, the side chain HB seems to be favoured as, for example, in compound **1**. In fact, compound **7** could form the HB in an unfavourable 8-term cycle preferring the extended structure that maximizes HB interaction with water. Similar considerations can be made for other **2-10** compounds.

Pharmacology

Even though the "*in vitro*" studies on human PBMC are difficult because of the genetic variability of different donors and the heterogeneity between and within mononuclear

phagocytes, they are necessary in order to understand the relationships between viruses/immune cells and antiviral compounds.

Figure 3 summarizes results on cytotoxicity test of compounds **1**-**10** on WISH cell lines. These data demonstrate that tested molecules displayed different degrees of cytotoxicity. In particular compounds **2**, **6**, **8**, **9** and **10** induced a higher percentage of cytotoxicity at concentrations of 100 and 50 μg/mL compared to compounds **1**, **3**, **4**, **5** and **7**.

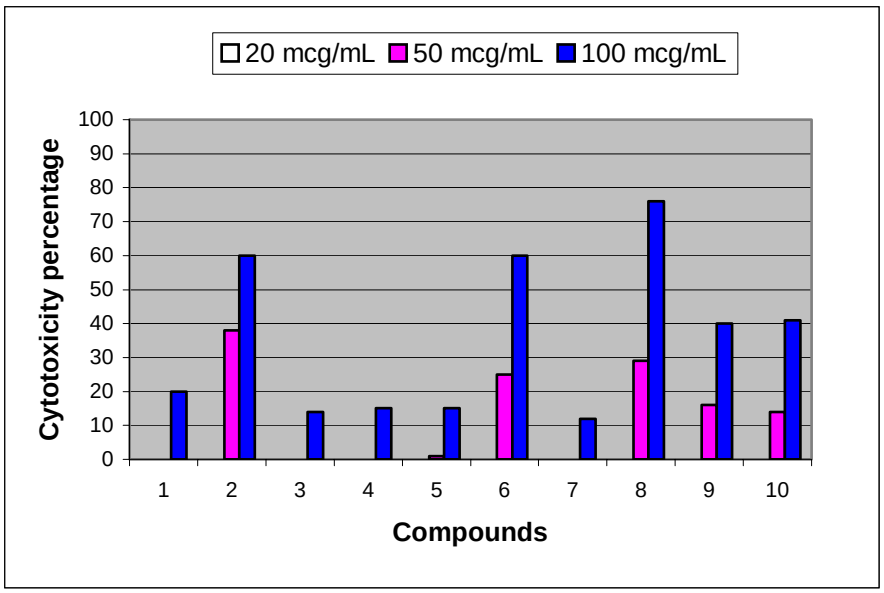


Figure 3. Cytotoxicity percentage on Wish cell lines. In all the experiments the Standard Deviation (S.D.) is less than 10%. (mcg = μg).

Furthermore, the cytotoxicity on PBMC was performed using trypan blue exclusion test; the results showed the same cytotoxicity obtained on WISH cell lines (data not shown).

On the basis of cytotoxicity results, in the further experiments we tested the highest non-cytotoxic concentration for each molecule; in particular, compounds **1**, **3**, **4**, **5** and **7** were tested at a concentration of 50 μg/mL whereas compounds **2**, **6**, **8**, **9** and **10** at a concentration of 20 μg/mL.

Figure 4 shows the effect of the compounds on HSV-2 replication. Our data demonstrate that only molecules **1**, **2**, **4**, **7**, **9** and **10** possess antiviral activity on PBMC ($p<0.05$).

In order to understand if this antiviral activity resulted by direct interaction with viral replication or by stimulation of PBMC to produce selected cytokines network, we analyzed the production kinetics of different cytokines involved in antiviral immune mechanisms such as IFN-γ, IFN-α, TNF-α.[15]

IFN-γ is an important regulator of immune responses. It is a product of activated T lymphocytes and NK cells and plays a critical role in the generation of Th1-type immune responses. IFN-γ stimulates phagocytosis, the oxidative burst, intracellular killing of

microorganisms, stimulates antigen presentation to T lymphocytes, induces other cell types to secrete proinflammatory cytokines such as TNF-α and chemokines.[27-29]

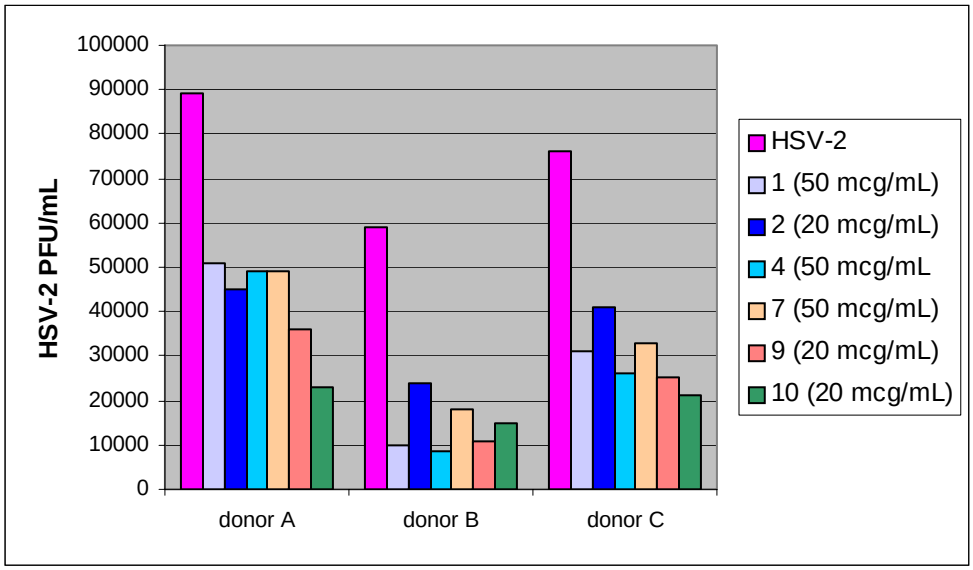


Figure 4. Anti-HSV-2 activity on PBMC. In all the experiments the S.D. is less than 10%.

The kinetics of IFN-γ release by PBMC from three different donors (A, B, C) under treatment with different compounds (**1**, **4**, **9** and **10**) are reported in Figure 5. No IFN-γ basal production was detected in all the experiments.

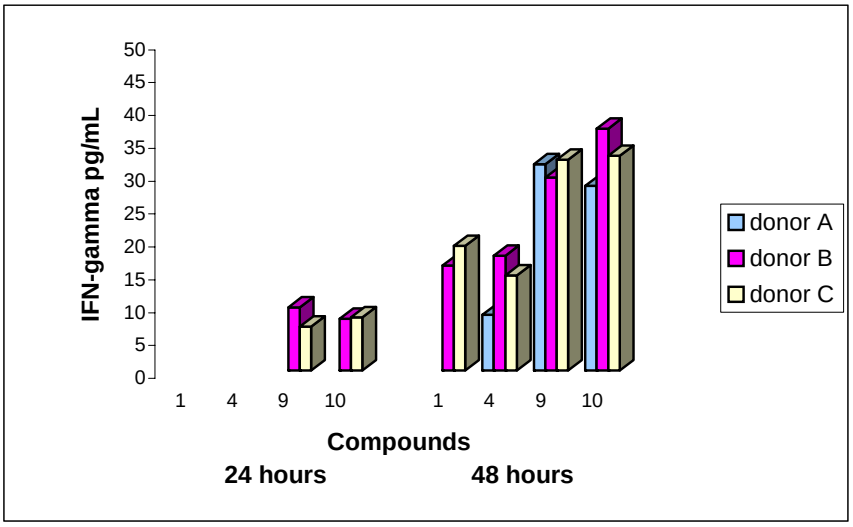


Figure 5. Kinetics of IFN-γ release by PBMC. For used doses see Figure 4. In all the experiments the S.D. is less than 10%.

The compounds **9** and **10** induced an higher IFN-γ production at 48 hrs post treatment compared to that obtained at 24 hrs p.t. ($p<0.05$). Whereas, the addition of compounds **1** and **4** to PBMC resulted in an increase of IFN-γ production at 48 hrs p.t. ($p<0.05$).

In Figure 6 the results concerning TNF-α release by PBMC after carboxamides treatment are reported.

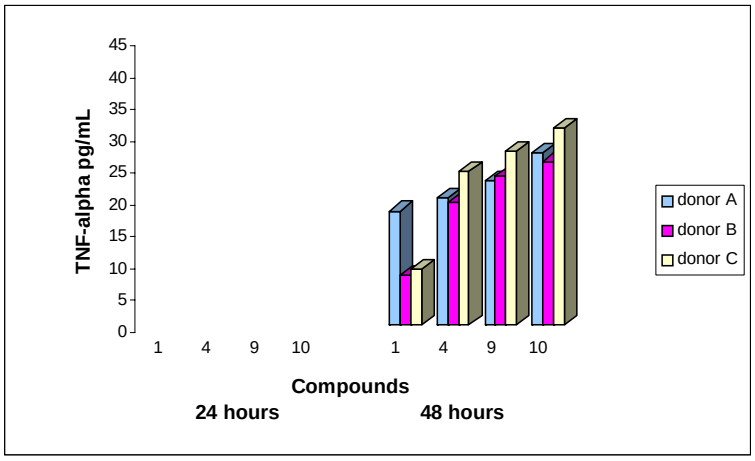


Figure 6. Kinetics of TNF-α release by PBMC. For used doses see Figure 4. In all the experiments the S.D. is less than 10%.

Our data demonstrate that molecules **1**, **4**, **9** and **10** trigger PBMC to produce TNF-α 48 hrs post treatment ($p<0.05$). No basal production was found in untreated PBMC.

These data supported the hypothesis that compounds **1**, **4**, **9** and **10** explain their antiviral activity by stimulating PBMC in releasing IFN-γ which in turn stimulates TNF-α production.

Figure 7 summarizes data on IFN-α production by PBMC. Consistent amounts of the cytokine were found after 24 and 48 hrs post treatment in supernatants of PBMC treated with compounds **9** and **10** ($p<0.05$). This up-regulation of IFN-α production was time-dependent, in fact at 48 hrs post treatment the amounts of IFN-α was higher than those observed at 24 hrs.

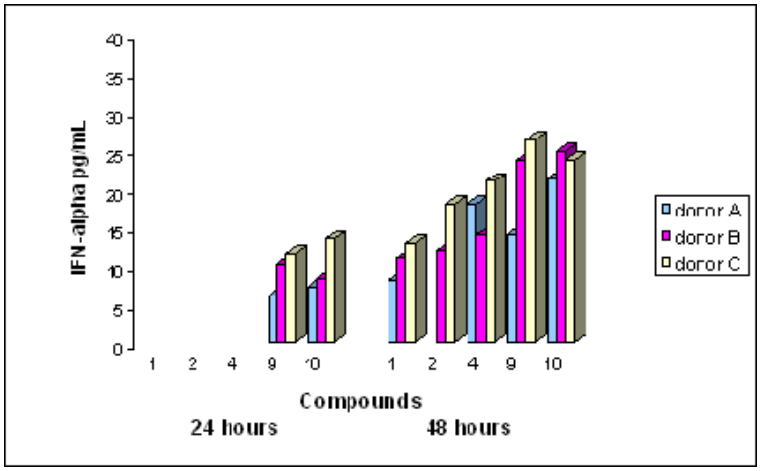


Figure 7. Kinetics of IFN-α release by PBMC. For used doses see Figure 4. In all the experiments the S.D. is less than 10%.

Compounds **1**, **2** and **4** trigger PBMC to release IFN-α only 48 hrs post treatment ($p<0.05$). No IFN-α basal production was found in untreated PBMC.

Compound **2** did not stimulate IFN-γ and TNF-α production whereas it induced IFN-α production and inhibited virus replication. This effect could be due to the presence of IFN-γ produced in the microenvironment by treated PBMC.

Compounds **3**, **5**, **6** and **8** did not show any significant effect either on HSV-2 replication or on cytokines production.

*Taken together our results allow to hypothesize that, by inducing IFN-γ, IFN-α and TNF-α secretion, compounds **1**, **4**, **9** and **10** hinder viral replication.*

Conclusions

A new series of tilorone like compounds, based on various substitutions in position 4 on the 9-fluorenone skeleton, has been proposed as potential anti-HSV-2 agents. Synthesis and chemical-physical characterization have been coupled with a conformational analysis focusing on the tricyclic surface exposed to the solvent and potentially able to make intercalation in the DNA duplex.

Moreover the pharmacological evaluation demonstrated that carboxamides **1**, **4**, **9** and **10**, inducing cytokines such as IFN-γ, IFN-α, and TNF-α, play beneficial roles against virus replication.

Although some SAR considerations could be envisaged we prefer to await the results of the forthcoming DNA intercalating and docking experiments that will give more insight into these derivatives and will suggest rational structural modifications to improve their pharmacological profiles.

Furthermore the observed combined effect both on antiviral activity and on cytokines stimulation could represent a strategy worth considering as a general therapeutic approach to viral infections.

Experimental Section

General Procedures. ^{1}H-NMR spectra were recorded on a Varian 300 spectrometer at 300 MHz, using the residual $CHCl_3$ peak at 7.26 ppm as reference line. Coupling constants (*J*) are given in Hz. Melting points were recorded on a Kofler hot-stage apparatus and are uncorrected. TLC controls were carried out on precoated silica gel plates (F 254 Merck). GC-MS spectra were recorded with Carlo Erba QMD 100 spectrometer. Elemental analyses were ± 0.4 % of theoretical values and were performed by CHNS-O Elementary Analizer 2004 serie II Perkin Elmer.

CH_2Cl_2 and triethylamine were dried following standard procedures.

Synthesis of *N*-(2-hydroxyethyl)-9-oxo-9H-fluorene-4 carboxamide (1)

A solution of 9-fluorenone-4-carbonyl chloride (300 mg, 1.2 mmol) in anhydrous CH_2Cl_2 (10 mL) was added dropwise to a stirred solution of 2-aminoethanol (217.4 mL, 3.6 mmol) and distilled Et_3N (0.26 mL, 1.8 mmol). The mixture was stirred at room temperature for 12 hrs (TLC monitoring), then the solution was washed with distilled water (4×15 mL) and the organic layer dried over anhydrous Na_2SO_4 and evaporated under vacuum. The residue was crystallized from MeOH. Pure **1** was obtained in 75% yield, then characterized by analytical and ^{1}H-NMR data.

Compounds **2**-**10** were similarly prepared and characterized.

***N*-(2-Hydroxyethyl)-9-oxo-9*H*-fluorene-4-carboxamide (1).** 75% yield; m.p. = 170-172 °C; M.W.= 267.29. Anal. Calcd for $C_{16}H_{13}NO_3$: C: 71.90, H: 4.90, N: 5.24. Found: C: 71.74, H: 4.72, N: 5.18. ^{1}H-NMR ($CDCl_3$) δ: 7.85–7.30 (m, 7H, ArH); 6.49 (br s, 1H, NH); 3.94 (t, 2H, CH_2OH, J= 4.5 Hz); 3.74 (m, 2H, NHCH_2); 1.63 (br s, 1H, OH). GC-MS: m/z (%); 267 ($M^{+\cdot}$, 12), 248 (49), 224 (43), 207 (100), 179 (66), 151 (64)

***N*-(3-Hydroxypropyl)-9-oxo-9*H*-fluorene-4-carboxamide (2).** 80% yield; m.p. = 148-150 °C; M.W.= 281.31. Anal. Calcd for $C_{17}H_{15}NO_3$: C: 72.58, H: 5.37, N: 4.98. Found: C: 72.72, H: 5.25, N: 4.90. ^{1}H-NMR ($CDCl_3$) δ: 7.85–7.30 (m, 7H, ArH); 6.53 (br s, 1H, NH); 3.84 (t, 2H, CH_2OH, J= 5.4 Hz); 3.73 (m, 2H, NHCH_2); 1.89 (m, 2H, NHCH$_2CH_2$); 1.59 (br s, 1H, OH)

***N*-(2-Hydroxypropyl)-9-oxo-9*H*-fluorene-4-carboxamide (3).** 73% yield; m.p. = 159-161 °C; M.W.= 281.31. Anal. Calcd for $C_{17}H_{15}NO_3$: C: 72.58, H: 5.37, N: 4.98. Found: C: 72.28, H: 5.24, N: 5.05. ^{1}H-NMR ($CDCl_3$) δ:7.83-7.29 (m, 7H, ArH); 6.59 (br s, 1H, NH, X part of an ABMX system); 4.13 (m, 1H, C*H*OH, M part of an ABMX system); 3.77 (m, 1H, NHCH_2, A part of an ABMX system); 3.39 (m, 1H, NHCH_2, B part of an ABMX system); 1.63 (br s, 1H, OH), 1.33 (d, 3H, CH_3, J= 6.3 Hz). GC-MS: m/z (%); 282 ($M^{+\cdot}$, 17), 237 (56), 224 (100), 207 (100), 179 (100), 151 (100), 76 (59)

***N*-(4-Hydroxybutyl)-9-oxo-9*H*-fluorene-4-carboxamide (4).** 75% yield; m.p. = 134-136 °C; M.W.= 295.34. Anal. Calcd for $C_{18}H_{17}NO_3$: C: 73.20, H: 5.80, N: 4.74. Found: C: 73.12, H: 5.78, N: 4.80. ^{1}H-NMR ($CDCl_3$) δ: 7.85–7.31 (m, 7H, ArH); 6.32 (br s, 1H, NH); 3.77 (t, 2H, CH_2OH, J= 6.0 Hz); 3.61 (m, 2H, NHCH_2); 1.78 (m, 5H, NHCH$_2(CH_2)_2$ and O*H*)

***N*-[2-(2-Hydroxyethoxy)ethyl]-9-oxo-9*H*-fluorene-4-carboxamide (5).** 89% yield; m.p. = 99-101 °C; M.W.= 311.34. Anal. Calcd for $C_{18}H_{17}NO_4$: C: 69.44, H: 5.50, N: 4.50. Found: C: 69.52, H: 5.46, N: 4.60. ^{1}H-NMR ($CDCl_3$) δ: 7.76–7.16 (m, 7H, ArH); 6.88 (br s, 1H, NH); 3.68 (m, 9H, $CH_2CH_2OCH_2CH_2$ and O*H*)

***N*-[2-(Dimethylamino)ethyl]-9-oxo-9*H*-fluorene-4-carboxamide (6).** 88% yield; m.p. = 103-105 °C; M.W.= 294.36. Anal. Calcd for $C_{18}H_{18}N_2O_2$: C: 73.45, H: 6.16, N: 9.52. Found: C: 73.40, H: 6.20, N: 9.60. ^{1}H-NMR ($CDCl_3$) δ: 7.89–7.30 (m, 8H, ArH and NH), 3.79 (m, 2H, NHCH_2); 2.91 (m, 2H, NHCH$_2CH_2$); 2.55 (s, 6H, N$(CH_3)_2$). GC-MS: m/z (%); 207 (4), 179 (6), 150 (14), 59 (100)

***N*-[3-(Dimethylamino)propyl]-9-oxo-9*H*-fluorene-4-carboxamide (7).** 91% yield; m.p. = 105-107 °C; M.W.= 308.38. Anal. Calcd for $C_{19}H_{20}N_2O_2$: C: 74.00, H: 6.54, N: 9.08. Found: C: 74.15, H: 6.45, N: 9.16. ^{1}H-NMR ($CDCl_3$) δ: 7.99 (s, 1H, NH); 7.95–7.21 (m, 7H, ArH); 3.67 (m, 2H, NH*CH*$_2$); 2.56 (t, 2H, *CH*$_2$N(CH_3)$_2$, J= 5.8 Hz); 2.29 (s, 6H, N*(CH*$_3$*)*$_2$); 1.86 (m, 2H, NCH$_2$*CH*$_2$)

***N*-[2-(Diethylamino)ethyl]-9-oxo-9*H*-fluorene-4-carboxamide (8).** 96% yield; m.p. = 220-222 °C; M.W.= 322.42. Anal. Calcd for $C_{20}H_{22}N_2O_2$: C: 74.51, H: 6.88, N: 8.69. Found: C: 74.60, H: 6.80, N: 8.65. ^{1}H-NMR ($CDCl_3$) δ: 7.87–7.27 (m, 7H, ArH), 6.89 (br s, 1H, NH), 3.65 (m, 2H, NH*CH*$_2$); 2.79 (t, 2H, NHCH$_2$*CH*$_2$, J= 5.4 Hz), 2.66 (q, 4H, N(*CH*$_2$CH$_3$)$_2$, J= 6.9 Hz), 1.07 (t, 6H, N(CH$_2$*CH*$_3$)$_2$, J= 6.9 Hz)

***N*-[2-(4-Morpholinyl)ethyl]-9-oxo-9*H*-fluorene-4-carboxamide (9).** 83% yield; m.p. = 198-200 °C; M.W.= 336.39. Anal. Calcd for $C_{20}H_{20}N_2O_3$: C: 71.41, H: 5.99, N: 8.33. Found: C: 71.14, H: 5.88, N: 8.28. ^{1}H-NMR ($CDCl_3$) δ: 7.83–7.26 (m, 7H, ArH); 6.69 (br s, 1H, NH); 3.68 (m, 6H, NH*CH*$_2$ and *O(CH*$_2$*)*$_2$ morphol.); 2.67 (t, 2H, NHCH$_2$*CH*$_2$, J= 6.3 Hz); 2.54 (m, 4H, N*(CH*$_2$*)*$_2$ morphol.). GC-MS: m/z (%); 207 (12), 179 (23), 151 (38), 100 (100)

***N*-[3-(4-Morpholinyl)propyl]-9-oxo-9*H*-fluorene-4-carboxamide (10).** 78% yield; m.p. = 132-134 °C; M.W.= 350.42. Anal. Calcd for $C_{21}H_{22}N_2O_3$: C: 71.98, H: 6.33, N: 7.99. Found: C: 71.70, H: 6.40, N: 7.85. ^{1}H-NMR ($CDCl_3$) δ: 8.12 (br s, 1H, NH); 7.79–7.23 (m, 7H, ArH); 3.59 (m, 2H, NH*CH*$_2$), 3.38 (m, 4H, *O(CH*$_2$*)*$_2$ morphol.), 2.50 (t, 2H, NHCH$_2$CH$_2$*CH*$_2$, J= 6.0 Hz); 2.34 (m, 4H, N*(CH*$_2$*)*$_2$ morphol.); 1.78 (m, 2H, NHCH$_2$*CH*$_2$)

Molecular modelling

Compounds **1-10** were graphically built by the Maestro interface of the Macro Model package v. 7.2.[26] Following our previous communication regarding similar compounds,[1] the molecular mechanics force field adopted in the conformational search study was AMBER* with united atom notation. Water solvation conditions were simulated by the GB/SA implicit method of solvation[25] as implemented in Macro Model package. The exploration of the internal torsional degrees of freedom of each molecule was performed generating 5000 conformations by a Monte Carlo (MC) search, followed by a energy minimization with a convergence criterion set to 0.01 kcal/mol·Å. After checking the average number of duplicates found during each simulation, the examination of the results was performed computing the averaged solvent accessible surface area (ASASA) computed on the fluorenone moiety as described in our previous work[1] and analysing the most stable conformers.

Pharmacology

Isolation of human peripheral blood mononuclear cells (PBMC)

PBMC were isolated from freshly collected buffy coats of healthy blood donors (Centro Trasfusionale, Policlinico Universitario "G. Martino", Messina, Italy), after centrifugation over Ficoll-Hypaque gradient (Pharmacia, Milan, Italy). PBMC were then washed three times in RPMI 1640 medium and cultured in 24 well plates (Corning, Bibby srl, Milan, Italy) at a

concentration of $2x10^6$ cells/mL per well in RPMI 1640 medium. PBMC were cultured at 37 °C in 5% CO_2 atmosphere, in RPMI 1640 (Sigma, Milan, Italy) supplemented with 50 μg/mL gentamicin and 5% fetal calf serum (FCS) (Biochrom KG Seromed, Milan, Italy).

PBMC were then treated with the new compounds at different concentrations. Lipopolysaccharide (LPS) from *E.coli* strain 055:B5 (Sigma, Milan, Italy) was used as positive control (data not shown). The molecule concentrations used did not show any cytotoxicity. 24 hours post treatment, the supernatants were harvested, aliquoted and stored at –80 °C until cytokine analysis. The remaining cells were infected with HSV-2 at a multiplicity of infection (MOI) 0.1for a further incubation of 24 hours at 37 °C in 5% CO_2, then the plates were frozen and thawed three times in order to release the virus. Cell lysates and supernatants were kept at –80 °C until virus titration.

Virus. HSV-type 2 Nahamias strain was used throughout the study. HSV-2 infection was propagated on WISH cell lines. Viral stocks were prepared by pelletting infected cells exhibiting cytopathic effect, and freezing aliquots at -80 °C. The virus titer was assessed on Wish cells and expressed as plaque forming unit (PFU) per mL. The input multiplicity of infection (MOI), used in all the experiments, was 0.1 PFU/cell.

Cytotoxicity test. All the compounds were diluted in dimethyl sulphoxide (DMSO) Hybri-Max for cell culture (Sigma, Milan, Italy) at a concentration of 1 mg/50 μL.

To determine the effect of different concentrations of molecules on cells viability a colorimetric assay was used as described by Mosmann.[30] The assay is based on the tetrazolium salt 3-(4,5 dimethylthiazol-2-yl)2,5diphenyltetrazoliumbromide (MTT), (Sigma, Milan, Italy) a pale yellow substrate that is cleaved by active mitochondria to produce a dark blue formazan product. Briefly, WISH cell lines were seeded in 96-microwell plates at $2x10^4$ per well, then treated with molecules at different concentrations (100, 50 and 20 μg/mL) for 24 hours. MTT diluted in saline solution was added to the cells and incubated for 4 hours, then acid propan-2-ol (0.04M HCl in propan-2-ol) (Sigma, Milan, Italy) was used to solubilize the formed crystals. The plates were read with a microelisa reader using a wavelength of 570 nm. Cytotoxicity percentage was calculated as follows:

1– [(experiment OD - lysis control OD) / (cell control OD - lysis control OD)] x 100.

Trypan blue exclusion test was performed on PBMC to confirm the cytotoxicity data obtained on WISH cell lines.

Limulus test. Culture media and reagents tested for the presence of endotoxin by E-Toxate kit (Sigma, Milan, Italy) were found to contain ≤10 pg of endotoxin per mL.

IFN-γ, IFN-α and TNF-α evaluations

Supernatants from PBMC, in different experimental conditions, were collected and analysed for the presence of IFN-γ IFN-α and TNF-α by an immunoenzymatic method (ELISA): human IFN-γ immunoassay; human IFN-α immunoassay and human TNFα immunoassay, all from Bender Medsystems (Milan, Italy); the limit of detection of the assay was 1.5 pg/mL for IFN-γ 4.8 pg/mL for IFN-α and 5.8 pg/mL for TNF- α.

Statistical evaluation

Results are expressed as the means of three experiments ± standard deviation (S.D.). Data were analysed by one way analysis of variance (ANOVA) and the Student-Newman-Keults test.

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