N,O-Nucleoside Analogues: Metabolic and Apoptotic Activity

Andrea Marraffa,[a] Piero Presenti,[a] Beatrice Macchi,[b] Francesca Marino-Merlo,[c] Mariella Mella,[a] and Paolo Quadrelli*[a]

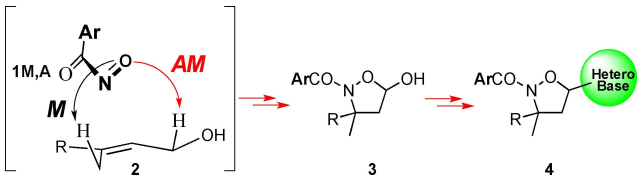
Two new families of *N,O*-nucleoside analogues containing the anthracene moiety introduced through the nitrosocarbonyl ene reaction with allylic alcohols were prepared. The core structure is an isoxazolidine heterocycle that introduces either atom either a phenyl ring or dimethyl moiety at the C3 carbon. Different heterobases were inserted at the position 5 of the heterocyclic ring. One of the synthesized compounds demonstrated a good capacity to induce cell death and an appreciable nuclear fragmentation was evidenced in treated cells.

1. Introduction

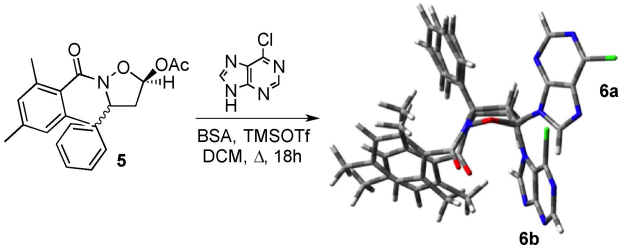
Isoxazolidines are privileged heterocyclic structures that find numerous applications in organic synthesis and in medicinal chemistry specifically.[1] The ene reactions of aromatic nitrosocarbonyl[2] intermediates **1** with allylic alcohols offered an alternative pathway for the synthesis of isoxazolidines, which were found valuable synthons to *N,O*-nucleoside analogues.[3] This methodology relies upon the mild oxidation of aromatic nitrile oxides with tertiary amine *N*-oxides to generate the nitrosocarbonyl intermediates **1**.[4] When these intermediates are *in situ* generated in the presence of the reactive allylic alcohols **2**, they undergo ene reaction.[5] The reactions proceed to the ene adducts in accordance with the prevailing $HOMO_{(alcohol)}$-$LUMO_{(nitrosocarbonyl)}$ interaction, somewhat enforced by the polarization of the C=C double-bond induced by the slightly electron withdrawing group CH_2OH.[6] When sterically demanding nitrosocarbonyl mesitylene **1 M** (**M** = Mesityl), the Markovnikov (*M*) directing effect is relieved and the anti-Markovnikov (*AM*) pathway becomes competitive and the preferred one. The selectivity drift is further increased when the bulkier anthracene nitrosocarbobonyl intermediate **1 A** (**A** = Anthryl) is used.[3,7]

The *AM* route preludes to the enol formation and subsequent cyclization to the isoxazolidines **3**, which are the synthons for the preparation of libraries of *N,O*-nucleosides **4** containing uracils and purines heterobases inserted by adapting to the scope the Vorbrüggen protocol (Scheme 1).[8]

In a previous work, we investigated the metabolic and induced cell death by apoptosis of a family of 6-chloropurine *N,O*-nucleoside analogues derived from the ene reaction of the cinnamyl alcohol and the mesityl nitrosocarbonyl intermediate **1 M**.[9] Among the various 6-chloropurine regio- and stereoisomers obtained through the Vorbrüggen derivatization of the 5-acetoxy-isoxazolidine **5**, it was demonstrated that compound **6 a**, showing the 6-chloropurine ring *cis*-related to the phenyl ring of the isoxazolidine moiety, was more cytotoxic than that the corresponding **6 b** in the pro-apoptotic, metabolic and cytotoxic activities, tested on the human monocytoid U937 and the lymphoblastoid MOLT-3 cell lines (Scheme 2). Collectively, the induction of apoptosis and/or the inhibition of metabolic



Scheme 1. Ene reaction of nitrosocarbonyl intermediates **1** with allylic alcohols **2**: synthetic pathway toward isoxazolidine *N,O*-nucleoside analogues.



Scheme 2. Synthesis through Vorbrüggen protocol of the compounds **6 a**, **b** and superimposed DFT calculated structures.

[a] *Dr. A. Marraffa, Dr. P. Presenti, Prof. M. Mella, Prof. P. Quadrelli*
Department of Chemistry
University of Pavia
Viale Taramelli 12, 27100 – Pavia (Italy)
E-mail: paolo.quaddrelli@unipv.it
[b] *Prof. B. Macchi*
Department of Chemical Science and Technologies
University of Rome Tor Vergata
Via della Ricerca Scientifica 1, 00133 Rome, Italy
[c] *Dr. F. Marino-Merlo*
Department of Chemical, Biological, Pharmaceutical and Environmental Sciences
University of Messina
Viale Ferdinando Stagno d'Alcontres 31, 98166 – Messina (Italy)

Supporting information for this article is available on the WWW under https://doi.org/10.1002/open.202000034

and cytotoxic activity of the tested compounds at 10 μM were similar to that exhibited by the positive control Etoposide.

In terms of structure-activity relationship (SAR) it was hypothesized that the stoichiometric structure including the substitution of chlorine atom versus nitrogen associated to phenyl group in *cis* and or in *trans* might influence the apoptotic and metabolic activity of the compounds toward U937, MOLT-3 cells and normal tissues.

On pursuing our research on this field, we have synthesized two new families of *N,O*-nucleoside analogues (Figure 1) which contain the anthracene moiety in place of the mesityl; in the group of compounds (A) the phenyl ring is maintained at the C3 isoxazolidine moiety while in the group (B) two methyls are inserted.

At the heterobases level, both the groups contain the 6-chloropurine ring for comparison with the data already known[9] and pyrimidine- and purine-types bases are also included. The biological assays will furnish further details on the SAR for these types of isoxazoline analogues.

2. Results and Discussion

2.1. Cinnamyl Alcohol Derivatives

Addition of a dichloromethane (DCM) solution of anthracenenitrile oxide **7** to a stirred solution of *N*-methyl-morpholine *N*-oxide (NMO, 1.2 equiv.) in DCM in the presence of an excess (5 equiv.) of *trans*-cinnamyl alcohol afforded, after 48 h at room temperature, the ene adduct **8** that was isolated upon chromatographic purification from the reaction mixture in 44% yield (Scheme 3), as inseparable mixture of diastereoisomers.

Besides the excess of cinnamyl alcohol, from the reaction mixture consistent portions of non-identified anthracene derivatives were collected, as a result of decomposition of the nitrosocarbonyl intermediate **1 A**.[10]

The structure of the 5-hydroxy-isoxazolidine **8** relies upon the corresponding analytical and spectroscopic data. In the IR spectrum the hydroxyl group gives a band at 3195 cm^{-1} while the carbonyl group C=O was found at 1637 cm^{-1}. The NMR spectra reveal that the product is a mixture of diastereoisomers and the relative signals do not give coalescence upon increasing the experiment temperature. In particular, in the ^1H NMR spectrum (DMSO) the hemiacetal proton is clearly found at δ 5.40 while the benzylic proton is observed at δ 5.83 for the major component of the 3:1 mixture of diastereoisomers. The definitive confirmation of the structure of **8** came from the X-ray analysis and Figure 2 reports the ORTEP view of the compound.

The ene adduct **8** was then acetylated according to the established procedure[11] and compound **9** was obtained in 98% yield as a mixture of diastereoisomers in a nearly 4:1 ratio. The structure of **9** was confirmed by the relative spectroscopic data; in the ^1H NMR spectrum (DMSO), the acetate group is clearly show by the singlet at δ 1.55 corresponding to the methyl as well as by the presence in the IR spectrum of the C=O band at 1756 cm^{-1} and the absence of the OH band. The other signals are in the expected range for the given isoxazolidine structure.

We then performed the functionalization of the isoxazolidine **9** with the commercially available uracil **U** and thymine **T** by adapting the standard protocol for the insertion of heterobases on isoxazolidine rings.[3,9] The acetylated isoxazolidine **9** was added under nitrogen atmosphere at r.t. to a solution of uracil **U** or thymine **T** (2.2 equiv.) and BSA (4 equiv.) and the solutions became clear after boiling in DCM for a couple of hours. The mixtures were then ice-cooled at 0 °C and TMS-OTf (4 equiv.) was added and the reactions refluxed overnight (Scheme 4).

The desired compounds **10 a**, **b** (**U**, **T**) were obtained in good yields by chromatographic separation of the diasteroisomeric mixtures and fully characterized through their analytical and spectroscopic data. The relevant spectroscopic and physical-chemical data are gathered in Table 1 and details can be found in the experimental section. Compounds **10 a**, **b** (**U**, **T**) are diastereoisomers of racemic mixtures and structurally can be

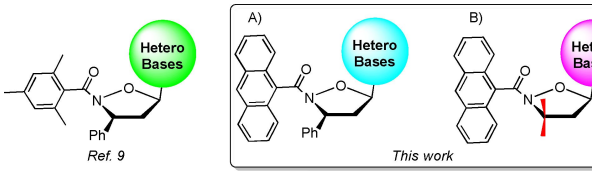
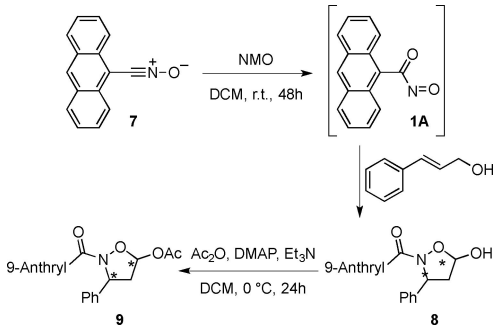


Figure 1. Structures of *N,O*-nucleoside analogues.



Scheme 3. Ene reaction of nitrosocarbonyl anthracene **1 A** with cinnamyl alcohol and acetylation reaction of the 5-hydroxy-isoxazolidine **8**.

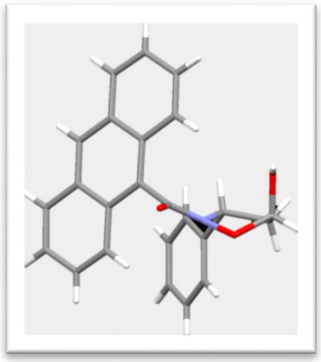


Figure 2. X-Ray structure of compound **8**.

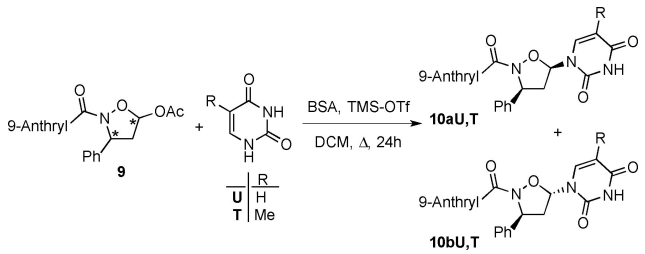
Table 1. Yields, physical-chemical and spectroscopic data of compounds **10a**, **b** (**U**, **T**).

Compound	m.p. [°C][a]	Yield [%]	IR $\nu_{C=O}$ [cm^{-1}]	^{1}H NMR [δ, DMSO] CH=/CH3	O–CH–N	Ph–CH	CH=	NH
10aU	166 (dec)	49	1698, 1708	5.46 (d)	6.18 (t)	6.34 (t)	7.87 (d)	11.25 (s)
10aT	>250 (dec)	40	1698	1.53 (s)	6.21 (t)	6.34 (t)	7.66 (s)	11.23 (s)
10bU	>250 (dec)	52	1693, 1716	5.75 (d)	6.17 (t)	6.22 (d)	7.93 (d)	11.09 (s)
10bT	171–175	49	1698, 1700	1.95 (s)	6.23 (d)	7.31 (t)	7.72 (s)	11.04 (s)

[a] White crystals from ethanol.

divided into two series: *cis*, **10a** (**U**, **T**) show the heterobase on the same side of the phenyl ring; *trans*, **10b** (**U**, **T**) show the heterocyclic and phenyl rings on opposite sides with respect to the isoxazolidine moieties (Figure 3).

The stereochemical features were established through NOESY experiments and Figure 3 reports the correlations between the protons located on the carbons C3, C4 and C5 of the isoxazolidine moieties that are responsible for the stereochemical outcome. In all the cases the relative position of the benzylic protons and methylene protons allowed for the correct determination of the stereochemistry of the products as a consequence of the possible correlation with the acetalic protons.



Scheme 4. Synthesis of uracil **U** and thymine **T** isoxazolidine nucleoside analogues through Vorbrüggen protocol.

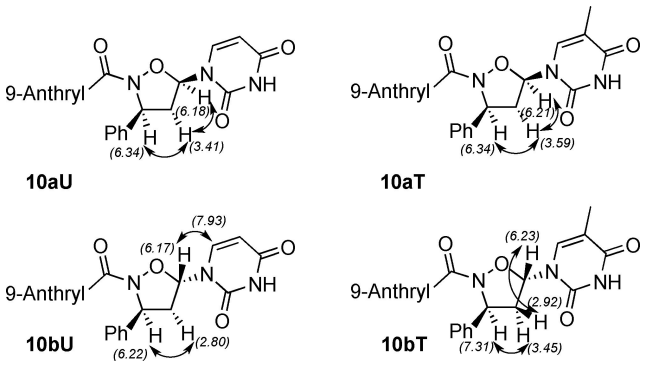
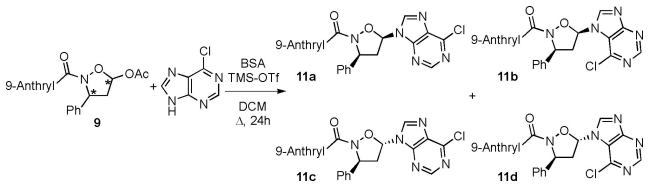


Figure 3. Structure of compounds **10a**, **b** (**U**, **T**) and NOE correlations. Values in parentheses are chemical shifts (δ, ppm) of the protons giving NOE correlations (indicated by the arrows).

An analogous synthetic procedure was applied for the preparation of the 6-chloropurine adducts. The acetylated isoxazolidine **9** was added under nitrogen atmosphere at r.t. to a solution of 6-chloropurine (2.2 equiv.) and BSA (4 equiv.) and the solutions became clear after boiling in DCM for a couple of hours. The mixtures were then ice-cooled at 0 °C and TMS-OTf (4 equiv.) was added and the reactions refluxed overnight (Scheme 5).

The desired compounds **11a–d** were obtained as white solids separated by column chromatography. The purine nucleoside analogues **11a–d** were isolated in fair yields (range 22–36%). On the basis of previous observations,[3,9,12] the reaction gave a mixture of four isomeric products where the purine ring can be linked at the isoxazolidine moiety through the N7 and/or N9 nitrogen atoms.

The structures of compounds **11a–d** were attributed on the basis of the corresponding analytical and spectroscopic data and Table 2 collects the relevant physical-chemical and spectroscopic data.



Scheme 5. Synthesis of 6-chloropurine isoxazolidine nucleoside analogues **11a–d** through Vorbrüggen protocol.

Table 2. Yields, physical-chemical and spectroscopic data of 6-chloropurine compounds **11a–d**.

11	m.p. [°C][a]	Yield [%]	IR [cm^{-1}] $\nu_{C=N}/\nu_{C=O}$	^{1}H NMR [δ, DMSO] O–CH–N	H–CH=N	H–CH=N
a	200–201	22	1592/1654	6.59 (t)	8.51	8.69
b	201–205	36	1595/1633	6.56 (d)	8.65	8.89
c	145 (dec.)	24	1652/1714	6.55 (d)	8.32	9.06
d	230 (dec.)	24	0000/1654	6.67 (t)	8.50	9.48

[a] White crystals from iPr_2O/EtOH.

These data confirm that the 6-chloropurine ring replaced the acetate group of the starting material **9** but say little on the stereochemical arrangements of the products.

Compounds **11 a–d** are diastereoisomers of racemic mixtures and structurally can be divided into two series: *cis*, **11 a**, **b** show the 6-chloropurine on the same side of the phenyl ring; *trans*, **11 c**, **d** show the heterocyclic and phenyl rings on opposite sides with respect to the isoxazoline moieties (Figure 4). Compounds **11 a** and **11 b** differ for the orientation of the purine ring, being the first linked through the N9 nitrogen atom to the isoxazolidine ring and the second through the N7 nitrogen atom. The same connections to the isoxazolidine ring are found in compounds **11 c** and **11 d**.

These two different stereo- and regiochemical aspects were solved by performing different NMR experiments. NOESY experiments allowed for the correct assignment of the stereochemical outcome of the reaction and Figure 4 reports the correlations between the protons located on the carbons C3, C4 and C5 of the isoxazoline moieties. The relative position of the benzylic protons and methylene protons allowed for the correct determination of the stereochemistry of the products as a consequence of the possible correlation with the acetalic protons.

To determine the correct orientation of the purine ring, HMBC and HSQC NMR experiments were conducted and positive correlations were found just in some cases. Within the *trans* series of products, in compound **11 c** the acetalic proton at δ 6.55 correlates with the carbon atom at δ 151.3 (annotated with a red dot in Figure 4). This carbon atom also correlates with both the H–CH=N protons of the purine ring at δ 8.32 and 9.06 ppm. In compound **11 d** the acetalic proton at δ 6.67 correlates with the carbon atom at δ 121.3 that also correlates with just one of the H–CH=N protons of the purine ring. In this way the N7 and N9 orientations are determined. Within the *cis* series, the structure attribution of compound **11 b** was consequently attributed as shown in Figure 4 along with that of **11 a**.

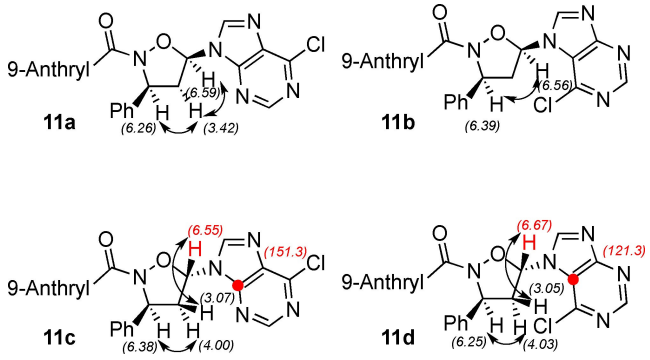


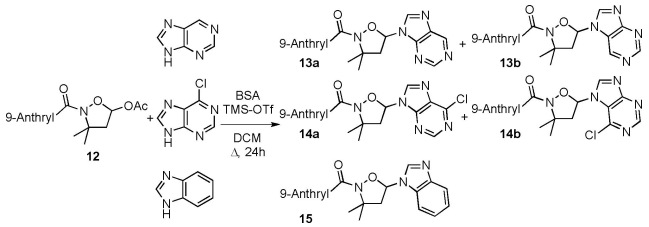
Figure 4. Structure of compounds **11 a–d** and NOE correlations. Values in parentheses are chemical shifts (δ, ppm) of the protons giving NOE correlations (indicated by the arrows). In red the HMBC and HSQC correlations between H and C atoms are annotated with relative chemical shifts.

2.1.1. 3-Methyl-2-buten-1-ol derivatives

The isoxazolidine **12**, prepared according to reported procedure,[3,12] was derivatized with the commercially available purine, 6-chloropurine and benzoimidazole rings by adapting the standard protocol for the insertion of heterobases on isoxazolidine rings.[3,9,12] The acetylated isoxazolidine **12** was added under nitrogen atmosphere at r.t. to a solution of 2.2 equiv. of the selected heterobases and BSA (4 equiv.) and the solutions became clear after boiling in DCM for a couple of hours. The mixtures were then ice-cooled at 0 °C and TMSO–Tf (4 equiv.) was added and the reactions refluxed overnight (Scheme 6).

In the reaction with the simple purine, two products were obtained as a racemic mixture, **13 a**, **b** (yields: 51 % and 45 %, respectively); they differ by the N7 and N9 orientation of the purine ring. Their structures were determined on the basis of the corresponding analytical and spectroscopic data. In particular the ^{1}H NMR spectrum (DMSO) of compound **13 a** shows the acetalic proton at δ 6.86 is a double doublet, coupled with the adjacent methylene of the isoxazolidine ring and the singlets corresponding to the purine ring are also found at δ 8.62 and 8.75. The N7/N9 orientation in compound **13 a** was determined by X-ray analysis and Figure 5 reports the ORTEP view of the compound.

In the ^{1}H NMR spectrum (DMSO) of compound **13 b** the acetalic proton is found at δ 6.80 (dd), coupled with the adjacent methylene of the isoxazolidine ring and the singlets corresponding to the purine ring are also found at δ 8.59 and 8.69, with the reversal orientation of the purine ring with respect to **13 a**.



Scheme 6. Synthesis of isoxazolidine nucleoside analogues **13 a**, **b**, **14 a**, **b** and **15** through Vorbrüggen protocol.

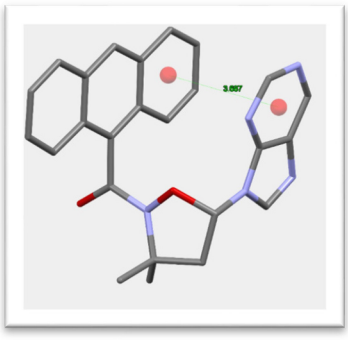


Figure 5. X-ray structure of compound **13 a**. π-Stacking between aromatic ring is indicated by red dots (centroids) with distance (3.69 Å).

Similarly, the reaction with the 6-chloropurine gives two racemic products with different regiochemical orientation of the purine ring **14a**, **b** in 56% and 40% yields, respectively. The structures were attributed on the basis of the relative analytical and spectroscopic data as well as specific NMR experiments for determining the regiochemical orientation of the products.

NOESY and HMBC experiments (DMSO) allowed for the definition of the regiochemical outcome. As shown in Figure 6, the acetalic proton of compound **14a** is clearly defined for its stereochemistry with respect to the methylene and methyl protons. Furthermore, the acetalic proton at δ 6.42 correlates in the HMBC experiment with both the carbon atoms at δ 145.4 and 151.3, relative to the H–CH=N protons (δ 8.53 and 9.75). On the other side, the acetalic proton of compound **14b** found at δ 6.48 does not correlate efficiently with none of the same carbon atoms. The structures are consequently defined.

Finally, the benzoimidazole derivative **15** was obtained as single product in 40% yield and fully characterized. In the ^{1}H NMR spectrum (DMSO) the acetalic proton was found at δ 6.48 and the H–CH=N–R proton at δ 8.43.

2.1.2. Biological assays

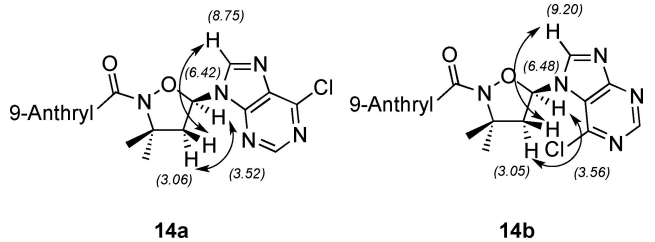
Samples of compounds **10a**, **b** (**U**, **T**), **11a–d**, **13a**, **b**, **14a**, **b** and **15** were subjected to biological assays to assess their metabolic and pro-apoptotic activities. For the scope the human monocytoid cell line U937, was grown in RPMI (Life Technologies, Paisley, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Life Technologies), 2 mM glutamine (Hyclone, Cramlington, UK), 50 U/ml penicillin and 50 U/ml streptomycin (Hyclone). The metabolic inhibition was evaluated by MTS assay. Inhibition of cell metabolic activity was detected through formazan product formation, using a commercial colorimetric kit (MTS [3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt]).[13] Apoptosis was detected by microscopy analysis of cellular (apoptotic bodies) or nuclear (chromatin condensation, nuclear fragmentation) apoptotic morphology following Hoechst 33342 staining. For compound **11a**, also cell viability was assessed through Trypan Blue exclusion assay.

Concerning the biological activity of compounds **10a**, **b** (**U**, **T**), **11a–d**, **13a**, **b**, **14a**, **b** and **15**, a dose effect assay using three or four different concentrations of the compounds, within the range from 100 to 1.56 μM, assessed the metabolic inhibitory activity, expressed as IC_{50}, showing that the diastereoisomers **10a**, **b** (**U**, **T**), were endowed with a high IC_{50} except for **10b T**. Conversely, the compounds **11a–d** were more effective in inhibiting the metabolic activity, showing lower IC_{50} values (Table 3).

Interestingly, compounds **11a**, **b**, **c** were also able to induce appreciable levels apoptosis, showing lower CC_{25} values in comparison with **10a**, **b** (**U**, **T**), being **11a** the most potent apoptosis inducer. Compounds **13**, **14** and **15** showed lack of metabolic inhibitory activity or ability to induce apoptosis except for **15**, which exhibited IC_{50} and CC_{25} values similar to that of **11a–d** compounds. Thus, the comparative analysis of the biological activities of the different diastereoisomer series, compound **11a** was found the most effective one exerting, in particular, a remarkable pro-apoptotic activity. We then focused our attention on the capacity of compound **11a** to induce cell death by comparing in details its ability to induce apoptosis, as assessed by Hoechst staining, with its ability to induce also other forms of cell death, as assessed by Trypan blue exclusion assay, at different concentrations after 18 hours (Table 4).

The results, expressed as percentage of Trypan blue positive and apoptotic cells ± SD, show that increasing concentrations of **11a** led to increased percentages of both Trypan blue and apoptotic cells, being the former always lower than the later. This implies that apoptosis was the exclusive form of cell death induced by **11a** treatment.

As an example, Figure 7 shows the nuclear morphology of apoptotic cells after treatment with **11a**. Vehicle treated U937 cells showed few sporadic apoptotic nuclei (Figure 7a). Conversely, following treatment with 6.25 and 25 μM **11a** (Figure 7b and 7c, respectively), an evident increase of fluorescence, due to increased stain absorption by condensed chromatin, and



14a **14b**

Figure 6. Structures of compounds **14a**, **b** and NOE correlations. Values in parentheses are chemical shifts (δ, ppm) of the protons giving NOE correlations (indicated by the arrows).

Table 3. Effects of compounds on metabolic activity, as assessed by the MTS assay and apoptotic cell death, as assessed by microscopy analysis, in U937 cells.[a]

Compound	IC_{50} [μM] ± SD MTS[b]	CC_{25} [μM] ± SD Apoptosis
10aU	> 200	> 1000
10bU	74.4 ± 15.9	281.1 ± 42.1
10aT	> 200	329.5 ± 68.2
10bT	15.1 ± 20.1	663.6 ± 115.2
11a	20.1 ± 1.1	8.7 ± 1.2
11b	42.7 ± 1.7	17.6 ± 1.4
11c	20.1 ± 0.2	12.3 ± 0.5
11d	15.1 ± 2,5	81.4 ± 26.8
13a	115.1 ± 1.1	93.7 ± 39.1
13b	> 200	> 1000
14a	> 200	n.d.[c]
14b	> 200	428.1 ± 259.4
15	45.1 ± 1.2	28.7 ± 2.4

[a] Data are expressed as the concentrations of the compounds capable to inhibit by 50% the metabolic activity at the MTS assay and to induce 25% cytotoxicity by apoptosis in U937 cells, respectively, as calculated using four parameter logistic 4pl curve regression analysis of data obtained at three or four different concentrations in three or four different experiments. [b] MTS = 3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo phenyl)-2H-tetrazolium salt; SD = standard deviation. [c] n.d. = not done

Table 4. Effects of compound **11a** on cell death, as assessed by the Trypan blue dye exclusion test and by microscopy detection of apoptosis in U937 cells.

Compound concentration [μM]	% Trypan Blue positive cells ± SD	% Apoptotic cells ± SD[a]
CTR[b]	3.69 ± 1.70	4.47 ± 1.55
1.56	10.22 ± 1.45	11.43 ± 0.81
3.12	n.d.[c]	13.27 ± 1.62
6.25	14.32 ± 1.24	17.53 ± 3.29
25	29.28 ± 6.09	73.97 ± 6.90
50	36.11 ± 8.24	n.d.[c]

[a] Apoptotic cells as detected by microscopy analysis of cellular (apoptotic bodies) or nuclear (chromatin condensation, nuclear fragmentation) apoptotic morphology following Hoechst 33342 staining. [b] Values obtained in cells treated with vehicle alone (DMSO) at the highest concentration utilized to dilute the compound. [c] n.d. = not done.

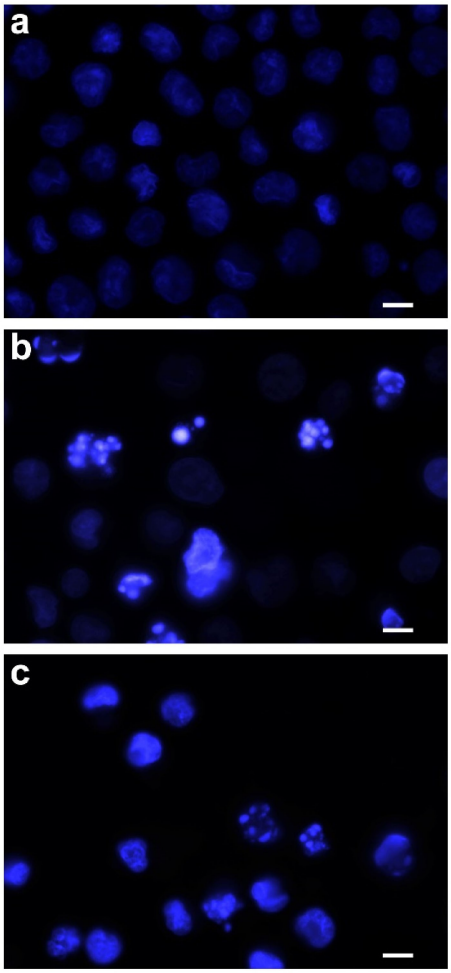


Figure 7. Fluorescence microscopy images of representative slides showing the effects of **11a** treatment on U937 cells. Control cells (a) and cells treated with 6.25 μM (b) and 25 μM (c) 11a were analysed at 18 h incubation after staining with 10 μM of the fluorescent DNA-binding dye Hoechst 33342. The majority of control, vehicle treated U937 cells exhibit normal nuclear morphology, with only a few sporadic cells showing signs of apoptosis. In contrast, the nuclear morphology of **11a** treated cells (b and c) demonstrated intense fluorescence resulting from increased stain absorption by condensed chromatin and visible nuclear fragmentation, characteristic of early and late apoptosis, respectively. Original magnification, 400X. Scale bar, 10 μM. Samples were analysed and captured by Observer Z1 fluorescence microscope (Zeiss, Jena, Germany).

well appreciable nuclear fragmentation are shown. Taken together, these data suggest that the diasteroisomer series **11a–d** stands out, in comparison with **10a–d** and **13–15**, in inducing metabolic inhibition and apoptosis. Previous data have shown that 6-chloropurine N,O-nucleoside analogues were endowed with biological activity. This study demonstrates that apparently their activity was not affected by replacing the mesityl with anthracenyl groups.[9]

Interestingly, here we demonstrate that compound **11a** induced considerable levels of cell death in U937 cells exclusively ascribed to apoptosis. In addition, it poorly inhibited metabolism of healthy lymphomonocytes (data not shown). Although **11a** and **11b** are diasteroisomers, they did not show the same biological activity. This might be due to a different stoichiometry since both **11a**, **b** show the 6-chloropurine on the same side of the phenyl ring, but **11a** is linked through the N9 nitrogen to the isoxazolidine ring, while **11b** is linked through the N7 nitrogen atom. This could affect a different interaction with a molecular cell target that should be involved in apoptosis induction. Further studies are necessary to identify this target.

Nonetheless it has to be underlined that the 6-chloropurine, *cis*-related to the phenyl ring of the isoxazolidine structure is endowed with a biological activity as previously shown for compound **6a**.[9] Therefore the scaffold of **11a** could be an important initial step to synthesize new compounds with broader antitumor activity.

In order to look for a SAR between the best active compound **11a** and compound **6a** reported in a previous work,[9] we have conducted a conformational analysis by performing *ab initio* calculations by means of DFT methods at the B3LYP(6-31G)(d) level.[15] Figures 8 shows the gas-phase optimized conformations of compounds **6a**[9] and **11a**.

The reported structures, the most populated according to the Boltzmann distribution, show the 6-chloropurine rings in

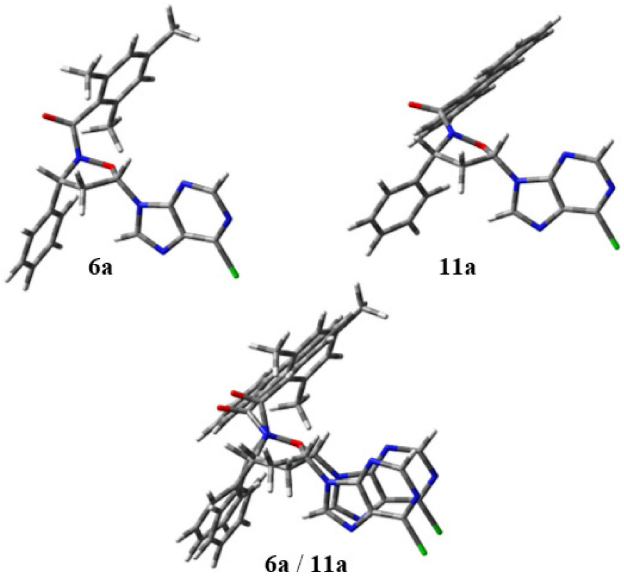


Figure 8. Optimized structures of compounds **6a** [see ref. 9: *ACS Omega* **2018**, *3*, 7621–7629] and **11a** (top). Overlap of **6a** and **11a** (bottom).

pseudo-equatorial positions, *cis*-related with the phenyl rings. Both compounds are the most stable conformers and belong to the N9 derivative series, *i.e.* the 6-chloropurine ring is attached to the isoxazolidine ring through the N9 nitrogen atom. They are reasonably stabilized by nonclassical intramolecular purine H-bond between the H–C=N–R proton and the phenyl ring.[9]

On the basis of the present as well as previous biological data, the structure comparison between compounds **6a** and **11a** leads to considering the structure overlap that is shown in Figure 8 (bottom) where the isoxazolidine rings were fused at the N–O heteroatoms. The resulting fitting of the other parts of the two molecules is substantially excellent. While the purine and phenyl rings are slightly apart each other, the overlap of the anthryl and mesityl moieties is quite perfect. These observations corroborate the nice fitting of the biological results and represent a strong contribution in the SAR analysis.

In order to broaden the antitumor activity, we are actively pursuing new syntheses of isoxazolidine derivatives and the strategies we are following are summarized in Figure 9. We wish to clarify the roles of the three main substituents around the isoxazolidine ring. First, we will replace the chlorine atom on the purine ring aiming to verify the role of the Cl atom itself and the potential effect of replacement with nitrogen, oxygen or other. Second, the substituent at the position C3 of the isoxazolidine ring seems to be a key element and needs to be verified by deleting the substituent and leaving on the heterocyclic ring a simple methylene group.[16] Third and finally, the aromatic moieties linked to the carbonyl group are normally characterized by a not negligible steric demand due to the nitrosocarbonyl ene reaction mechanism that requires the activation of the anti-Markovnikov pathway through steric effects.[2,5] For these reasons we plan to modify the synthetic approach for the insertion of simple phenyl rings on some isoxazolidine derivatives and even simple aliphatic substituents to shine some light on the role of this portion of the molecule in the biological activity. This last point will be pursued by means of catalyzed syntheses between hydroxamic acids and unsaturated aldehydes.[16]

3. Conclusions

In conclusion, we have synthesized two new families of *N,O*-nucleoside analogues (Figure 10) which contain the anthracene moiety introduced through the nitrosocarbonyl ene reaction with allylic alcohols, using the stable anthracenenitrile oxide as synthon of the fleeting intermediate; in the group of compounds (A) the phenyl ring is maintained at the C3 isoxazolidine moiety while in the group (B) two methyls were inserted.

At the heterobase level, both the groups of isoxazolidines were functionalized with the 6-chloropurine ring for comparison with the data already known.[9] Pyrimidines and other bases were also included to expand the scope of the synthesis and the biological frame.

From the synthetic point of view the methodology was found to be robust and reliable and gave the desired products in good yields. The structures were definitively demonstrated on the basis of analytical and spectroscopic data as well as specific experiment that contributed in the stereochemical definition of every single compound. X-Ray diffractometric analyses also corroborated the spectroscopic attributions.

Biological assays were conducted to investigate the metabolic and pro-apoptotic activities of the designed compounds. Compound **11a** demonstrated a good capacity to induce cell death and an appreciable nuclear fragmentation was evidenced in cells treated with **11a**.

These results nicely fit with some previous observations[9] regarding the role of the heterobases and the relative stereochemistry with substituents in C3 of the isoxazolidine ring and these SAR observations pave the way to further studies that require the synthesis of new compounds, even through different synthetic methods already located in our research activities.

We are confident in gaining rapidly new results able to give the isoxazolidine derivatives a solid background as antitumor promoters.[17]

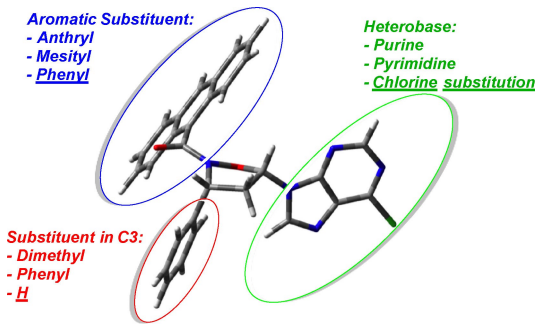


Figure 9. SAR: structural modifications on the **11a** scaffold.

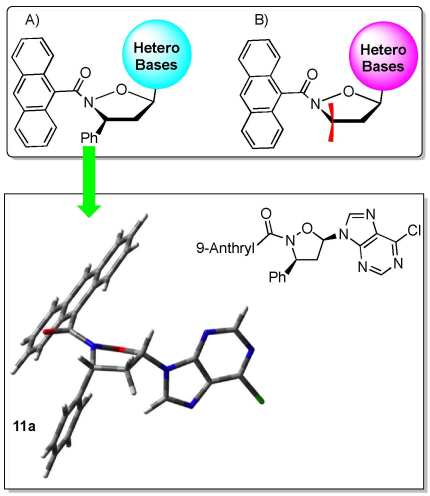


Figure 10. Synthesized nucleoside analogues; compound **11a**.

Experimental Section

All melting points (m.p.) are uncorrected. Elemental analyses were done on an elemental analyzer available at the Department. ^{1}H and ^{13}C NMR spectra were recorded on a 300 MHz and 400 MHz spectrometers (solvents specified). Chemical shifts are expressed in ppm from internal tetramethylsilane (δ) and coupling constants (J) are in Hertz (Hz): b, broad; s, singlet; bs, broad singlet; d, doublet; t, triplet; qi, quintet; m, multiplet. IR spectra (nujol mulls) were recorded on a spectrophotometer available at the Department and absorbtions (ν are in cm^{-1}. Column chromatography and tlc: silica gel H60 and GF_{254}, respectively; eluants: cyclohexane/ethyl acetate 9:1 to pure ethyl acetate; when specified, pure $CHCl_3$ to $CHCl_3$/MeOH 9/1 for the nucleosides syntheses.

Starting and Reference Materials: Cinnamyl alcohol (98%) and 3-methyl-2-buten-1-ol (99%) were purchased from chemical supplier.

Anthracene oxide **1 A** was obtained by oxidation of anthracenaldoxime with NCS.[14]

Other reagents and solvents were purchased from chemical suppliers and used without any further purification.

Ene Reaction of Nitrosocarbonyl Anthracene 1 A with Cinnamyl Alcohol: To an ice-cooled DCM (200 mL) solution of cinnamyl alcohol (32 mL, 5 equiv.), 3.04 g (1.2 equiv.) of NMO were added under stirring. Solid anthracenenitrile oxide **7** (4.74 g, 21.6 mmol) was added portionwise and the reaction left under stirring at r.t. for 48 h. After dilution with an equivalent volume of DCM, the organic phase was washed with water and dried over anhydrous Na_2SO_4. After filtration, the solvent was then evaporated and the reaction mixture separated on column chromatography, affording the ene adduct **8** as an inseparable mixture of diastereoisomers in the ratio 3:1. In the NMR spectra of **8** the signals of the minor diastereoisomer are reported in brackets.

Compound 8: Yield 3.51 g (44%). M.p. 185–186 °C (EtOH/iPr_2O 1:2 V/V). IR: ν = 3195 (OH), 1637 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 2.37 [2.25] (m, 1H, CH_2), 2.81 [2.58] (m, 1H, CH_2), 5.40 [4.62] (t, 1H, J = 5 Hz, CH), 5.83 [5.98] (t, 1H, J = 8 Hz, CH–Ph), 6.86 (d, 1H, J = 5 Hz, O–CH–O), 7.40–7.70 (m, 8H, arom.), 7.96–8.18 (m, 4H, arom.), 8.68 [8.62] (s, 1H, Anthr.). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 44.9 [45.2], 58.6 [60.5], 97.3 [97.5], 124.8 [124.7], 125.4 [125.3], 125.6 [125.9], 126.2 [126.4], 126.6 [126.5], 126.8 [127.3], 127.5 [127.4], 127.7 [127.9], 128.0 [128.1], 128.6 [128.7], 128.8 [130.4], 130.8, 142.0 [140.3], 168.2 [164.6]. $C_{24}H_{19}NO_3$ (369.14): calcd. C 78.03, H 5.18, N 3.79; found C 78.01, H 5.19, N 3.80.

Synthesis of 2-(Anthracene-9-carbonyl)-3-Phenylisoxazolidin-5-yl Acetate 9: To an ice-cooled anhydrous DCM (150 mL) solution of compound **8** (1.00 g, 2.7 mmol.), 2.2 equiv. of Ac_2O were added under stirring along with 0.3 equiv. of DMAP and 2.2 equiv. of Et_3N. The reaction is left under stirring at r.t. for 24 h. After dilution with an equivalent volume of DCM, the organic phase was washed with a saturated solution of $NaHCO_3$ and dried over anhydrous Na_2SO_4. After filtration, the solvent was evaporated and an oily residue is obtained corresponding to the inseparable mixture of the two diastereoisomers **9**, purified by column chromatography and fully characterized. In the NMR spectra of **9** the signals of the minor diastereoisomer are reported in brackets.

Compound 9: Yield 1.09 g (98%). M.p. 143–145 °C (EtOH). IR: ν = 1652 (C=O), 1756 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.55 [2.32] (s, 3H, CH_3), 2.66 [2.55] (m, 1H, CH_2), 3.10 [2.90] (m, 1H, CH_2), 5.92 [4.61] (t, 1H, J = 8 Hz, CH), 6.21 [6.40] (d, 1H, J = 5 Hz, CH–Ph), 7.10–8.30 (m, 13H arom.), 8.72 [8.69] (s, 1H, Anthr.). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 20.4 [21.1], 43.1 [44.1], 58.3 [60.2], 95.6 [96.0], 124.7 [124.4], 125.4 [124.8], 125.6 [125.3], 125.9 [126.0], 126.2 [126.4], 126.5 [126.8], 126.6 [127.3], 127.5 [127.9], 127.7 [128.1], 128.0 [128.7], 128.6 [130.4], 128.8, 130.4 [140.3], 130.8 [164.6], 140.9 [138.8], 168.1 [165.0], 168.5 [169.0]. $C_{26}H_{21}NO_4$ (411.46): calcd. C 75.90, H 5.14, N 3.40; found C 75.91, H 5.12, N 3.41.

Synthesis of Nucleosides 10 a, b (U, T) by Coupling of Isoxazolidine 9 and Uracil and Thymine: A solution of 2.2 equiv. of uracil or thymine and 4 equiv. of bis(trimethylsilyl)acetamide (BSA) in anhydrous DCM (50 mL) is refluxed under nitrogen atmosphere for 1 hour. A solution in DCM (10 mL) of isoxazolidine **9** (0.40 g, 0.97 mmol) is added dropwise and cooled to 0 °C and additioned of 4 equiv. of TMSO–Tf. The reaction is refluxed under stirring overnight and finally quenched with a saturated solution of $NaHCO_3$ at pH = 7. The mixture is diluted with an equivalent volume of DCM and washed with water and finally dried over Na_2SO_4. From the residues, nucleosides **10 a**, **b** (**U, T**) are isolated through column chromatography and fully characterized.

Compound 10 aU: Yield 0.22 g (49%). M.p. 166 °C (dec.) (EtOH). IR: ν = 1698 (C=O), 1708 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 2.61 (m, 1H, CH_2), 3.41 (m, 1H, CH_2), 5.46 (d, 1H, J = 8 Hz, CH=), 6.18 (t, 1H, J = 8 Hz, CH), 6.34 (t, 1H, J = 6 Hz, CH–Ph), 7.25–8.25 (m, 13H, arom.), 7.87 (d, 1H, J = 8 Hz, CH=), 8.72 (s, 1H, Anthr.), 11.25 (s, 1H, NH). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 60.4, 84.9, 103.0, 124.2, 124.8, 125.7, 125.9, 126.2, 127.0, 127.1, 127.2, 127.6, 127.8, 128.1, 128.5, 128.7, 129.0, 129.6, 130.5, 139.4, 140.6, 149.8, 162.3, 168.6. $C_{28}H_{21}N_3O_4$ (463.49): calcd. C 72.56, H 4.57, N 9.07; found C 72.57, H 4.59, N 9.04.

Compound 10 bU: Yield 0.23 g (52%). M.p. > 250 °C (dec.) (EtOH). IR: ν = 1693 (C=O), 1716 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 2.80 (m, 1H, CH_2), 3.45 (m, 1H, CH_2), 5.75 (d, 1H, J = 8 Hz, CH=), 6.17 (t, 1H, J = 8 Hz, CH), 6.22 (d, 1H, J = 7 Hz, CH–Ph), 7.25–8.25 (m, 13H, arom.), 7.93 (d, 1H, J = 8 Hz, CH=), 8.67 (s, 1H, Anthr.), 11.09 (s, 1H, NH). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 58.3, 85.2, 101.8, 124.0, 125.1, 126.1, 126.3, 126.4, 127.7, 128.6, 130.1, 134.2, 149.9, 170.5, 173.7, 174.1. $C_{28}H_{21}N_3O_4$ (463.49): calcd. C 72.56, H 4.57, N 9.07; found C 72.55, H 4.56, N 9.08.

Compound 10 aT: Yield 0.19 g (40%). M.p. > 250 °C (dec.) (EtOH). IR: ν = 1698 (C=O), 1708 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.53 (s, 3H, CH_3), 2.65 (m, 1H, CH_2), 3.59 (m, 1H, CH_2), 6.21 (t, 1H, J = 8 Hz, CH), 6.34 (t, 1H, J = 6 Hz, CH–Ph), 7.44–8.20 (m, 13H, arom.), 7.66 (s, 1H, CH=), 8.72 (s, 1H, Anthr.), 11.23 (s, 1H, NH). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 11.8, 60.3, 84.7, 110.6, 124.2, 124.6, 125.7, 125.9, 126.2, 127.0, 127.1, 127.13, 127.2, 127.8, 128.1, 128.5, 128.7, 129.0, 129.7, 130.4, 130.5, 134.6, 140.5, 149.8, 163.0, 168.9. $C_{29}H_{23}N_3O_4$ (477.52): calcd. C 72.94, H 4.86, N 8.80; found C 72.97, H 4.87, N 8.79.

Compound 10 bT: Yield 0.23 g (49%). M.p. 171–175 °C (EtOH). IR: ν = 1698 (C=O), 1700 (C=O) cm^{-1}. ^{1}H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.95 (s, 3H, CH_3), 2.92 (m, 1H, CH_2), 3.45 (m, 1H, CH_2), 6.23 (d, 1H, J = 8 Hz, CH), 7.31 (t, 1H, J = 8 Hz, CH–Ph), 7.35–8.20 (m, 13H, arom.), 7.72 (s, 1H, CH=), 8.66 (s, 1H, Anthr.), 11.07u (s, 1H, NH). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 11.6, 58.3, 84.8, 109.7, 123.8, 124.2, 125.0, 125.1, 125.3, 126.1, 126.3, 127.0, 127.6, 127.7, 128.1, 128.3, 128.6, 129.9, 130.0, 135.6, 140.9, 149.9, 163.0, 163.9. $C_{29}H_{23}N_3O_4$ (477.52): calcd. C 72.94, H 4.86, N 8.80; found C 72.96, H 4.85, N 8.81.

Synthesis of Nucleosides 11 a–d by Coupling of Isoxazolidine 9 and 6-Chloropurine: A solution of 2.2 equiv. of 6-chloropurine and 4 equiv. of bis(trimethylsilyl)acetamide (BSA) in anhydrous DCM (50 mL) is refluxed under nitrogen atmosphere for 1 hour. A solution in DCM (10 mL) of isoxazolidine **9** (0.40 g, 0.97 mmol) is added dropwise and cooled to 0 °C and additioned of 4 equiv. of TMSO–Tf. The reaction is refluxed under stirring overnight and finally quenched with a saturated solution of $NaHCO_3$ at pH = 7.

The mixture is diluted with an equivalent volume of DCM and washed with water and finally dried over Na_2SO_4. From the residues, nucleosides **11 a–d** are isolated through column chromatography and fully characterized.

Compound 11 a: Yield 0.11 g (22%). M.p. 200–201 °C (iPr_2O/EtOH). IR: ν = 1592 (C=N), 1654 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 3.42 (m, 1H, CH_2), 3.64 (m, 1H, CH_2), 6.26 (t, 1H, J = 8 Hz, CH–Ph), 6.59 (t, 1H, J = 6 Hz, CH), 7.43–7.86 (m, 10H, arom.), 8.10–8.20 (m, 3H, arom.), 8.51 (s, 1H, H–C=N), 8.57 (s, 1H, Anthr.), 8.69 (s, 1H, H–C=N). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 40.1, 60.5, 84.4, 124.0, 124.9, 125.6, 125.8, 126.4, 127.0, 127.1, 127.2, 127.6, 127.8, 128.1, 128.5, 128.6, 128.9, 129.4, 130.4, 130.5, 131.1, 140.1, 145.1, 149.4, 151.4, 151.8, 168.2. $C_{29}H_{20}ClN_5O_2$ (505.96): calcd. C 68.84, H 3.98, N 13.84; found C 68.87, H 3.99, N 13.84.

Compound 11 b: Yield 0.18 g (36%). M.p. 201–205 °C (iPr_2O/EtOH). IR: ν = 1595 (C=N), 1633 (C=O) cm^{-1}. ^1H NMR (400 MHz, [D6]DMSO, 25 °C): δ = 3.43 (m, 1H, CH_2), 4.00 (m, 1H, CH_2), 6.31 (t, 1H, J = 8 Hz, CHPh), 6.56 (d, 1H, J = 7 Hz, CH), 7.40–7.70 (m, 9H, Ph and Anthr.), 7.85 (m, 1H, Anthr.), 8.12 (m, 1H, Anthr.), 8.21 (m, 2H, Ph), 8.65 (s, 1H, H–C=N), 8.74 (s, 1H, Anthr.), 8.89 (s, 1H, H–C=N). ^{13}C NMR (100 MHz, [D6]DMSO, 25 °C): δ = 40.1, 60.4, 85.2, 121.9, 124.1, 125.1, 125.6, 125.8, 126.4, 127.1, 127.2, 127.4, 127.9, 128.3, 128.7, 129.0, 129.5, 130.4, 130.6, 140.1, 142.0, 148.1, 152.3, 161.5, 168.8. $C_{29}H_{20}ClN_5O_2$ (505.96): calcd. C 68.84, H 3.98, N 13.84; found C 68.85, H 3.96, N 13.86.

Compound 11 c: Yield 0.12 g (24%). M.p. 145 °C (dec.) (iPr_2O/EtOH). IR: ν = 1652 (C=N), 1714 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 3.07 (m, 1H, CH_2), 4.00 (m, 1H, CH_2), 6.38 (t, 1H, J = 8 Hz, CH–Ph), 6.55 (d, 1H, J = 7 Hz, CH), 6.77 (m, 2H, CH, Anthr.), 7.21 (m, 1H, CH, Anthr.), 7.46–7.84 (m, 9H, arom.), 8.10 (d, 1H J = 8 Hz, CH, Anthr.), 8.32 (s, 1H, H–C=N), 8.52 (s, 1H, Anthr.), 9.06 (s, 1H, H–C=N). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 40.9, 58.8, 83.8, 122.0, 124.2, 124.6, 125.5, 125.8, 126.4, 126.9, 127.3, 127.9, 128.0, 128.1, 128.7, 129.1, 129.9, 130.2, 131.4, 141.1, 145.1, 149.3, 151.27, 151.3, 165.9. $C_{29}H_{20}ClN_5O_2$ (505.96): calcd. C 68.84, H 3.98, N 13.84; found C 68.82, H 3.97, N 13.83.

Compound 11 d: Yield 0.12 g (24%). M.p. 230 °C (dec.) (iPr_2O/EtOH). IR: ν = 1651 (C=N), 1684 (C=O) cm^{-1}. ^1H NMR (400 MHz, [D6]DMSO, 25 °C): δ = 3.05 (m, 1H, CH_2), 4.03 (m, 1H, CH_2), 6.25 (t, 1H, J = 8 Hz, CH–Ph), 6.67 (t, 1H, J = 8 Hz, O–CH–N), 7.09 (m, 1H, CH, Anthr.), 7.45–7.85 (m, 10H, arom.), 8.13 (d, 1H J = 8 Hz, Anthr.), 8.50 (s, 1H, H–C=N), 8.58 (s, 1H, Anthr.), 9.48 (s, 1H, H–C=N). ^{13}C NMR (100 MHz, [D6]DMSO, 25 °C): δ = 41.0, 58.9, 85.1, 121.3, 121.8, 124.2, 124.7, 125.4, 126.0, 126.3, 126.4, 126.9, 127.5, 127.8, 128.0, 128.2, 128.5, 128.7, 129.1, 129.8, 130.2, 140.8, 142.0, 147.7, 151.7, 162.1, 166.6. $C_{29}H_{20}ClN_5O_2$ (505.96): calcd. C 68.84, H 3.98, N 13.84; found C 68.83, H 3.99, N 13.82.

Synthesis of Nucleosides 13–15 by Coupling of Isoxazolidine 12 and Purine, 6-Chloropurine and Benzoimidazole: A solution of 2.2 equiv. of 6-chloropurine and 4 equiv. of bis(trimethylsilyl) acetamide (BSA) in anhydrous DCM (50 mL) is refluxed under nitrogen atmosphere for 1 hour. A solution in DCM (10 mL) of isoxazolidine **12** (0.50 g, 1.38 mmol) is added dropwise and cooled to 0 °C and additioned of 4 equiv. of TMSO–Tf. The reaction is refluxed under stirring overnight and finally quenched with a saturated solution of $NaHCO_3$ at pH = 7. The mixture is diluted with an equivalent volume of DCM and washed with water and finally dried over Na_2SO_4. From the residues, nucleosides **13–15** are isolated through column chromatography and fully characterized.

Compound 13 a: Yield 0.30 g (51%). M.p. > 200 °C (dec.) (MeOH). IR: ν = 1574 (C=N), 1634 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.88 (s, 3H, CH_3), 2.21 (s, 3H, CH_3), 3.07 (m, 1H, H–CH), 3.53 (m, 1H, HC–H), 6.45 (m, 1H, arom.), 6.86 (dd, 1H J = 8 Hz, O–CH–N), 7.24 (t, 1H, arom.), 7.46 (d, 1H, arom.), 7.57 (m, 2H, arom.), 7.89 (d, 2H, arom.), 8.09 (d, 1H, arom.), 8.53 (s, 1H, CH=N), 8.62 (s, 1H,CH=N), 8.75 (s, 1H,CH=N), 9.08 (s, 1H, arom.). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 25.6, 25.7, 45.8, 63.3, 81.7, 123.4, 124.3, 125.2, 125.5, 125.8, 126.2, 126.7, 126.8, 127.5, 128.0, 128.6, 130.1, 130.4, 130.5, 134.1, 145.2, 148.1, 150.7, 151.9, 164.5. $C_{25}H_{21}N_5O_2$ (423.17): calcd. C 70.91, H 5.00, N 16.54; found C 70.93, H 5.02, N 16.54.

Compound 13 b: Yield 0.26 g (45%). M.p. > 200 °C (dec.) (MeOH). IR: ν = 1592 (C=N), 1626 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.91 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 3.03 (m, 1H, H–CH), 3.49 (m, 1H, HC–H), 6.46 (m, 1H, arom.), 6.80 (dd, 1H J = 8 Hz, O–CH–N), 7.16 (m, 2H, arom.) 7.60 (m, 2H, arom.), 7.73 (s, 1H,CH=N), 7.89 (t, 2H, arom.), 8.15 (d, 1H, arom.), 8.59 (s, 1H, CH=N), 8.69 (s, 1H,CH=N), 9.02 (s, 1H, arom.). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 25.6, 25.7, 45.0, 62.0, 83.2, 122.1, 123.9, 124.7, 125.2, 125.6, 126.2, 126.5, 127.2, 127.7, 128.3, 129.7, 129.8, 129.9, 140.3, 146.4, 151.9, 159.9, 163.6. $C_{25}H_{21}N_5O_2$ (423.17): calcd. C 70.91, H 5.00, N 16.54; found C 70.90, H 4.98, N 16.56.

Compound 14 a: Yield 0.35 g (56%). M.p. > 200 °C (dec.) (MeOH). IR: ν = 1560 (C=N), 1636 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.87 (s, 3H, CH_3), 2.19 (s, 3H, CH_3), 3.06 (m, 1H, H–CH), 3.52 (m, 1H, HC–H), 6.42 (dd, 1H J = 8 Hz, O–CH–N), 6.87 (t, 1H, arom.), 7.24 (t, 1H, arom.), 7.32 (d, 1H, arom.), 7.59 (m, 2H, arom.) 7.86 (m, 2H, arom.), 8.10 (d, 1H, arom.), 8.45 (s, 1H, CH=N), 8.53 (s, 1H, arom.), 8.75 (s, 1H,CH=N). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 25.6, 25.9, 45.6, 63.0, 82.2, 122.9, 124.3, 125.0, 125.5, 125.6, 125.9, 126.8, 127.5, 128.0, 128.6, 130.1, 130.4, 130.5, 131.4, 145.4, 149.2, 151.3, 164.4. $C_{25}H_{20}ClN_5O_2$ (457.92): calcd. C 65.57, H 4.40, N 15.29; found C 65.53, H 4.42, N 15.29.

Compound 14 b: Yield 0.25 g (40%). M.p. > 200 °C (dec.) (MeOH). IR: ν = 1590 (C=N), 1635 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6]DMSO, 25 °C): δ = 1.89 (s, 3H, CH3), 2.10 (s, 3H, CH3), 3.05 (m, 1H, H–CH), 3.56 (m, 1H, HC–H), 6.48 (dd, 1H J = 8 Hz, O–CH–N), 6.68 (t, 1H, arom.), 6.97 (d, 1H, arom.) 7.11 (t, 1H, arom.), 7.59 (m, 2H, arom.), 7.84 (m, 2H, arom.), 8.13 (d, 1H, arom.), 8.54 (s, 1H, CH=N), 8.58 (s, 1H, arom.), 9.20 (s, 1H,CH=N). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 25.9, 26.6, 45.0, 62.4, 83.7, 121.8, 122.0, 124.3, 124.9, 125.5, 125.7, 125.9, 126.7, 126.8, 127.5, 127.9, 128.1, 130.1, 130.3, 130.4, 142.2, 147.7, 151.7, 162.0, 164.2. $C_{25}H_{20}ClN_5O_2$ (457.92): calcd. C 65.57, H 4.40, N 15.29; found C 65.58, H 4.38, N 15.30.

Compound 15: Yield 0.23 g (40%). M.p. > 200 °C (dec.) (MeOH/iPr_2O). IR: ν = 1587 (C=N), 1652 (C=O) cm^{-1}. ^1H NMR (300 MHz, [D6] DMSO, 25 °C): δ = 2.00 (s, 3H, CH_3), 2.09 (s, 3H, CH_3), 2.97 (m, 1H, H–CH), 3.26 (m, 1H, HC–H), 6.48 (dd, 1H, J = 7 Hz, O–CH–N), 6.76 (d, 1H, arom.), 6.84 (t, 1H, arom.), 7.05 (t, 1H, arom.), 7.21 (t, 1H, arom.), 7.33 (t, 1H, arom.), 7.61 (m, 4H, arom.), 7.97 (m, 2H, arom.), 8.13 (d, 1H, arom.), 8.43 (s, 1H,CH=N), 8.57 (s, 1H, arom.). ^{13}C NMR (75 MHz, [D6]DMSO, 25 °C): δ = 25.3, 26.3, 30.7, 45.9, 63.5, 82.7, 110.7, 119.4, 122.2, 122.6, 123.9, 124.4, 125.4, 125.5, 126.4, 126.5, 126.7, 126.9, 127.5, 128.0, 128.6, 130.3, 130.4, 132.8, 142.6, 143.4, 163.7. $C_{27}H_{23}N_3O_2$ (421.50): calcd. C 76.94, H 5.50, N 9.97; found C 76.95, H 5.48, N 10.00.

Biological Assays

Human monocytic U937 cells, originally obtained from the Istituto Zooprofilattico, Brescia (Italy) were cultured at 37 °C in a 5% CO_2 incubator.

For detection of metabolic activity by the MTS assay and of cell death by the Trypan blue dye exclusion test, standard methods were utilized. For detection of apoptosis following Hoechst 33342 staining, fluorescence microscopy analysis was performed as previously described.[9]

IC_{50} and CC_{25} values were calculated using the (MLA) "Quest Graph™ Four Parameter Logistic (4PL) Curve Calculator." AAT Bioquest, Inc, 09 Jan. 2020, https://www.aatbio.com/tools/four-parameter-logistic-4pl-curve-regression-online-calculator.

X-Ray Crystallographic Analysis of Compounds 8 and 13 a

Unit-cell dimensions for compounds **8** and **13 a** were obtained by least-squares fit of 2θ values for 25 reflections, using an Enraf-Nonius CAD4 diffractometer with graphite-monochromated Mo-Kα radiation at the Centro Grandi Strumenti (CGS) of the University of Pavia, Italy.

The structure was solved by direct method and the E-map correctly revealed the non-hydrogen atoms in the molecules. The positions of the hydrogen atoms were located from a difference Fourier synthesis, compared with those calculated from the geometry of the molecules, and refined isotropically in the subsequent least-squares refinement. The programs SHELXL[18] is used to solve the structure. The ORTEP[19] program is used for molecular graphics.

CCDC depositions numbers: **8**, 1981058; **13 a**, 1981060.

Acknowledgments

Financial support by the University of Pavia is gratefully acknowledged. We warmly thank Dr. Massimo Boiocchi (CGS – UniPV) for X-Ray data collection. Thanks are also due to "VIPCAT – Value Added Innovative Protocols for Catalytic Transformations" project (CUP: E46D17000110009) for valuable financial support.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: isoxazolidines · *N,O*-nucleosides · ene reactions · apoptosis · structure–activity relationship

[1] M. Berthet, T. Cheviet, G. Dujardin, I. Parrot, J. Martinez, *Chem. Rev.* **2016**, *116*, 15235–15283.
[2] M. G. Memeo, P. Quadrelli, *Chem. Rev.* **2017**, *117*, 2108–2200.
[3] P. Quadrelli, M. Mella, S. Carosso, B. Bovio, *J. Org. Chem.* **2013**, *78*, 516–526.
[4] P. Quadrelli, M. Mella, A. Gamba Invernizzi, P. Caramella, *Tetrahedron* **1999**, *55*, 10497–10510.
[5] a) P. Quadrelli, M. Mella, A. Piccanello, S. Romano, P. Caramella, *J. Org. Chem.* **2007**, *72*, 1807–1810; b) P. Quadrelli, S. Romano, A. Piccanello, P. Caramella, *J. Org. Chem.* **2009**, *74*, 2301–2310.
[6] W. Adam, O. Krebs, *Chem. Rev.* **2003**, *103*, 4131–4146.
[7] a) M. Baidya, H. Yamamoto, *Synthesis* **2013**, *45*, 1931–1938; b) S. Carosso, B. Bovio, E. Valletta, B. Macchi, P. Quadrelli, *Synthesis* **2017**, *49*, 1972–1982.
[8] a) H. Vorbrüggen, K. Krolikiewicz, B. Bennua-Skalmowski, *U. S. Pat.* **1998**, Pat. N. 5,750,676; b) H. Vorbrüggen, K. Krolikiewicz, B. Bennua, *Chem. Ber.* **1981**, *114*, 1234–1255; c) H. Vorbrüggen, G. Höfle, *Chem. Ber.* **1981**, *114*, 1256–1268; d) H. Vorbrüggen, B. Bennua, *Chem. Ber.* **1981**, *114*, 1279–1286; For a survey on the nucleoside synthesis see: e) H. Vorbrüggen, C. Ruh-Pohlenz, *Handbook of Nucleoside Synthesis* Wiley, J. & Sons; New York: 2001.
[9] M. G. Memeo, E. Valletta, B. Macchi, A. Porta, B. Bovio, M. Moiola, P. Quadrelli, *ACS Omega* **2018**, *3*, 7621–7629.
[10] a) P. Quadrelli, G. Campari, M. Mella, P. Caramella, *Tetrahedron Lett.* **2000**, *41*, 2019–2022; b) P. Quadrelli, M. Mella, P. Caramella, *Tetrahedron Lett.* **1998**, *39*, 3233–3236.
[11] For acetylation of 5-hydroxy-isoxazolidines see: a) Y. Xiang, Y. Gong, K. Zhao, *Tetrahedron Lett.* **1996**, *37*, 4877–4880; b) X. Zhang, F.-L. Qing, Y. Yu, *J. Org. Chem.* **2000**, *65*, 7075–7082; c) X. Zhang, H. Xia, X. Dong, J. Jin, W.-D. Meng, F.-L. Qing, *J. Org. Chem.* **2003**, *68*, 9026–9033.
[12] P. Quadrelli, M. Mella, S. Carosso, B. Bovio, *Synthesis* **2013**, *45*, 1414–1420.
[13] M. Berridge, A. Tan, K. McCoy, R. Wang, *Biochemica* **1996**, *4*, 14–19.
[14] C. Grundmann, P. Grünanger, *The Nitrile Oxide* Springer-Verlag: Heidelberg, **1971**.
[15] Gaussian 09, Revision **E.01**, M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, G. Scalmani, V. Barone, B. Mennucci, G. A. Petersson, H. Nakatsuji, M. Caricato, X. Li, H. P. Hratchian, A. F. Izmaylov, J. Bloino, G. Zheng, J. L. Sonnenberg, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, T. Vreven, J. A. Montgomery, Jr., J. E. Peralta, F. Ogliaro, M. Bearpark, J. J. Heyd, E. Brothers, K. N. Kudin, V. N. Staroverov, R. Kobayashi, J. Normand, K. Raghavachari, A. Rendell, J. C. Burant, S. S. Iyengar, J. Tomasi, M. Cossi, N. Rega, J. M. Millam, M. Klene, J. E. Knox, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, R. L. Martin, K. Morokuma, V. G. Zakrzewski, G. A. Voth, P. Salvador, J. J. Dannenberg, S. Dapprich, A. D. Daniels, Ö. Farkas, J. B. Foresman, J. V. Ortiz, J. Cioslowski, D. J. Fox, Gaussian, Inc., Wallingford CT, **2009**.
[16] K. K. Aljaf, A. Amin, F. Hussain, P. Quadrelli, ARKIVOC *manuscript in preparation*.
[17] P. Quadrelli, in *"Modern Applications of Cycloaddition Chemistry"* Quadrelli, P. Eds.; Elsevier, Amsterdam: **2019**, pp. 85–152.
[18] G. M. Sheldrik, *SHELXL-93. Program for the Refinement of Crystal Structure,* University of Göttingen, Germany, **1993**.
[19] C. K. Johnson, *ORTEP.* Report ORNL-3793, Oak Ridge National Laboratory, Tennessee, USA, **1966**.

Manuscript received: February 10, 2020
Revised manuscript received: March 4, 2020