A multitarget approach toward the development of a novel series of 8-substituted purines for photoprotection and prevention of ultra violet (UV) related damages

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ABSTRACT

In environmental abundance, UV is the most important modifiable risk factor for skin cancer and many other skin diseases such as early photo-aging. Among solar radiation, ultraviolet (UV) is mainly responsible for inducing skin problems. In search of novel photoprotective purines, a new series of 8-substituted purines were synthesized from commercially available 6-hydroxy-4,5-diaminopyrimidine hemisulfate or 4,5-diaminopyrimidine. All the title compounds were investigated for their UV-filter, antioxidant, antifugal and antiproliferative activities. For the photoprotective assays we used a diffuse transmittance technique to determine the SPF in vitro, and DPPH and FRAP tests for antioxidant activity evaluation of the more potent compounds. Compound **26** proved, among the others, to be a good radical-scavenger and was also endowed with broad-spectrum UVA filtering capabilities, suitable to be developed as a protective molecule.

Abbreviations: AcOEt, Ethylacetate; CPD, cyclobutane pyrimidine dimmers; EtO_2, Diethyl ether; DCM, Dichloromethane; DMSO, Dimethylsulfoxide; DNA, Deoxyribonucleic acid; DPDT, disodium phenyl dibenzimidazole tetrasulfonate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FDA, Food and Drug Administration; FRAP, Ferric Reducing Antioxidant Power; HCl, Hydrochloric acid; H_3PO_4, Phosphoric acid; MED, minimal erythemal dose; MeOH, Methanol; NaOH, Sodium hydroxide; PBSA**,** Phenyl-benzimidazole sulfonic acid; $POCl_3$, Phosphoryl chloride; ROS, reactive oxygen species; SPF, Sun Protection Factor.

Keywords: antioxidant activity, benzimidazole, polyphenols, 8-substitutedpurines, UV-filtering.

1. Introduction

Excessive and repeated exposure to solar ultraviolet radiation (UV) can cause several skin damages, such as: sunburn,[1] skin tumors,[2] cancer, and photoaging.[3] However, UV also benefits human health by mediating natural synthesis of vitamin D and endorphins in the skin;[4] therefore UV has complex and mixed effects on human health. There is substantial evidence that UV protection is important in order to reduce the risk of squamous cell carcinoma, actinic keratosis and probably also the risk of melanoma.[5]

A recent study about sunlight-induced melanoma can arise from cyclobutane pyrimidine dimers (CPDs) generated into the melanocytes and CPDs are generated for more than 3 hours after exposure to UVA. This study confirms that the chemi-excitation of melanin derivatives induces DNA photoproducts long after UV exposure.[6]

In 1934, Friedrich Ellinger firstly reported the concept of the minimal erythemal dose (MED) for protected and unprotected skin, proposing a coefficient of protection that decreased in value to the extent that protection increased.[7,8] Only in 1974 Greiter introduced the term Sun Protection Factor (SPF).[9]

The SPF is defined as the UV energy required for producing a MED on protected skin, divided by the UV energy required to produce a MED on unprotected skin.[10,11]UV filters and antioxidants are the first line of defense against skin cancer and early photo-ageing; prevention is the most effective strategy. For these reasons there is an increasing interest in developing novel molecules especially if endowed with UV filtering and antioxidant activity. Interestingly, this work is mainly conduced at the industrial level. As a matter of fact, sun filters and dyes are the most "patented" ingredients in the cosmetic field, as a proof of the industrial/economic interest (577 patent applications. In the period of 2011-2015, search conducted on the ORBIT.COM data base using "sunscreen and preparation" as key words). Literature in this regard is much scarce(151 hits by search with the same key words and same years on Scopus database).

Thus, chemists and cosmetics-producing companies are interested in developing new molecules, which have photoprotective activities that can be incorporated into sunscreens counteracting actinic damage by both shielding and quenching reactive oxygen species (ROS). The UV-radiation (UVR) that reaches the earth's surface consists mainly of long wavelength ultraviolet A (UVA) (320 – 400 nm) and short wavelength ultraviolet B (UVB) (280 - 320 nm). UVC (200 – 280 nm) does not reach the surface of the earth, as it is absorbed by atmospheric oxygen and well-absorbed by the ozone layer. However, some researches demonstrated that among different types of solar radiation, UVB is the most cytotoxic and mutagenic waveband and it is able to induce skin cancer by mutation of epidermal cells.[12] Although it has a low level of skin penetration, it can readily affect macromolecules in the epidermal layer, thus altering cellular functions via DNA damage and generation of reactive oxygen species (ROS).[13] It is also known to up-regulate gene expression through intracellular signal transduction pathways, which may contribute to developing skin cancer at the tumor promotion stage.[14]

Nowadays, diagnostic tools showed that one over three cases of cancer is a skin cancer.[15]Ultraviolet light A (UVA) and UVB radiation has proven to produce DNA damage directly and/or indirectly through DNA adducts or ROS generation which promote tumor formation through different pathways.[16] However, the phototoxic effect of UVA radiation is much lower than

UVB radiation, because UVA is efficient at generating reactive oxygen species that can damage DNA via indirect photosensitizing reactions and such DNA, which causes molecular rearrangements forming the specific photoproducts, directly absorbs UVB.DNA bases directly absorb incident photons within a narrow (290–400 nm) wavelength range. It is not a chromophore for UVA radiation, however, it could be damaged by a photosensitization reaction initiated through absorption of UVA by an unidentified chromophore.[14,17] High levels of ROS can cause damage to the cell structure, nucleic acids, membrane lipids and proteins.[18]They also damage purine and pyrimidine bases of the DNA molecule, thus leading to mutation.[19Some scientific studies show that organic UV filters contain a chromophore that is conjugated by the π-electron system. Therefore, an increase of the conjugation, until certain limits, shifts the absorption maximum to higher wavelengths, and enhances the molecule's ability to absorb UV radiation.[20] In addition, heterocyclic ring systems have emerged as powerful scaffolds for many biological activities.[21]

Heterocycles are organic compounds that have a ring structure containing heteroatoms in addition to carbon such as sulfur, oxygen or nitrogen. Heterocycles, play an important role in biochemical processes because the side groups of the most typical and essential constituents of living cells, DNA and RNA, are based on aromatic heterocycles.[22] A multi-target approach, which include not only a better balance between activity and side effects but also a reduction of costs and time of development to reach the market, offers several benefits.[23] When considering the concept of a multi-target approach it is possible to think about it as dualistic. A multi-target drug can be designed to act on different levels of the same pathways. In this context, we have recently discovered a novel series of polyphenols endowed with interesting antitumor, antifungal and antioxidant activities[24] and benzimidazole polyphenols endowed with potent antioxidant activities but devoid of significant UV filtering activity (Figure 1).[25]

Figure 1

This discovery prompted us do deepen our knowledge on the mechanistic aspect of this class of molecules thus starting a structure-activity relationship study using phenyl-benzimidazole sulfonic acid (**PBSA**) and disodium phenyl dibenzimidazole tetrasulfonate (**DPDT**) as model commercial filters. It has to be stressed that, beside the above stated activities, benzimidazole is a very interesting class of biologically active molecules, often endowed with important properties. Purine is the most widely distributed N-heterocycle in nature, constituting of a pyrimidine ring fused with an imidazole ring synthesized for the first time in 1899 by the German chemist Emil Fischer.[26] As stated above, compounds with an imidazole ring system have many pharmacological properties and can play an important rolein biochemical processes.[27] Two of the five bases in nucleic acids, adenine and guanine, are purines. In DNA, these bases form hydrogen bonds with their complementary pyrimidines, thymine and cytosine, respectively. The pyrimidine ring in purines can be considered as a bio-isoster of a phenyl ring and thus a good candidate for our structure-activity study.Purine bases modified in the 6-position and their derivatives and analogues possess a wide variety of biological properties such as antitumor, antitubercular and fungicidal effects.[28] It was reported that some substituted purines are good antioxidant compounds that can prevent ROS-induced DNA damage.[29]

Thus, the present study describes the synthesis of different 6,8-disubstituted purines bearing an OH-group at position 6 and phenol or polyphenol at position 8 in order to yield novel photoprotectors endowed with UV-filtering and antioxidant properties. In order to explore the

possibility of a multi-target approach, we have taken into account that mycosis and tumors are commonly associated with inflammation. It is easy to understand the relevance and reasoning behind our research towards multi-target molecules that are able to act on both pathologies.

2. Results and Discussion

2.1. Chemistry

Chemistry of heterocyclic compounds is one of the leading lines of investigations in the organic chemistry. Various procedures have been developed for the synthesis of poly-substituted purines.[30,31] However, some derivatives with aryl or hydroxy-aryl substituents at position 8 are more difficult to obtain.[32]

After having evaluated the synthetic procedures described in the literature, we finally turned our attention to simple and effective procedures also suitable for industrial scale-up. The target compounds were prepared following the synthetic pathway described in Scheme 1. The intermediate 4-amino-5-arylamidopyrimidine **4** and N-(4-amino-6-hydroxypyrimidin-5-yl)-4-methoxybenzamide**5** were prepared according to the literature method,[33] by an acylation reaction between6-hydroxy-4,5-diaminopyrimidine hemisulfate**1** and respectively benzoyl chloride **2**orp-methoxybenzoyl chloride **3** in sodium hydroxide 1 N at room temperature. These 5-acylamino compounds are subsequently dehydro-cyclized to the purines**6**, **7, 10, 11, 12, 14.** Substitutions on the C-6 positions of the pyrimidine ring had influence on cyclization, depending on the ring closure method.

For the dehydro-cyclization we first used phosphoryl chloride but in this case we obtained several competing products: **4, 5, 6, 7, 10, 11, 12, 14** (Scheme 1). This competition reaction is due to the presence of the –OH group in the C-6 position. Using the phosphoric acid we also obtained simultaneous cyclization but not 6-chloropurine. Finally, heating 4-amino-5-arylamidopyrimidine to about 130-150°C without solvent, affording selectively compounds **6** and **7**.

Preparation of 8-mono and poly-phenol-purine

Like several heterocyclic compounds, the new class of compounds was synthesized through free-solvent reaction and the principle is based on the melting point's difference. In one step we directly obtained the expected compounds. Condensation reaction of 6-hydroxy-4,5-diaminopyrimidine hemisulfate **1** or 4,5-diaminopyrimidine **15** with the corresponding mono- and poly-benzaldehyde by heating under free solvent condition gave products **19, 20, 21, 22, 26, 28, 29, 31, 33** (Table1). These products were purified by crystallization using appropriate solvents.

In the synthesis of various purines we usually performed the combination of 4,5-diaminopyrimidines, which may also have other substituents (i.e. amino, hydroxyl, thiol) at position 2 and/or 6, with suitable acid derivatives.[33] Wilson and others[34,35] have demonstrated that the acylation of poly-aminopyrimidines occurs on the 5-amino group, whereas other amino groups can also be acylated but only under more drastic condition.[35]The 5-acylamino compound is subsequently cyclized to the purine. Substitutions on the C-2 and C-6 positions of the pyrimidine ring had little or no influence on cyclization, depending on thering closure method. In literature it has been shown that the cyclization is performed using phosphoryl chloride or bromide.[36]

Scheme 1: Synthesis of compounds **4** and **5**. Reagents and conditions: a) NaOH; b) heat, 150°C,48h; c) $POCl_3$; d) H_3PO_4.

Table 1: Synthesis of some 8- substituted purines through free solvent reactions.

2.2. Biological Evaluation

2.2.1.Calculation of the in vitro SPF

As by the definition, art. 2, EU-regulation 1223/2009, a UV-filter "means substances which are exclusively or mainly intended to protect the skin against certain UV radiation by absorbing, reflecting or scattering UV radiation".Since its introduction in 1974 the SPF has been used as a number related to the filter compositions present in the sunscreens, to define the hours of allowed exposition. It should be taken into account that the traditional and only officially accepted method is the in vivo method of determination of SPF (i.e. FDA, United States; former COLIPA, European Union). All of these involve 10 to 20 human volunteers of both sexes, with appropriate skin types. The in vivo method is always accompanied by in vitro measurement during explorative phases. The broadly applied in vitro method is the well-known Diffey-Robson approach.[37] It is a spectrophotometrically-based measurement of transmission. Its determination was developed using the SPF as a relative index. The SPF was calculated using the following equation:

Equation 1.

Where d(λ) is 1 nm, A(λ) represents the erythema action spectrum, E(λ) represents the sun's radiation power, MPF is the inverse of the transmission (1/T) at a given wavelength and MPF(λ) represents how much radiation is absorbed and the ability of the skin to be damaged.

Therefore, we synthesized new candidates for innovative sunscreens and determined their theoretical SPF, the Critical Wavelength Value (λ_c) and the UVA/UVB ratio.

The theoretical SPF, λ_c and UVA/UVB ratio values are obtained by an SPF calculator software[38] as preliminary data.

Some of the synthesized molecules exhibited significant photoprotection, considering they are a single UV-filter substance (Table 2).

The electronic transition responsible for the absorption spectra of substituted purines, depends on the HOMO-LUMO energy gap. As reported in previous studies[39,40],substituted purines are characterized by π-π* and n- π*; these transitions that are centered generally in the UV region between 220 and 300 nm. [41] The presence of different substituents may cause a red shift or a blue shift in the transition and may generate novel bands. The appearance of novel fine structures reflects both the possibility that the system will assume new conformations, whether electronic transitions take place between the different vibrational energy levels possible for each electronic state.

The -OH group in C6 of purine is reported to have a band of absorption at 240 nm.[41] The substitution of R2 with an aromatic group gives a bathochromic effect for all the components proposed and novel transitions appear as consequence of a highly conjugated system.

The -H group in C6 has a major influence in the band-shift: comparing **7** and **33**, the spectrum presents a red-shift of the transition and higher energy transition disappears; while **26**and **28**, the two higher energy transitions of **26** disappear, but on the contrary the transition band at lower energy has a blu-shift in **28**.The presence in C2 of an electron donor group, such as -OH, show positive mesomeric effect when it releases electrons to the rest of the molecule by delocalization.

Also the position of the functional group in the phenyl ring influence the number and the wavelength of the bands. Comparing **19**, **20** and **21**, the *ortho* and *para* substitutions have an higher resonance effect respect to the *meta* position. The same behavior is registered for **26** and **27**. The mesomeric effect reflects on a red-shift of the transition and a finest structure of bands. The combination of the inductive effect of several -OH groups on the molecule gives a red-shift that indicates a lower energy level of the ground state and novel conformation seem to be possible as reason of the appearance of novel bands on the spectrum.

Comparing each other **21** and **22**, and **26** and **28**, the major influence on the wavelength and the structure of the band depends on C2 substituent. The contribution given in the inductive and mesomeric effect makes this position crucial in the definition of the energy asset of the entire molecules.

Table 2: Filtering activity of 8-substituted purines versus two commercial UV filters (DPDT and PBSA).

The order of SPF is the following:**21≤20, 27, 28, 29<26, 31, DPDT, 7, 19< PBSA<<33<22.** The UV-filtering activities for compounds **22** and **33** is remarkable as compared to the others but also with some commercial UV filters (i.e. **PBSA** and **DPDT** taken as reference compounds). Any other changes decreased filtering activity. This demonstrates that substitution of a phenyl sulphonic acid by a pyrimidine is compatible and even improves UV filtering capabilities as compared to the model **PBSA**.

Of interest that compound **21** had a lower value in comparison with**7**(methoxy-substitution at the 8-phenyl ring), indicating that the *p*-methoxy is better as compared with hydroxyl. Compounds **29** and **28** have two OH's in position 3,5 and 2,5, respectively, they had a lower value of SPF, being in agreement with the fact that solar filters based on phenyl moieties are *ortho/para* substituted.[42] However, compounds **19** and **31** had the same value of protection in spite of the presence of *p*-OH and *m*-OCH_3, *p*-OH on the aromatic group, respectively. But compound **27** was less active than **31**. Compounds **27**, **26** and **29** featured two OH's on the aromatic moiety, on different position, and also a 6-OH on the purine ring and their activity order was **26**, **29** and **27**.In this case we may advance the hypothesis that, having an OH group at position 6 increases the compound's activity.

In conclusion, compounds endowed with the best SPF (**22** and **33**) were those unsubstituted at the pyrimidine ring and featured by a methoxy or hydroxyl moiety in *para* position. Any other modification decreased activity.

Another parameter important for UV protection (UVA) is the critical wavelength λ_c, classified by the United States Food and Drug Administration into five numerical categories, as follows: 0 ($\lambda_c < 325$ nm), 1($325 \leq \lambda_c \leq 335$), 2 ($335 \leq \lambda_c \leq 350$), 3 ($350 \leq \lambda_c < 370$) and 4 ($\lambda_c \geq 370$).[43] According to this classification, compounds **21**, **26**, **28** and **29** were rated as '4' while compounds **7, 19, 22, 27, 31** and **33** were rated as '2' and compounds **20**was rated as '1'. Springsteen et al.[44] devised another classification affirming that the broad-spectrum sunscreen product should have a λ_C value greater than 370 nm. Therefore, the sole interesting compounds were **21**, **26**, **29** and **28**. The UVA/UVB absorbance ratio is also an important parameter to assess the wide range of the absorbance through the entire UV range. Following the latest EU recommendation, this ratio should be at least 1/3. In this regard, almost all compounds, beside **22** and **33**, followed this indication, with the best compounds being **26**=**29**>**28**.

Finally, UVA-PF, determined following ISO-24443 guidelines ranked the compounds with the most suitable UVA filter properties as **DPDT>26**>**29**>**28**=**33**>**21**=**19, 22.**

Taken together, these compounds were designed using **PBSA** as model compound. Compounds **26** and **29** fulfill 3 requisites out of 4, and thus can be defined as the best candidates being provided with both UVB and, although less efficient, also UVA protection. However, considering only UVB absorbance **22** and **33** were really good candidates for further studies. Thus, the possible use strongly depends on the final application, only UVB or a wider range. An interesting final consideration is that the lack of the OH-group at the purine ring strongly increases UVB protection but reduces UVA filtering capabilities.

In order to confirm the in vitro data, the in vivo SPF capability of the most interesting compounds will be examined in depth in further studies on sunscreen formulations.

2.2.2. Radical scavenging Activity

All the synthesized purines were evaluated for their in vitro antioxidant activity by DPPH and FRAP assays. The results are expressed respectively in IC_{50} value (μg/ml)and μmol TE/g, and summarized in Table 2. Antioxidant activity varies with substitution of number and position of the OH on the phenyl group. The hydroxyl group at position 6 of the purine ring is very important: when not present the activity considerably decreases. A compound with two hydroxyl groups on the phenyl ring and a hydroxyl on C-6 of the purine ring showed the highest potency in the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay (**26, 27, 7**), and the best result is obtained with**27**, while compounds with only one OH on the phenyl ring were moderately potent (**19, 20, 21**). In agreement with these results we could confirm that the feature responsible of the increase in antioxidant activity is the presence of an additional OH-group on the phenyl ring. Of interest, when the OH-group of the phenyl ring of **21** is substituted by a methoxy (compound **7**) activity increased but, however, when the same replacement is applied at position 4 activity decreased (i.e. **27**vs**31**). This is difficult to explain but certainly related to the presence of the OH-group in the *para* position. The poor antioxidant capacity of **22, 31, 33** confirms the importance of the OH-group at the C-6 of the purine ring. The mono phenolic compounds **19, 21** have the OH- at the *ortho* and *para* position and are more active. The *para* position is favored because the *ortho* OH moiety can establish a hydrogen bond between the nitrogen of the purine ring and the phenolic-OH. This hypothesis is confirmed by the result obtained with **26, 27.** In both DPPH and FRAP assays, compounds with methoxy moieties in the *para* position of the aromatic ring (compound **7**) give better results, comparable to that of molecules with a hydroxyl at the same position (compound **21**). Considering

FRAP assay, the molecule with a better antioxidant profile is **27,** bearing two hydroxyl groups in positions 3 and 4 of the phenyl ring. Finally it was impossible to evaluate compound **29** because its insolubility in the test conditions.

Table 3. Antioxidant activity of the compounds.

Because the parent benzymidazoles are known for their interesting antifungal and antitumor activity, synthesized compounds were also evaluated in such assays.

2.2.3. Antifungal activity.

Because of the possible application to the dermatologic field, synthesized compounds were investigated for their antifungal activities against six dermatophytes responsible for the most common dermatomycoses: *Microsporum gypseum*, *Arthroderma cajetani*, *Trichophyton mentagrophytes*, *Epidermophyton floccosum*, *Trichophyton tonsurans* and *Microsporum canis*. Inhibition of the dermatophytes was evaluated daily by measuring the colony diameter at each disk (Table 4). All the tested compounds showed inhibitory activities toward the six species of fungi. Compounds **20** and **21** at the concentration of 100 µg/ml were moderately active against *A. cajetani*, *T. mentagrophytes* and *M. canis*. Compound **33** at 100 µg/ml displayed good activity against *T. mentagrophytes*, *T. tonsurans* and *M. canis*. Compounds **28** and **31** showed good activity against *E. floccosum*.

Compound concentrations should deserve special attention because of the poor solubility in the medium for many of the compounds. Indeed **7, 19, 21, 26, 27, 28** and **29** at a concentration of 100 µg/ml plunged into the plates, therefore only the next lower concentration could be taken into consideration.

2.2.4. Antiproliferative activity

Antiproliferative activities were determined for five cancerous cell lines: murine leukemia L1210, human CD_4^+ T-lymphoma CEM, human cervix carcinoma HeLa, human pancreatic Mia Paca-2 and human endothelial HMEC-1 cells (Table 5). Compound **7**displayed good cytostatic activity against all the assayed cell lines, thus, $–OCH_3$ in *para* position of the phenyl group seems important for the antiproliferative activity. Compounds **22**, **26**, **27**, **31** and **33** showed good inhibitory activity onHMEC-1. However, compound**27** is also active against L1210. Compound **28** showed a lower activity against all the assayed cell lines, likely due to the lack of –OH in position 6 of the purine. None of the compounds were cytotoxic against primary human embryonic lung fibroblast cell cultures (Table 5).

Table 4. Percent growth inhibition of dermatophytes treated with the substituted purine derivatives at 20 or 100 μg/ml. Each value is the mean of three measurements.

Table 5. Inhibitory effects of substituted purine derivatives on the proliferation of L1210, CEM, HeLa, Mia Paca-2 and HMEC-1 cells.

3. Conclusion

A novel series of 6,8-disubstituted purines were synthesized by a condensation reaction between pyrimidine and benzaldehyde derivatives. Their structure was determined by the IR and UV spectroscopy, ^{1}H and ^{13}C NMR and mass spectroscopy. These compounds were evaluated for their antioxidant, UV-filter, antifungal and antiproliferative activities with the aim to discover multifunctional molecules. Among these compounds **26, 27**and **7** showed the highest antioxidant capacity followed by **21**. The most potent in the DPPH assay was compound **26** and using FRAP, the most potent was **27**. From the results of the UV filter activity, it can be concluded that the four new derivatives **21**, **26**, **28** and **29** were potential broad-spectrum UVA filters while **22** and **33** were suitable candidates as potent UVB filters, being more effective than the commercial **PBSA** used as reference filter compound. Compound **26** was endowed with both antioxidant and wide range UV-filter activity, meaning that the presence of the hydroxyl groups in position two and five of the phenyl ring is very important to display these two concomitant properties. Regarding the antiproliferative activity, compound **7**displayed good activity against L1210, CEM, HeLa, Mia-Paca-2 and HMEC-1 cells. Taken together the best multifunctional molecule was **26**, which well performed as wide range UV filter, antioxidant and antiproliferative activity. Also **21** is of interest for future development having both antifungal and UVA protective activity. Thus, we confirmed that this class of heterocycles is endowed with very interesting concomitant biological activity, that might be useful to develop novel multifunctional molecules endowed with inhibitory activity in several important health-related domains (i.e. skin diseases) and suitable of further investigations.

Finally, these data are already very promising for practical use, because our findings confirm the possibility to design multifunctional molecules. As an example compound **26** might have possible application as a drug candidate in the development of UV radiation-protective molecules in case of skin neoplastic diseases such are the common actinic hyperkeratosis or melanoma. Further studies are currently ongoing to verify this hypothesis.

4. Experimental protocols

4.1. General

Reagents and solvents were purchased from Sigma-Aldrich and Carlo ErbaReagenti (Italia). The TLC layers are Macherey-Nagel Poligram SIL G/UV254 0.20 mm; to visualize spots, we are used 1% solution of $KMnO_4$, solution of $FeCl_3$ (1% in H_2O) and UV light. Chromatographic purifications were run over silica gel Macherey-Nagel 60M 230400 mesh. ^{1}H-NMR and ^{13}C-NMR were registered withVarian spectrometers at 300 MHz and 400 MHz at room temperature. Chemical shifts are measured with respect to tetramethylsilane and relative to residual solvent peaks as an

internal standard set to D_2O, $(CD_3)_2SO$ and CD_3OD in the following manner: chemical shift (multiplicity, coupling constants, integer value). Signal multiplicity is shortened in the following manner: s for singlet; d for doublet; t for triplet; q for quartet; br for broad signal; m for multiplet; dd for double doublet. The molecular weights of the compounds were determined by ESI (Micromass ZMD 2000), and the values are expressed as [MH]+. UV spectrophotometric analyses were carried out on a UV-VIS spectrophotometer (Shimadzu UV-2600).

4.2. Synthesis

4.2.1. General procedure for 4-Amino-6-hydroxy-5-arylamidopyrimidines.

The mixture of 0.26 g of 6-hydroxy-4,5-diaminopyrimidine hemisulfate and 2 molar equiv. of 1 N NaOH was stirred at 0°C for 10 min. and an equimolar quantity of acyl chloride was then added drop by drop. The mixture was stirred vigorously for an additional 0.5 hour at room temperature. The reaction mixture was then acidified,with 2 N HCl, to pH 5 at 0°C. After cooling at 4-8° for 1 hour, the precipitate was filtered and washed with cold water and ether.

4.2.2. Synthesis of N-(4-amino-6-hydroxypyrimidin-5-yl)benzamide (4)

This compound was prepared by acetylation of 6-hydroxy-4,5-diaminopyrimidine hemisulfate with benzoyl chloride in NaOH solution, according to the general procedure. The obtained yellow product was purified by recrystallization from H_2O/CH_3OH:2/1 (93% yield).

IR (KBr) cm^{-1}:3350-2408 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1688,08-1450 (N-C, C=C, N=C of aromatic and purine ring)

1H NMR (DMSO): δ (ppm) 7.60 (dd, 2H, 2CH) 7.72 (dd, 1H, CH); 7.89 (s, 1H, CH); 8.10 (d, 2H, 2CH); 5.83 (br s, 1H, NH);

ESI-MS (m/z): 231.32 $[M+H]^+$, $C_{11}H_{10}N_4O_2$

4.2.3. Synthesis of N-(4-amino-6-hydroxypyrimidin-5-yl)-4-methoxybenzamide (5)

The obtained yellow solid was washed with DCM and then recrystallized from H_2O/MeOH: 2/1 (95% yield).

IR (KBr) cm^{-1}:3352-2410 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1675,08-1433 (N-C, C=C, N=C of aromatic and purine ring)

1H NMR (DMSO): δ (ppm) 7.60 (dd, 2H, 2CH) 7.72 (dd, 1H, CH); 7.89 (s, 1H, CH); 8.10 (d, 2H, 2CH); 5.83 (br s, 1H, NH);

ESI-MS (m/z): 261.22 $[M+H]^+$, $C_{12}H_{12}N_4O_3$

4.2.4. General procedure to obtain the 8-substituted-7H-purin-6-ol

In a round-bottomed-flask, 0.52 g (1.48 mmol) of 4.5-diamino-6-hydroxypyrimidine hemisulfate salt and 1.48mmol of the corresponding benzaldehyde were mixed. Under free solvent and vigorous stirring, the mixture was heat using oil bath for 6 h. Caution should be made to the high temperature, the round-bottomed-flask was equipped with a condenser. During this time, the temperature was increased by small intervals until the maximum desired temperature value is reached (200°C).The residue was then cooled at room temperature, dissolved in HCl 2N, concentrated with vacuum. Purification was effected by column chromatography or re-crystallization from an appropriate solvent to afford the desired products.

4.2.4.1. Synthesis of 8-(4-methoxyphenyl)-7H-purin-6-ol (7)

The residue was washed with H_2O, the solution was filtered and solid part was washed again with MeOH to obtain 0.29 g of yellow solid **7** with a yield of 81%. Mp> 250 (°C)dec.

IR (KBr) cm^{-1}: 3400-2250 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1682,60 (N_3-C_4), 1603.47 (C_4=C_5), 1520.05 (N_7=C_8or C_8=N_9), 1500,27(C=C-Arom), 1296.12 (C_6-O), 1243.46 (C-O-Ar), 1187.37 (N_1-C_2).

^{1}H-NMR (400MHz, D_2O) δ: 8.30 (1H, s), 8.00(2H, d),7.02 (2H, d), 3.90 (3H, s).

^{13}C-NMR (400 MHz, D_2O) δ: 163.11 ($\underline{C}_4$=C_5), 161.67(C_8=N), 159.53 (C_{para} -O), 146.27 (C_2=N), 145.93 (C_6-O), 129.45 (C_{orto}-Ar), 128.55 (C_4=$\underline{C}_5$), 117.90 (C_8-$\underline{C}$-Ar), 114.98 (C_{meta}-Ar), 54.94 (CH_3-O).

ESI-MS (m/z): 243.15 $[M+H]^+$, $C_{12}H_{10}N_4O_2$

4.2.4.2. Synthesis of 8-(2-hydroxyphenyl)-7H-purin-6-ol (19).

Recrystallization with AcOEt/ MeOH, 53.49% yield, grey solid. Mp260-265 (°C)dec.

IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1688.46 (N_3-C_4), 1604.51 (C_4=C_5), 1572.55 (N_7=C_8or C_8=N_9), 1503.47(C=C-Arom), 1294.94 (C_6-O), 1240.69 (O-Ar), 1187.99 (N_1-C_2).

^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.02 (1H, s), 7.98(2H, d),6.80 (2H, d).

^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar).

ESI-MS (m/z): 229.03 $[M+H]^+$, $C_{11}H_8N_4O_2$.

4.2.4.3. Synthesis of 8-(2-hydroxyphenyl)-7H-purin-6-ol (20).

Recrystallization with AcOEt/ MeOH, 78% yield, grey solid. Mp> 240 (°C)dec.

IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1689.69 (N_3-C_4), 1604.41 (C_4=C_5), 1575.65 (N_7=C_8or C_8=N_9), 1503.60 (C=C-Arom), 1294.76 (C_6-O), 1242.14 (O-Ar), 1188.19 (N_1-C_2);

^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.18 (1H, s), 7.46(1H, d), 7.44 (1H, s), 7.06 (2H, m)

^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar).

ESI-MS (m/z): 229.05 $[M+H]^+$, $C_{11}H_8N_4O_2$.

4.2.4.4. Synthesis of 8-(4-hydroxyphenyl)-7H-purin-6-ol (21)

Recrystallization with AcOEt/ MeOH, 73% yield, grey solid. Mp 278-281 (°C)dec.

IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1688,08 (N_3-C_4), 1604.56 (C_4=C_5), 1560.65 (N_7=C_8or C_8=N_9), 1505,68(C=C-Arom), 1365.84 (C_6-O), 1240.30 (O-Ar), 1186.95 (N_1-C_2).

^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.02 (1H, s), 7.98(2H, d),6.80 (2H, d).

^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar).

ESI-MS (m/z): 229.18 $[M+H]^+$, $C_{11}H_8N_4O_2$.

4.2.4.5. Synthesis of 8-(4-hydroxyphenyl)-7H-purine (22).

Recrystallization with H_2O/ DCM, 60% yield, brown solid.Mp> 280 (°C)dec.

IR (KBr) cm^{-1}: 3400-2250 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1682,67 (N_3-C_4), 1604.85 (C_4=C_5), 1560.05 (N_7=C_8or C_8=N_9), 1504.81(C=C-Arom), 1294.93 (C_6-O), 1242.99 (C-O-Ar), 1187.33 (N_1-C_2)
^{1}H-NMR (400MHz, DMSO-d_6) δ: 9.00 (1H, s), 8.90(1H, s),7.90 (2H, d), 6.92 (2H, d)
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 162.01 ($\underline{C}_4$=C_5), 160.98 (C_8=N), 158.89 (C_{para} -O), 146.92 (C_2=N), 134.83 (C_6), 129.89 (C_{orto}-Ar), 128.42 (C_4=$\underline{C}_5$), 117.12 (C_8-$\underline{C}$-Ar), 115.76 (C_{meta}-Ar).
ESI-MS (m/z): 213.31 $[M+H]^+$,$C_{11}H_8N_4O$

4.2.4.6. Synthesis of 8-(2,5-dihydroxyphenyl)-7H-purin-6-ol (26).
Recrystallization with AcOEt /MeOH,75% yield, brown solid. Mp240-245 (°C)dec.
IR (KBr) cm^{-1}: 3400-2250 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1687.42 (N_3-C_4), 1604.42 (C_4=C_5), 1582.25 (N_7=C_8or C_8=N_9), 1503.68(C=C-Arom), 1294.82 (C_6-O), 1242.95 (C-O-Ar), 1187.95 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 9.07 (1H, s), 8.06(1H, d), 6.82 (2H, s).
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar), 55.8 (CH_3-O).
ESI-MS (m/z): 245.39 $[M+H]^+$,$C_{11}H_8N_4O_3$

4.2.4.7. Synthesis of 8-(3,4-dihydroxyphenyl)-7H-purin-6-ol (27).
Recrystallization with AcOEt/ MeOH, and DCM, 75 % yield, grey solid. Mp253-260 (°C)dec.
IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1689.46 (N_3-C_4), 1604.83 (C_4=C_5), 1504,93 (C=C-Arom), 1294.84 (C_6-O), 1243.42 (O-Ar), 1187.21 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.05 (1H, s), 7.59(2H, d), 6.88 (2H, d).
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar), 55.8 (CH_3-O).
ESI-MS (m/z): 245.38 $[M+H]^+$, $C_{11}H_8N_4O_3$

4.2.4.8. Synthesis of 8-(2,5-dihydroxyphenyl)-7H-purine (28).
Chromatography with AcOEt/ MeOH, 2/5, 51% yield, grey solid.Mp>250 (°C)dec.
IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1687.36 (N_3-C_4), 1604.87 (C_4=C_5), 1504.51 (C=C-Arom), 1294.87 (C_6-O), 1236.31 (O-Ar), 1188.44 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 9.07 (1H, s), 8.06(1H, d), 6.82 (2H, s).
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar), 55.8 (CH_3-O).
ESI-MS (m/z): 229.16 $[M+H]^+$, $C_{11}H_8N_4O_2$.

4.2.4.9. Synthesis of 8-(3,5-dihydroxyphenyl)-7H-purin-6-ol (29).
Chromatography with AcOEt/ MeOH, 1/5, 54% yield, grey solid. Mp285-300 (°C)dec.
IR (KBr) cm^{-1}: 3300-2305(N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1688.75 (N_3-C_4), 1604.49 (C_4=C_5), 1502.71 (C=C-Arom), 1294.87 (C_6-O), 1241.44 (O-Ar), 1188.12 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.15 (1H, s), 6. 53(2H, s), 6.03 (1H, s).

^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar), 55.8 (CH_3-O).
ESI-MS (m/z): 245.08 $[M+H]^+$, $C_{11}H_8N_4O_3$.

4.2.4.10. Synthesis of 8-(3-hydroxy-4-methoxyphenyl)-7H-purin-6-ol (31).
Recrystallization with AcOEt / MeOH and EtO_2/DCM, 83% yield, grey solid. Mp180-185 (°C)dec.
IR (KBr) cm^{-1}: 3400-2250 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1688.08 (N_3-C_4), 1604.48 (C_4=C_5), 1720.15 (N_7=C_8or C_8=N_9), 1503.77 (C=C-Arom), 1294.74 (C_6-O), 1241.63 (C-O-Ar), 1188.22 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.25 (1H, s), 7.50(1H, d),7.20 (1H, s), 6.85 (1H, d) 3.90 (3H, s).
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 171.9 (C_6-O), 161.7(C_8=N), 160.6 (Ar-C-O), 152.3 ($\underline{C}_4$=C_5), 148.9 (C_2=N), 137.7 (C_4=$\underline{C}_5$), 127.0 (C_8-$\underline{C}$-Ar), 130.3 (C_{orto}-Ar), 114.8 (C_{meta}-Ar), 55.8 (CH_3-O).
ESI-MS (m/z): 259.37 $[M+H]^+$, $C_{12}H_{10}N_4O_3$

4.2.4.11. Synthesis of 8-(4-methoxyphenyl)-7H-purine (33).
Recrystallization with H_2O/ DCM. 80% yield, amorphous solid.
IR (KBr) cm^{-1}: 3400-2250 (N-H, O-H, C_2-H of purine ring, O-H C-H of aromatic ring), 1689.46 (N_3-C_4), 1604.83 (C_4=C_5), 1504.93 (C=C-Arom), 1294.84 (C_6-O), 1243.42 (C-O-Ar), 1187.21 (N_1-C_2).
^{1}H-NMR (400MHz, DMSO-d_6) δ: 8.96 (1H, s), 8.94(2H, d),7.90 (2H, d),6.92 (2H, d), 3.90 (3H, s).
^{13}C-NMR (400 MHz, DMSO-d_6) δ: 163.11 ($\underline{C}_4$=C_5), 161.67(C_8=N), 159.53 (C_{para} -O), 146.27 (C_2=N), 133.93 (C_6), 130.00 (C_{orto}-Ar), 128.55 (C_4=$\underline{C}_5$), 117.90 (C_8-$\underline{C}$-Ar), 114.61 (C_{meta}-Ar), 55.46 (CH_3-O).
ESI-MS (m/z): 227.18 $[M+H]^+$, $C_{12}H_{10}N_4O$

4.3. Antioxidant Activity Assays

4.3.1. Free radical Scavenging Activity on DPPH.

The DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging assay is widely used to evaluate antioxidant capacity in a short time[45] and it is ideal for phenolic compounds. This assay measures the hydrogen donation ability of antioxidant to convert stable DPPH free radical in 1,1-diphenyl-2-picrylhydrazyl, which is accompanied by a change of color from deep-violet to light –yellow. This ability can be evaluated by measuring the decrease in absorbance at 517 nm of the solution after the radical reaction with products to be tested. The percent was calculated using the following equation:

$$\text{DPPH radical-scavenging capacity (\%)} = [1 - (A_1 - A_2) / A_0] \times 100\%$$

where A_0 was the absorbance of the control (without sample), A_1 was the absorbance in the presence of the sample, and A_2 was the absorbance without DPPH. The IC_{50}values, defined as the amount of antioxidant necessary to decrease the initial $DPPH^{\cdot}$ concentration by 50%, were calculated from the results. To a DPPH methanolic solution (1.5 mL) was added 0.750 mL of

compound solution (methanol + DMSO) at different concentration (1, 0.5, 0.25, 0.125 and 0.0625 mg/mL) and the absorbance was measured by a UV-VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, UK) at 517 nm, according to a described procedure.[46] IC_{50} values expressed as nmol/mL were determined by regression analysis of the results obtained at different concentrations of the sample.

4.3.2. Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP method measures the ferric reducing ability of plasma and it is based on the reduction of ferric ions (Fe^{III}) to ferrous ions (Fe^{II}) under acid conditions in the presence of TPTZ (2,4,6-tripyridyl-striazine).[47] In the presence of an antioxidant, the ferric-tripyridyltriazine (Fe^{III}-TPTZ) complex is reduced to the ferrous (Fe^{II}) form and an intense blue color with an absorption maximum at 593 nm was observed. The analysis reagent was prepared by mixing the following solutions in the reported ratio 10/1/1 (v:v:v) i) 0.1 M acetate buffer pH 3.6, ii) TPTZ 10 mmol/L in 40 mmol/ HCl, iii) ferric chloride 20 mmol/L. To 1.9 mL of reagent were added 0.1 mL of sample proper diluted or solvent when blank was performed. Readings at fixed wavelength of the absorption maximum (593 nm) were done after 30 min, using a UV-VIS spectrophotometer; it was evaluated as the absorbance increase of the sample solution against the absorbance of the blank reaction as parameter to calculate the antioxidant activity. The antioxidant activity is given as Trolox activity since this standard was used to perform the calibration curves.

4.4. Evaluation of filtering parameters

Sun protection factor evaluation was performed by means of a UV-VIS spectrophotometer SHIMADZU UV-2600 240 V. Test compounds were dissolved in a dimethylsulfoxide /methanol (1:10) solution at the concentration of 0,0015 (±0,0005) % and the absorbance was measured between 250-600 nm using a 1 cm quartz cell at intervals of one 1 nm using a UV-Vis Spectrophotometer (SHIMADZU UV-2600 240 V). The absorbance data were transformed in transmission data applying the Equation 2.

Equation 2

The SPF was calculated by applying Equation 1 previously described.[48]

4.5. Microorganisms

The dermatophytes used were *Arthroderma cajetanum* (Netherlands), CBS 495.70 strain; *Epidermophyton floccosum* var. floccosum (Netherlands) CBS 358.93 strain; *Trichophyton violaceum* (Africa) CBS 459.61 strain; *Trichophytontonsurans* (Netherlands) CBS 483.76 strain; *Trichophyton mentagrophytes* (Netherlands) CBS 160.66 strain; *Microsporum canis* (Iran) CBS 131110strain; *Trichophyton rubrum* (Turkey) CBS 132252 strain; *Microsporum gypseum* (Iran) CBS 130948 strain; the phytopatogens used were *Botrytis cinerea* (Netherlands) CBS 1798.71 strain; *Pyricularia oryzae* (unknown) CBS 433.70 strain; obtained from the CBS-KNAW Fungal Biodiversity Centre, an institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, The Netherlands. All dermatophytes were maintained at 4°C as agar slants on Sabouraud dextrose agar (SDA; Sigma-Aldrich SRL, Milano, Italy).

4.6. Antifungal activity

Antifungal activity was determined as follows. Each test substance was dissolved in dimethylsulfoxide (DMSO) and aseptically mixed with sterile medium (SDA) at 45°C to concentrations of 20 and 100 μg/ml. The DMSO concentration in the final solution was adjusted to 0.1%. Controls were also prepared with equivalent concentrations (0.1% v/v) of DMSO. The cultures were obtained by transplanting mycelium disks (10 mm diameters) from a single mother culture in the stationary phase. They were incubated at 26 ± 1°C on SDA on thin sheets of cellophane until the logarithmic growth phase. Subsequently, the cultures were transferred to Petri plates with media containing 20 or 100 μg/ml of the single substance and incubated under growth conditions. The fungal growth was evaluated daily by measuring the colony diameters (in millimeters) during seven days from the treatment onset. The percent inhibition of growth was determined as the average of three different experiments.

4.7. Antiproliferative activity

4.7.1. Cell lines

Human cervical carcinoma (HeLa), human CD_4^+T-lymphoblast (CEM), mouse leukemia (L1210) cellsand human embryonic lung (HEL) cell fibroblasts were obtained from ATCC (Middlesex, UK). Human pancreatic carcinoma (Mia-Paca 2) cells were kindly provided by Prof. Anna Karlsson (Karolinska Institute, Stockholm, Sweden). HMEC-1 cells were obtained from CDC, Atlanta, GA, USA. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Gibso), 0.01M Hepes (Gibco) and 1 mM sodium pyruvate (Gibco) in a humidified 5% CO_2 incubator at 37°C.

4.7.2. Cell proliferation

Suspension (L1210 and CEM cells) were seeded in 96-well microtiter plates at 60,000 cells/well in the presence of different concentrations of the compounds. The cells were allowed to proliferate for 48 h or 72 h, respectively, and then counted in a Coulter counter. The 50% inhibitory concentration (IC_{50}) was defined as the compound concentration required to reduce cell proliferation by 50%. HeLa, Mia-Paca2, HMEC-1 and HEL cells were seeded in 96-well plates at 15,000 cells/well in the presence of different concentrations of the compounds. After 4 days (5 days for HMEC-1) of incubation, the cells were trypsinized and counted in a Coulter counter. The HEL cell cultures were inspected microscopically for altered cell morphology to determine the MIC of the test compounds.

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4.7.3. IC_{50} Determination

The compounds were dissolved in DMSO at 20 mM (stock solution) and kept in the refrigerator until use. Then, compound dilutions were made in cell culture medium, and serial compound concentrations (5-fold dilutions) were tested starting at 250 μM as the highest concentration. The DMSO concentration, present at the highest compound concentration was 1.25%, that is at a concentration that did not markedly affect the tumour cell proliferation. The IC_{50} values were calculated using following formula:

C1 -[50- N1%/N2% - N1%] - (C1 - C2)

wherein C1 is the compound concentration that inhibits cell proliferation more than 50%; C2 is the compound concentration that inhibits cell proliferation less than 50%; N1% represents the cell number (in percent of control in the absence of compound) obtained in the presence of C1 and N2% represents the cell number (in percent of control in the absence of compound) obtained in the presence of C2.

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