Parallel Synthesis and Cytotoxicity Evaluation of a Polyamine−Quinone Conjugates Library

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A library of 24 derivatives designed by combining two natural products-derived fragments was prepared and tested to determine their anticancer potential in HT29 colon cancer cells. All library members inhibit cell proliferation as measured by MTT mitochondrial functional assay, with IC_{50} values in the 1−100 μM range. Entry **1b** caused apoptotic EGFR-mediated intracellular signaling. Thus, polyamino−quinones emerged as readily accessible and easily diversified scaffolds for anticancer lead discovery.

Introduction

Proliferative disorders are expected to be the major cause of death in the 21st century. To identify new chemical entities for a more effective treatment of cancer, drug designers can follow many strategies, but the crucial decision is always the selection of a suitable starting point from the vast chemical space.[1] In this respect, natural products can be viewed as evolved privileged structures[2] and biologically prevalidated leads, in other words, as molecules that have probably evolved evolutionarily to exert highly specialized functions. Therefore, they represent the biologically relevant and prevalidated fractions of chemical space explored by nature so far.[3,4] Indeed, natural products continue to provide an important source of new anticancer leads, with about 74% of anticancer compounds being either natural or natural product-derived products.[5] Natural molecules have potent antitumor properties and have provided multiple active compounds in the past.[6] The chemical diversity, the structural complexity, and the inherent biologic activity of natural products make them ideal candidates for new therapeutics.[7−10] Natural products not only disrupt aberrant signaling pathways leading to cancer (i.e., proliferation, deregulation of apoptosis, angiogenesis, invasion, and metastasis) but also synergize with chemotherapy and radiotherapy.

All of these considerations prompted us to design a combinatorial library of potential anticancer agents by using as molecular building blocks two natural scaffolds with great anticancer potential such as quinones and polyamines.

Naphthoquinones[11] and anthraquinones[12,13] have a wide spectrum of anticancer activity: they covalently bind to and intercalate into DNA, inhibit DNA replication and RNA transcription, act as DNA topoisomerase II poisons, produce oxidative stress, and induce DNA breakage and chromosomal aberrations.[14,15] Moreover, quinones, such as emodin[16] and shikonin,[17] are able to modulate receptor tyrosine kinase (RTK)[a] activity, an innovative molecular target for successful mechanism-based cancer therapies. RTKs are mediators of cellular proliferation, and their mutations are often associated with hyperplasia and tumor development. Targeted inhibition of RTKs has therefore become an attractive therapeutic strategy in the treatment of cancer and has resulted in several small-molecule RTK inhibitors recently approved for clinical use worldwide.[18]

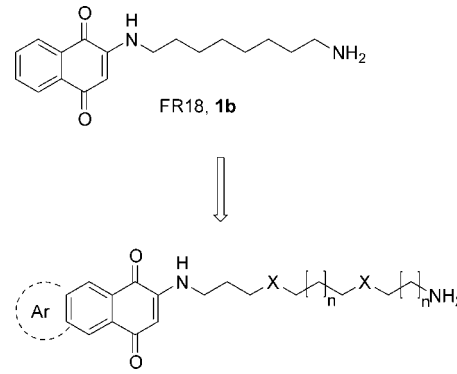

Figure 1. Early hit **1b** and general formula of the designed library compounds.

Polyamines have long been associated with cell growth and cancer: their important roles in angiogenesis and invasion have recently been identified, and now polyamine analogues are being developed as anticancer drugs to target polyamine metabolic enzymes and inhibit polyamine biosynthesis.[19,20] Besides modulating cell growth, some analogues also play an important role in cell apoptosis by regulating gene expression.[21]

Therefore, following the concept that a library built from selected natural products should yield better hits at a higher rate than classical compound libraries, we planned to design a small library combining aromatic quinone scaffolds with polyamines, varying from diamines to triamines and tetramines, as potential new chemical entities against cancer (Figure 1).

We recently reported preliminary biological results showing that a polyaminoquinone derivative designed following this rationale, FR18 (**1b**) (Figure 1), shows cytotoxic activity in a human colon carcinoma cell line by activating apoptosis.[22] This encouraged us to perform SAR studies, synthesizing a focused polyaminoquinone library. Herein, to our knowledge, we disclose the first parallel synthesis with a polymer-assisted purification of a polyamine−quinone conjugates library together with their antiproliferative properties.

Results and Discussion

In designing the library, we decided to combine three quinone building blocks with eight polyamines, generating a library of 24 compounds (Chart 1). To introduce diversity into the quinone subunit, we selected the naphthoquinone (**1**) of **1b**, and two higher quinone homologues, anthraquinone (**2**) and naphtacenequinone

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[a] Abbreviations: EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; PAT, polyamine transporter; RTK, receptor tyrosine kinase; SS, serum starved.

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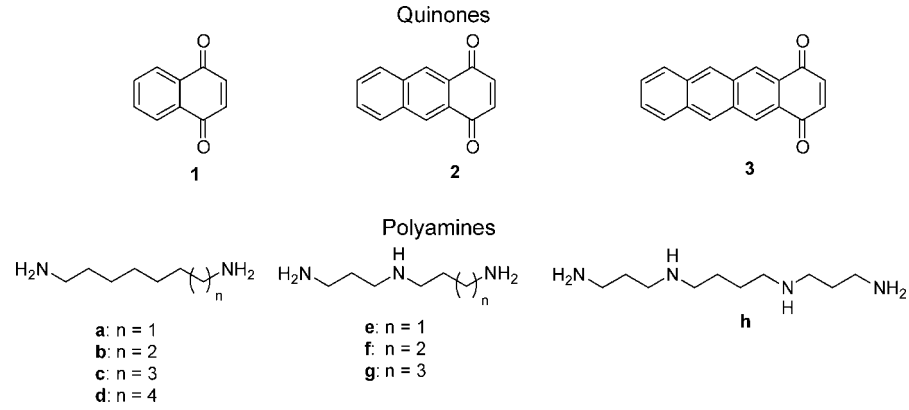
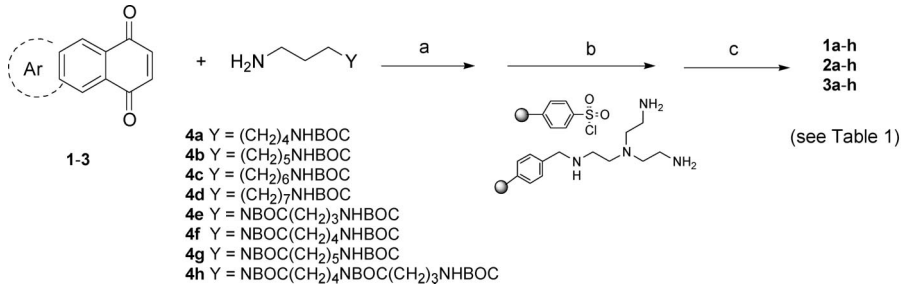
Chart 1. Building Blocks for the Designed Library



Scheme 1[a]



[a] Reaction conditions: (a) CH_2Cl_2, air, rt, 6 h; (b) CH_2Cl_2, rt, overnight; (c) CF_3COOH, CH_2Cl_2, rt, 4 h.

Table 1. IC_{50} Values for the Cellular Proliferation Inhibition (MTT Assay) of Library Compounds in HT29 Colon Cancer Cells

Entry	*IC50* (μM)	Entry	*IC50* (μM)	Entry	*IC50* (μM)
1a	62.4± 15.05	**2a**	9.2 ± 1.05	**3a**	7.0 ± 5.55
1b	57.7 ± 8.7	**2b**	14.6 ± 4.55	**3b**	15.5 ±2.45
1c	24.4 ± 3.9	**2c**	6.0 ± 2.55	**3c**	20.1 ± 2.6
1d	16.1 ± 4.7	**2d**	.1 ± 1.15	**3d**	103.5 ± 12.4
1e	10.4 ± 2.85	**2e**	16.0± 5.05	**3e**	21.9±1.23
1f	19.8 ± 5.35	**2f**	25.8 ± 9.7	**3f**	26.1± 7.95
1g	25.6 ± 7.15	**2g**	31.4± 14.2	**3g**	6.6 ± 0.95
1h	5.3 ± 2.7	**2h**	17.1± 3.05	**3h**	15.5 ± 5.95

(**3**). In contrast to the first two quinones, the latter has been less investigated as a molecular scaffold endowed with anticancer properties.

Natural polyamines spermidine (**f**) and spermine (**h**) and six synthetic variants (**a**−**e**, **g**) were chosen to explore the role of the polyamine backbone in relation to the quinone component. In fact, the selected polyamines differ in chain length and number of nitrogen atoms within the carbon chains, thus allowing study of the length and number of cationic sites required for optimal activity.

For the synthesis of the library, we initially focused on a solid-phase approach. Attachment of the polyamine to a solid support via one terminal amine function, allowing subsequent reaction of the other with the appropriate quinone, appeared to be advantageous because of a sort of selective asymmetric protection. Unfortunately,

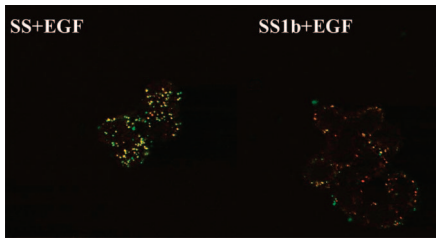


Figure 2. Confocal laser scanning micrographs of EGF and EGFR in control and **1b** treated cells. EGF fluorescence is shown in green, EGFR staining in red. White pixels represent the colocalization of the two proteins.

the bond between the amino group and the quinone is very labile, as observed in the synthesis of natural products having this functionality.[23] Thus, we decided to construct a library via solution-phase synthesis and examined the possible routes to incorporating a properly protected polyamine into the 2-position of the quinone.

In accordance with the widely reported reactivity of the quinone system in the 1,4-Michael addition, amino-quinones are usually synthesized from primary amines and quinone itself. The unsaturated nature of the quinone is maintained, using either excess quinone or other oxidizing agents such as atmospheric oxygen. The same transformation can also be achieved through nucleophilic substitution chemistry, starting from quinones bearing good leaving groups such as methoxy groups and halogen atoms.[24]

We explored both approaches and found the first more practical in terms of lower number of formed byproduct and greater commercial accessibility of starting materials. Moreover, the latter was reported to require very drastic conditions.[25] However, to obtain a quantitative conversion, the Michael addition should be carried out using an excess of starting amine. We circumvented this drawback using scavenger resins to facilitate the purification process. Amine excess was then scavenged through the use of polymer-supported sulfonyl chloride and trisamine to yield the library compounds, which, after deprotection, possessed adequate purity for direct biological screening. The developed synthetic route is shown in Scheme 1. More specifically, quinones **1**−**3** reacted with protected polyamines **4a**−**h** in a ratio 1:2 for 6 h. Upon completion of the reaction, resins were added to sequester the remaining reactants. Simple filtration and evaporation of the solvents afforded highly purified products. Deprotection of the BOC groups was accomplished by treatment with trifluoroacetic acid in CH_2Cl_2 to obtain the target compounds as trifluoroacetate salts. The overall yields ranged from 47% to 96% based on mass recovery, with an average of 90% and 75% for compounds of series **1** and **2**, respectively. Notably, the yield dropped (55%) for compounds of series **3** due to the very low solubility of naphtacene derivatives. The HPLC purities for **1a**−**h** and **2a**−**h** ranged from 80% to 99% with an average purity level of 94%. Because of their low solubility, it was impracticable to obtain HPLC analysis of compound of series **3**. Gratifyingly, the biological screening data obtained on some library compounds were highly reproducible and consistent with those obtained for the same compounds made by traditional chemistry.

A prerequisite for the chosen strategy was an easy access to large amounts of partially protected polyamines (**4a**−**h**), having only one free primary amine unmasked. The ready removal in mildly acidic conditions, compatible with the stability of the amino−quinone bond, made BOC a superior protecting group with respect to the others for the purpose of this library. Thus, norspermine (**e**),[26] spermidine (**f**),[27] and spermine (**h**)[28] derivatives were synthesized by following reported procedures, whereas protected homospermidine (**g**) was prepared using the acrylonitrile approach (see Scheme 1 in Supporting Information).[29] Michael addition of *N*-BOC-diaminopentane (**5**) to acrylonitrile provided in 91% yield derivative **6**, which was subsequently transformed into the BOC-protected nitrile **7**. Raney-nickel reduction of **7** gave the desired product **4g**. Regarding diamines **4a**−**d**, we adapted a protocol developed by Krapcho.[30] The success of this method relies on the use of a large excess of the diamine with respect to the BOC_2O (8:1). Amine excess was easily removed by washing the crude reaction repeatedly with warm water, affording the desired monoprotected diamines with yields ranging from 70% to 84% (Scheme 2 in Supporting Information).

To achieve an initial picture of the anticancer properties of the library members, **1a**−**h**, **2a**−**h**, and **3a**−**h** were evaluated for their antiproliferative activity in HT29 cells, a human colon adenocarcinoma cell line. The cells were treated with the test compounds at concentrations ranging from 0.1 to 400 μM for 24 h, and cell viability was assessed through measurements of mitochondrial dehydrogenase activities using the MTT method. To compare the antiproliferative potencies in terms of concentrations required for 50% of the effect, all analogues were tested in dose-dependent manner, and the obtained IC_{50} values are listed in Table 1. The results revealed that all compounds were capable of inducing a decrease of cell growth. No major differences were observed within the library, all members possessing similar IC_{50} values in the micromolar range. Decanediamine derivatives (series **d**) were less uniform in their behavior, with derivative **3d** being the less cytotoxic of the series and anthracene analogue **2d** standing out as the most efficacious compound (IC_{50} = 4.1 μM). Conversely, natural polyamine analogues belonging to the spermidine (**f**) and spermine (**h**) series showed only slight differences in the IC_{50} values. This might suggest that in these compounds the polyamine moiety is more relevant than the quinone one for biological activity. This finding might be rationalized by the fact that both are natural polyamines, in principle more capable of recognizing a biological counterpart. Of the remaining compounds, five of them possessed an IC_{50} between 1 and 10 μM, the others between 10 and 100 μM.

As mentioned above, we have previously shown that in HT29 **1b** is active at concentrations as low as 10 nM and inhibits EGF binding to its receptor (EGFR). Furthermore, flow cytometric analysis showed that **1b** induces an arrest in the S phase of the cell cycle and apoptotic death.[22] From this peculiar pharmacological profile, **1b** seems to have possible therapeutic applications in colon cancer. Because of the structural proximity, it was assumed that the synthesized compounds would similarly interfere with the binding mechanisms of EGF to its receptor. Fluorescence confocal microscopy was used to detect specific binding of **1b** to EGFR, and nine representative compounds were properly selected for further investigation using this technique. We designated **2b** and **3b** as close analogues of **1b**, and series **e** and **h** as prototypical examples of a triamine and a tetramine.

For confocal microscopy, HT29 were grown on glass coverslips for 24 h and serum starved (SS) for an additional 24 h before being exposed to a 1 μM concentration of each compound for 30 min. Untreated as well as series **1** compounds treated cells were then stimulated with fluorescent EGF, while samples treated with series **2** and **3** compounds were stimulated with nonfluorescent EGF because **2** and **3** compounds show intrinsic fluorescence. The analysis reveals that in EGF stimulated cells, EGF and EGFR form a granular complex localized in the plasma membrane, whose presence greatly diminishes in **1b** treated, EGF stimulated HT29. This indicates that concomitant treatment with **1b** and EGF prevents the formation of the complex (Figure 2). We have proposed that **1b** binds competitively to the extracellular domain of the receptor, and this interaction does not prevent the RTK activation and causes

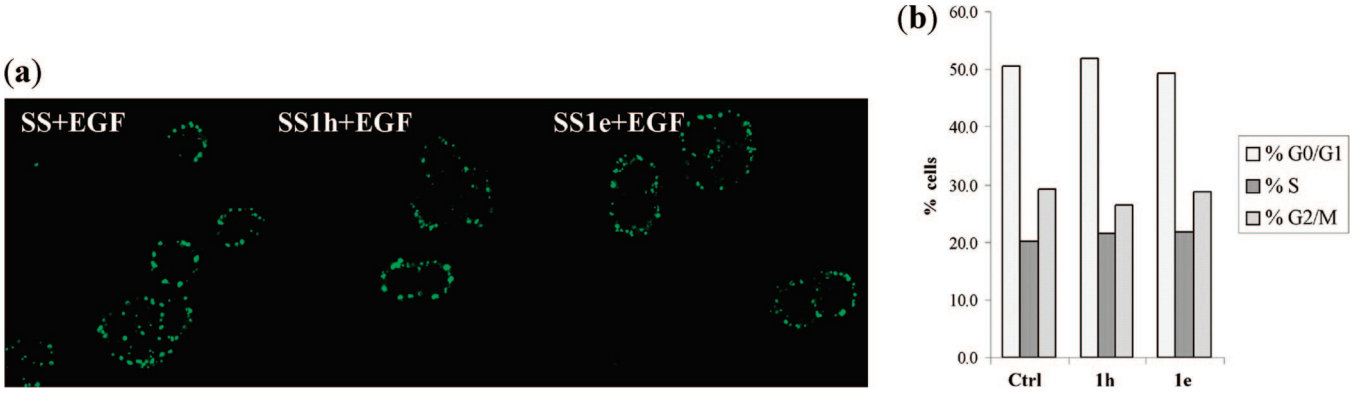


Figure 3. Confocal laser scanning micrographs (a) and cell cycle analysis (b) of control and **1e** and **1h** treated HT29 cells. (a) Cells were SS, treated with **1e** or **1h** and stimulated with fluorescent EGF (green). (b) Flow cytometric analysis of cells treated with **1e** or **1h** for 24 h.

apoptotic, EGFR-mediated intracellular signaling.[22] Surprisingly enough, confocal microscopy analysis showed that EGF binding to EGFR is not perturbed by **1e** and **1h** administration, as indicated by the equal intensity of EGF fluorescent signal in untreated and treated samples (Figure 3a). In addition, 24 h treatment with **1e** and **1h** does not affect HT29 proliferation, as shown by flow cytometric cell cycle analysis (Figure 3b). For class **2** and **3** compounds, fluorescence confocal analysis has been performed by taking advantage of their fluorescent properties and staining EGFR in indirect immunofluorescence (see Figure 1 in Supporting Information). Again, **2b**, **2e**, and **2h** and **3b**, **3e**, and **3h** do not colocalize with EGFR, suggesting that 1 μM administration of each of them does not interfere with the binding of EGF to EGFR. Accordingly, no effect on cell proliferation has been observed for these compounds. In conclusion, these studies indicate that the series **1** has no further experimental use, but for **1b**, given its proven ability to interfere with EGF/EGFR interactions. Series **2** and **3**, on the other hand, promise a successful use in cellular internalization and transport studies, given their fluorescent properties.

In this respect, cancer cells frequently demonstrate an elevated polyamine uptake activity, importing high level of exogenous polyamines through the polyamine transporter system (PAT).[31] Thus, it is a promising strategy for anticancer therapies to exploit toxic compounds that selectively can gain entry into the cell via PAT.[32] Notably, the conjugates of this study appear particularly intriguing, because they may utilize PAT to enter cells where they may exert cytotoxic effects through a pleiotropic mechanism of action. This suggests a potential for specific targeting of tumors that have enhanced PAT activity over their normal cell counterparts.

In this regard, notwithstanding the enormous financial investment and many efforts, effective drugs are not available yet for anticancer therapy, which is an urgent need and unmet goal. A possible reason might be that cancer, like other diseases such as neurodegenerative syndromes, diabetes, and cardiovascular diseases, is a disorder that involves multiple pathogenic factors.[33,34] Thus, a drug able to hit a single target, albeit with outstanding affinity and selectivity, may not be clinically effective for the treatment of a multifactorial disease.[35] Nowadays, besides therapies based on cocktails or combinations of drugs with different therapeutic mechanisms, to overcome this problem, a new design strategy is emerging based on the assumption that a single molecule is able to simultaneously modulate multiple targets relevant for a given complex pathology.[36,37]

All these considerations clearly establish the polyamine−quinone conjugates as promising structures for the development of new antitumor agents that, besides the modulation of EGFR, might have potential multiple anticancer activities (DNA intercalation, formation of covalent DNA adducts, topoisomerase poisoning, and free radical effects) in addition to a successful drug delivery via PAT. Therefore, compounds incorporating these natural product fragments are being synthesized and will be reported in due course.

Experimental Section

General Information. Chemical reagents and scavenger resins were purchased from Sigma Aldrich, Fluka, and Lancaster (Italy). Dichloromethane was distilled from calcium chloride. The library was synthesized using a Syncore Reactor manual synthesizer with evaporation system (BUCHI Italia) using a 24-well liquid-phase reaction block. Filtration was performed using 4 mL disposable polypropylene syringe Extract-Clean filter columns (Alltech). TLC were performed on 0.20 mm silica gel 60 F254 plates (Merck, Germany). Nuclear magnetic resonance spectra (NMR) were recorded at 200 and 300 MHz on Varian VXR 200 and 300 spectrometers and reported in parts per million. Low-resolution mass spectra EI-MS and ESI-MS were recorded on a VG707EH-F and Waters ZQ 4000 apparatus, respectively.

General Procedure for the Synthesis of Polyamine−Quinone Conjugate Library Members. Quinones **1**−**3** (0.3 mmol), dry CH_2Cl_2 (10 mL), and protected polyamines **4a**−**h** (0.6 mmol) were added in parallel in 24 vials (30 mL). The mixtures were shaken at 200 rpm in open vials at room temperature for 6 h. After the reaction was completed, polymer-supported sulfonyl chloride (100−200 mesh, loading 1.5−2.0 mmol/g) (440 mg) and polymer-supported trisamine (PS-trisNH, 200−400 mesh, loading 1.1 mmol/g) (270 mg) were added to each reactor to scavenge the excess of unreacted polyamine. After shaking overnight, the suspensions were filtered to remove the resins and the solutions recovered were evaporated. The crude products were dissolved in CH_2Cl_2 (4 mL), and CF_3COOH (4 mL) was added. After shaking for 4 h, the solvent was evaporated under reduced pressure, and the solids obtained were triturated with ether to afford the trifluoroacetate salts in acceptable yields (see Supporting Information).

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Supporting Information Available: Characterization data, a table of HPLC analysis data for target compounds, synthetic procedures for **4a**−**d** and **4g**, biological methods, and confocal laser scanning micrographs and cell cycle analysis for **2b**, **2e**, **2h**, **3b**, **3e**, and **3h**. This material is available free of charge via the Internet at http://pubs.acs.org.

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