Pergamon

Bioorganic & Medicinal Chemistry 7 (1999) 1873–1880

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Binding Properties of Photoactivable Biotin-Conjugated Verapamil Derivatives for the Study of P-170 Glycoprotein

Elisabetta Teodori,[a] Dominique Ettori,[b] Arlette Garnier-Suillerot,[b] Fulvio Gualtieri,[a,*] Dina Manetti,[a] Maria Novella Romanelli[a] and Serena Scapecchi[a]

[a]*Dipartimento di Scienze Farmaceutiche, Universita' di Firenze, via G. Capponi 9, 50121 Firenze, Italy*
[b]*Laboratoire de Physicochimie Biomoleculaire et Cellulaire (LPBC, UPRES-A 7033), Université Paris Nord, 74 rue Marcel Cachin, 93017 Bobigny, France*

Received 8 December 1998; accepted 11 March 1999

Abstract—The design and synthesis of two photoactivable biotin-labeled analogues of verapamil (**6** and **7**) is reported. Preliminary evaluation of the biological profile of **6** (EDP 137) and **7** (EDP 141) shows that they have comparable affinities to that of verapamil for P-170, the protein responsible for multidrug resistance (MDR). Since both appear to bind irreversibly to the protein and the presence of biotin in their structure makes them easily detectable by avidin, they promise to be of great help in studying the protein and its mechanism of action.

Introduction

A 170 KDa glycoprotein, known as P-170 (also referred to as Pg-170, GP-170, or Pgp) and a 190 KDa protein called MRP_1 (MDR-related protein) are known to play a critical role in the acquired resistance of cancer to chemotherapy, known as multidrug resistance (MDR).[1–3] Both belong to the super family of the ATP-binding cassette (ABC) of transport proteins[4] and operate as extrusion pumps, even though their mechanisms of action seem different.[5] Analogous systems of drug extrusion have been found in microorganisms[6,7] and it is becoming more and more evident that the multidrug efflux systems encountered in prokaryotic cells are very similar to those observed in eukaryotic cells.[8] Moreover, in addition to their role in cancer cell resistance, these proteins seem to have a physiological function as well, since they are expressed also in non cancer tissues: P-170 has been reported to be an important element of the blood–brain barrier (BBB),[9] of the intestinal epithelium[10] and, together with other ATP-dependent transporters, in the hepatobiliary secretion.[11]

The best known of these proteins is P-170 which is a membrane-associated 1280 aa protein composed of 12 putative transmembrane domains and two nucleoside binding sites[12] whose structure at 2.5 nm has been recently reported.[13] P-170 is overexpressed in cancer cells showing MDR. It is known that cells exhibiting MDR accumulate a lower intracellular concentration of drug and that this effect is associated with accelerated efflux of anti tumor agents by P-glycoprotein which pumps out the drug reducing its cellular concentration below cytotoxic levels.[1–3] In fact, while being present in many tissues, P-170 generally shows high levels in malignant cells and increasing amounts of it are found in patients with increasing resistance to chemotherapy, so that a significant correlation between P-170 expression and MDR, both in vitro and in vivo, can be detected.[14] As a consequence, P-170 has become the target of many studies aimed to identify drugs able to block its extrusive activity and restore the initial response of tumor cells to chemotherapy.[15–17]

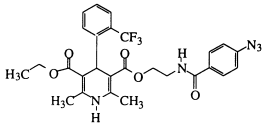
The role played by P-170 in physiology, pathology and pharmacology has stimulated studies aimed to clarify its structure, functions, and mechanism of action. In particular, knowledge of the structure of the binding site of substrates and modulators of the glycoprotein will be invaluable not only for an understanding of how drugs interact with it, but also to the design of better and more specific inhibitors. It has been suggested that P-170 antagonists fall into two groups: those that are transported themselves by the protein and those that are not.[18]

Key words: Verapamil analogues; biotin-labeled compounds; multidrug resistance; *P*-glycoprotein; photoaffinity labeling.
* Corresponding author. Tel.: +39-55-275-7295; fax: +39-55-240776; e-mail: gualtieri@farmfi.scifarm.unifi.it

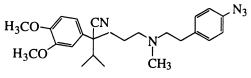
0968-0896/99/$ - see front matter
PII: S0968-0896(99)00104-2

In general however, the mechanism of action of P-170 and the binding mode of its inhibitors[19] are poorly understood and many things remain to be clarified. Toward this aim, potent and selective ligands of the protein, in particular those that can be labeled and can establish covalent bonds with the molecule, are necessary.

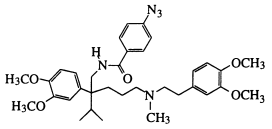
Several compounds that covalently bind to P-170 protein have been synthesized and studied;[20–26] few of them are dihydropyridine like **1**[23,25] and verapamil analogues like **2** and **3**[20,26] but representatives of other chemical classes have also been proposed.[21,22,24] Frequently they are photoactivable molecules, most of them carrying an azido group like azidopine **1**, one of the most used compounds,[27] and the vast majority are labeled with ^3H, or an equivalent radioisotope.



1



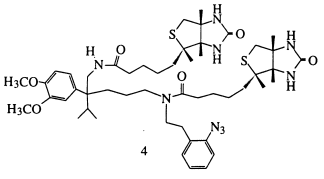
2



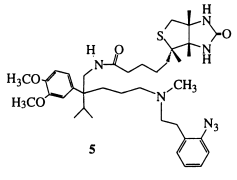
3

Radioisotope labeling of suitable molecules has provided precious tools for the progress of molecular biology, pharmacology and life sciences in general. However, these kinds of probes suffer from some relevant drawbacks such as expensive synthesis, short half-life, safety, and finally, the requirement of special facilities and disposal methods. To overcome these problems, biotin-labeling of suitable molecules represents an alternative approach that has been widely used.[28] In fact, two proteins, avidin (from egg white) and streptavidin (from *Streptomices avidini*), bind with outstanding affinity (Ka$\sim 10^{15}$ M^{-1}) to biotin and this can be exploited to construct sensitive methods[29] for the detection of biotin-labeled molecules.

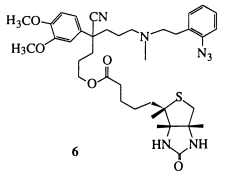
In this paper we report on the design, synthesis and preliminary evaluation of the affinity for P-170 of a few photoactivable biotin-labeled analogues of verapamil (**4**–**7**) which were designed following our previous work on compounds able to covalently bind to P-170.[26]



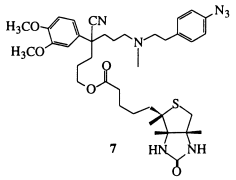
4



5



6



7

First, we designed a molecule where the biotin moiety was linked through an amide bond to the quaternary carbon atom of the *N*-desmethyl derivative of verapamil. As a matter of fact, while the nitrile group is considered critical for calcium activity of verapamil,[30] its role in P-170 protein binding is less well established. In any case, an analogous *p*-azido-benzoylderivative of nitrile-reduced verapamil (**3**) is an efficient photoaffinity labeler of P-170.[31] Moreover, *N*-desmethylderivatives are, in our experience, less potent as calcium antagonists but more potent as MDR reverters.[32] However, this molecule could not be obtained since the biotinyl group also binds to the nitrogen to give compound **4**. As a consequence, we turned to the synthesis of the corresponding *N*-methyl derivative **5**.

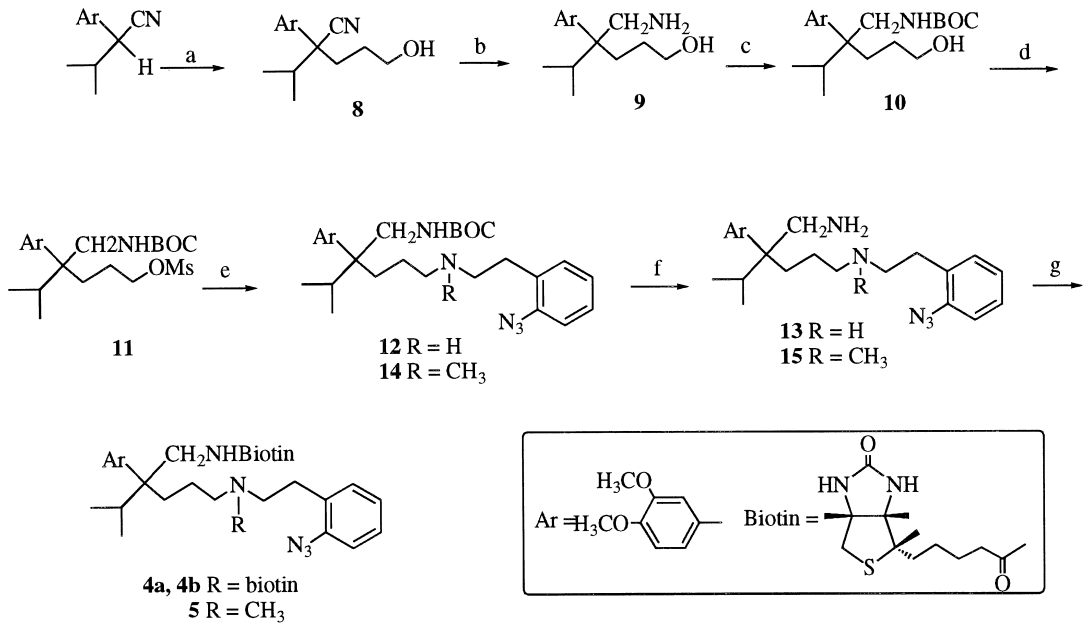
At the same time, to maintain the nitrile group, we designed compound **6** and **7** where the biotin moiety is linked, through a suitable spacer, to the same quaternary carbon atom of verapamil. Since the *ortho* azido group of **6** could suffer for steric hindrance problems to give irreversible binding to the P-170 protein, we also synthesized compound **7** which carries the azido group in a less hindered position of the benzene ring.

Chemistry

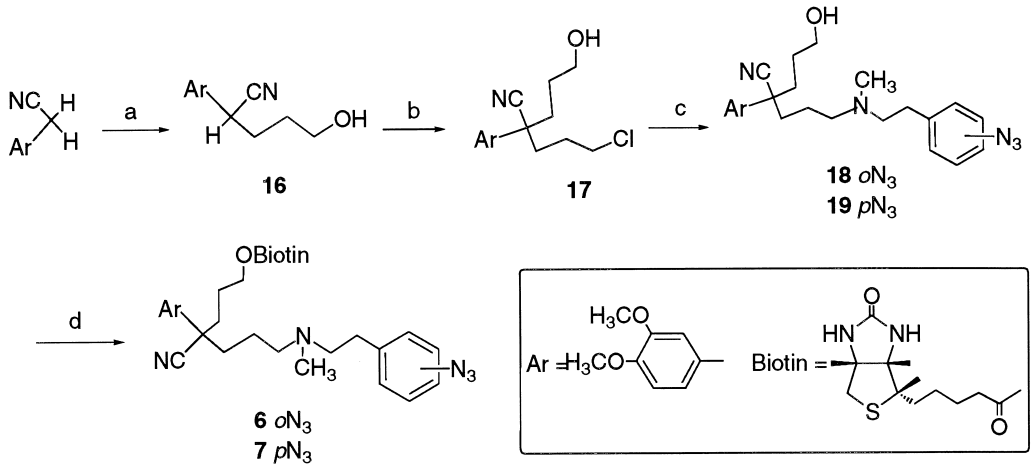
The synthetic pathway chosen to synthesize compounds **4** and **5** is shown in Scheme 1. 2-(3,4-Dimethoxyphenyl)-2-isopropylacetonitrile[33] was alkylated with 3-bromopropanol to give **8** which in turn was reduced with AlH_3. After protection of the amine group with di-*tert*-butyl dicarbonate, the resulting compound **10** was transformed into the methansulfonyl derivative **11**. The compound was smoothly transformed in the amines **12** and **14** and, by cleavage of the protecting group, in the amines **13** and **15**. At this point, while **15** gave the biotinyl compound **5**, the corresponding compound **13** reacted with two molecules of succinimido biotinate[34] to give two compounds of identical molecular weight (measured by FAB) separated by column chromatography. Most likely, the compounds are the diastereoisomers corresponding to the *R* and *S* forms of verapamil, that, unlike the diastereoisomers of compounds **5**–**7**, can be separated. However, no further studies to establish their absolute configuration were carried out due to the lack of affinity of both compounds for P-170.

In Scheme 2 the synthesis of compounds **6** and **7** is reported. The homoveratryl acetonitrile was alkylated in two steps to give compound **17**, which was transformed in **18** and **19** by reaction with *N*-methyl-2-(*o*-azidophenyl)ethylamine (**22**) and *N*-methyl-2-(*p*-azidophenyl)ethylamine (**23**) whose synthesis is described in Scheme 3. Finally, reaction with succinimido biotinate gave compounds **6** and **7**.

Compounds **5**, **6** and **7** are diastereomeric mixtures of the *R* and *S* forms of verapamil. However, at this step of the research, no attempt was made to separate the isomers.



Scheme 1. (a) BuLi, $BrCH_2CH_2CH_2OH$; (b) AlH_3; (c) di-*tert*-butyl dicarbonate; (d) methansulfonyl chloride; (e) 2-(*o*-azidophenyl)ethylamine[40] or **22**; (f) HCl; (g) succinimidobiotinate.[34]



Scheme 2. (a) BuLi, $BrCH_2CH_2CH_2OH$; (b) LICKOR (BuLi/*t*-BuOK), $BrCH_2CH_2CH_2Cl$; (c) **22** or **23**; (d) succinimidobiotinate.[34]

Biology

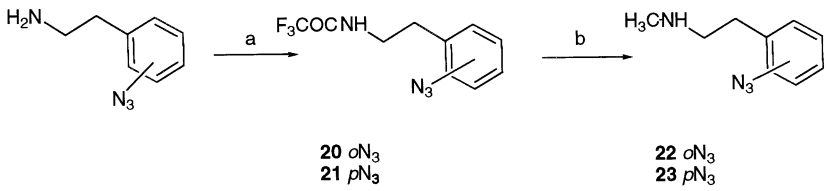
MDR reverting activity

The affinity of the compounds synthesized for P-170 protein was deduced by their ability to revert MDR. This was evaluated on anthracycline-resistant erythroleukemia K 562 cells, measuring the uptake of THP-adriamicin (pirarubicin) by continuous spectrofluorometric monitoring of the decrease of the fluorescence signal of the anthracycline at 590 nm ($\lambda_{ex} = 480$ nm) after incubation with cells. The decrease of fluorescence occurring during incubation with cells is due to quenching after intercalation of antracyclin between the base-pair of DNA. We have previously shown that this methodology allows accurate measurement of the concentration of anthracyclines bound to the nucleus in steady-state, their initial rates of uptake and kinetics of active efflux.[32,35–39]

The overall concentration, C_n, of pirarubicin bound to the nucleus of drug-resistant cells was determined, at the steady-state, in the presence of different concentrations of the drug to be tested. In all cases, C_n increased as the concentration of inhibitor increased, and this can be quantified using the following equation:

$$\alpha = [(C_n)_{Ri} - (C_n)_{Ro}]/[(C_n)_S - (C_n)_{Ro}]$$

where $(C_n)_S$ is the overall concentration of pirarubicin bound to the nucleus of sensitive cells and $(C_n)_{Ro}$ and $(C_n)_{Ri}$ are the overall concentrations of pirarubicin bound to the nucleus of resistant cells, in the absence and presence of a concentration [*i*] of inhibitor, respectively. The value of α represents the fold increase in the nuclear concentration of pirarubicin in the presence of the MDR-reverting agent; α varies between 0 (in the absence of inhibitor) and 1 (when the amount of pirarubicin in



20 *o*N_3
21 *p*N_3

22 *o*N_3
23 *p*N_3

Scheme 3. (a) Trifluoroacetic anhydride; (b) CH_3I, KOH.

resistant cells is the same as in sensitive cells). The potency of the compounds in reverting MDR is expressed by $[i]_{0.5}$ which represents the concentration of the inhibitor that causes a half-maximal increase in cellular pirarubicin ($\alpha = 0.5$), while its efficacy is expresed as α_{max}, the maximum increase that can be obtained in the concentration of pirarubicin in resistant cells with a given inhibitor.

Photolabeling

Irradiation was performed on resistant cells in the presence of the photolabelers, under conditions that did not damage the cells.[26] The level of pirarubicin incorporated into the nucleus after irradiation and accurate washing of unbound photolabeler, compared to that of sensible cells, gives a measure of P-170 functionality and consequently of the irreversible binding.

Results and Discussion

Compounds **4** and **5** did not show affinity for P-170 protein, as is shown by the fact that they do not reverse MDR in anthracycline-resistant erythroleukemia K 562 cells (Table 1). This result seems to suggest that, besides calcium antagonistic activity, the ciano group, at least in this case, is critical also for binding to P-170. This is confirmed by the fact that compounds **6** and **7** do show MDR reverting activity (Table 1), being able to restore the concentration of pirarubicin in resistant cells with identical efficacy with respect to verapamil ($\alpha_{max} = 0.8$ for **6** and **7**; $\alpha_{max} = 0.7$ for verapamil) at a slightly higher concentration ($[i]_{0.5} = 10$ μM for both **6** and **7**; verapamil, $[i]_{0.5} = 1.6$ μM). As a consequence, it can be reasonably accepted that both compounds show affinity for P-170 glycoprotein and can be used to label the protein itself.

Moreover, preliminary irradiation experiments, performed according to the protocol described previously,[26] have shown that both compounds are able to bind irreversibly to the protein, as indicated by the substantial reduction of P-170 protein functionality. In fact, under irradiating conditions that do not damage the cells and after accurate washing of unbound **6** and **7**, the amount of pirarubicin found in the cell nucleus was definitly increased.

Work is in progress to establish the eligibility of **6** and **7** as biotin-labeled photoactivable ligands of P-170 protein; the results will be referred in due time.

Table 1. MDR reverting activity of compounds **4**–**7** compared with verapamil[a]

N	α_{max}	$[i]_{0.5}$(μM) ($\alpha = 0.5$) (M ± SEM)
4	n.a.	n.a.
5	n.a.	n.a.
6	0.8	10 ± 2
7	0.8	10 ± 2
Verapamil	0.7	1.6 ± 0.3

[a] Evaluated on erthroleukemia K 562 cells line (see Experimental). n.a. = not active.

Experimental

Chemistry

All melting points were taken on a Büchi apparatus and are uncorrected. Infrared spectra were recorded with a Perkin–Elmer 681 spectrophotometer in a Nujol mull for solids and neat for liquids. NMR spectra were recorded on a Gemini 200 spectrometer. Chromatographic separations were performed on a silica gel column by gravity chromatography (Kieselgel 40, 0.063–0.200 mm, Merck) or flash chromatography (Kieselgel 40, 0.040–0.063 mm, Merck). Yields are given after purification, unless otherwise stated. Where analyses are indicated by symbols, the analytical results are within ±0.4% of the theoretical values (Table 2). CF-FAB spectrometric measurements in positive ion mode were carried out using a 7070-E mass spectrometer (VG Analitical, Manchester, UK).

Table 2. Elemental analyses

	Calculated			Found		
Compd	C%	H%	N%	C%	H%	N%
4a	60.17	7.24	14.36	60.34	7.41	14.09
4b	60.17	7.24	14.36	60.46	6.95	14.18
5	63.12	7.73	14.73	63.34	7.41	14.36
6	62.00	7.00	14.47	62.26	7.34	14.14
7	62.00	7.00	14.47	62.33	7.29	14.76
8	69.27	8.37	5.05	68.98	8.71	4.89
9	68.28	9.69	4.98	68.45	9.40	5.23
10	66.10	9.26	3.67	66.34	8.96	3.98
11	57.48	8.13	3.05	57.68	8.44	3.33
12	66.24	8.26	13.32	66.56	8.03	13.06
13	67.72	8.31	16.46	67.99	8.68	16.13
14	66.75	8.42	12.98	66.51	8.13	13.22
15	68.29	8.50	15.93	68.44	8.68	16.21
16	76.79	8.45	6.89	76.98	8.61	6.55
17	61.62	7.13	4.49	61.45	7.41	4.61
18	66.48	7.38	15.51	66.23	7.53	15.84
19	66.48	7.38	15.51	66.74	7.09	15.37
22	61.33	6.88	31.80	60.95	7.12	31.56
23	61.33	6.88	31.80	61.54	7.03	31.98

2-(3,4-Dimethoxyphenyl)-5-hydroxy-2-isopropylpentanenitrile (8). 3.5 mL (5.64 mmol) of BuLi (1.6 M in hexane) were added to a solution of 0.8 mL (5.64 mmol) of *i*-Pr_2NH in 10 mL of anhyd THF at 0°C. After 10 min the mixture was cooled to −20°C and 0.5 g (2.28 mmol) of 2-(3,4-dimethoxyphenyl)-2-isopropylacetonitrile[33] were added. Then the mixture was cooled to −50°C and 0.2 mL (2.28 mmol) of 3-bromo-1-propanol was added. The temperature was kept at −50°C for 2 h and then allowed to return to room temperature. The mixture was treated with NH_4Cl and extracted with ether. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by flash cromatography using ethylacetate:cyclohexane (80:20) as eluting system. Title compound **8** (0.5 g, 80% yield) was obtained as a thick oil. IR (neat) ν 3550 (OH), 2240 (CN) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.79 and 0.82 (2d, $J = 6.6$ Hz, 6H, $2CH_3$), 1.50–1.72 (m, 2H, CH and OH), 1.84–2.31 (m, 4H, $2CH_2$), 3.59 (t, $J = 5.6$ Hz, 2H, CH_2O), 3.87 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.81–6.97 (m, 3H, aromatics) ppm. Anal. ($C_{16}H_{23}NO_3$) C, H, N.

5-Amino-4-(3,4-dimethoxyphenyl)-4-isopropyl-1-pentanol (9). To a solution of 0.07 g (1.8 mmol) of $LiAlH_4$ and 0.24 g (1.8 mmol) of $AlCl_3$ in dry ether, 0.5 g (1.8 mmol) of **8**, dissolved in anhyd ethyl ether, were added. The mixture was left at room temperature for 16 h, then 5 mL of H_2O and 2 mL of H_2SO_4 were added and the mixture was extracted with ethyl ether. The aqueous solution was made basic with NaOH and extracted with ethyl ether. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by column cromatography using $CHCl_3$:MeOH (9:1) as eluting system. Title compound **9** (0.38 g, 75% yield) was obtained as a thick oil. IR (neat) ν 3500 (OH), 3400 (NH_2) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.74 and 0.76 (2d, $J = 5.6$ Hz, 6H, $2CH_3$), 1.42–1.58 (m, 1H, CH), 1.78–1.98 (m, 4H, CH_2CH_2), 2.05 (bs, 2H, NH_2), 3.03 and 3.15 (2d, $J_{gem} = 12.4$ Hz, 2H, CH_2NH_2), 3.62 (t, $J = 5.6$ Hz, 2H, CH_2O), 3.85 (s, 6H, $2OCH_3$), 6.81 (s, 3H, aromatics) ppm. Anal. ($C_{16}H_{27}NO_3$) C, H, N.

5-(*t*-Butoxycarbonyl)amino-4-(3,4-dimethoxyphenyl)-4-isopropyl-1-pentanol (10). To a solution of 0.38 g (1.35 mmol) of **9** in 5 mL of THF, 0.3 mL (2.10 mmol) of Et_3N and 0.36 g (1.65 mmol) of di-*tert*-butyl dicarbonate were added. The mixture was left at room temperature for 16 h then 20 mL of ethyl acetate were added and the organic layer washed with water. After drying with Na_2SO_4, the solvent was removed under reduced pressure. Title compound **10** (0.42 g, 81% yield) was obtained as a thick oil. IR (neat) ν 3460 (OH), 3380 (NH), 1740 and 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.76 and 0.78 (2d, $J = 5.6$ Hz, 6H, $2CH_3$), 1.50 (s, 9H, $(CH_3)_3$), 1.58–1.82 (m, 5H, $2CH_2$ and CH), 3.49–3.75 (m, 4H, CH_2O and CH_2N), 3.85 (s, 6H, $2OCH_3$), 6.80 (s, 3H, aromatics) ppm. Anal. ($C_{21}H_{35}NO_5$) C, H, N.

1-[5-(*t*-Butoxycarbonyl)amino-4-(3,4-dimethoxyphenyl)-4-isopropylpentyl] methansulfonate (11). To a solution of 0.42 g (1.10 mmol) of **10** and 0.18 mL (1.29 mmol) of dry Et_3N in 20 mL anhyd ethyl ether cooled to −20°C, a solution of 0.10 mL (1.29 mmol) of methansulfonyl chloride in 3 mL of anhydrous ethyl ether were added. The mixture was left for 1.5 h at −20°C, then filtered and the solvent was removed under reduced pressure. Title compound **11** (0.47 g, 94% yield) was obtained as a thick oil. IR (neat) ν 3400 (NH), 1740 and 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.78 and 0.82 (2d, $J = 6.6$ Hz, 6H, $2CH_3$), 1.41 (s, 9H, $(CH_3)_3$), 1.62–1.92 (m, 5H, $2CH_2$ and CH), 3.05 (s, 3H, CH_3), 3.49–3.75 (m, 2H, CH_2N), 3.88 (s, 6H, $2OCH_3$), 4.22 (t, $J = 5.6$ Hz, 2H, CH_2), 6.72–6.88 (s, 3H, aromatics) ppm. Anal. ($C_{22}H_{37}NO_7S$) C, H, N.

***N*^{1}-*t*-Butoxycarbonyl-2-(3,4-dimethoxyphenyl)-2-isopropyl-*N*^{5}-[2-(*o*-azido-phenethyl)] 1,5-pentyldiamine (12).** 0.40 g (0.87 mmol) of **11** were mixed with 0.28 g (1.74 mmol) of 2-(*o*-azidophenyl)ethylamine[40] with 1 mL of anhydrous CH_3CN. The mixture was heated at 60°C for 7 h. After cooling, $CHCl_3$ was added and the organic layer washed with NaOH 10%. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by column cromatography using $CHCl_3$: MeOH (9:1) as eluting system. Title compound **12** (0.15 g, 32% yield) was obtained as a thick oil. IR (neat) ν 3370 (NH), 2120 (N_3), 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.76 and 0.78 (2d, $J = 7.2$ Hz, 6H, $2CH_3$), 1.39 (s, 9H, $3CH_3$), 1.40–1.58 (m, 1H, CH), 1.71–1.89 (m, 4H, $2CH_2$), 2.62–2.75 (m, 2H, CH_2), 2.84 (bs, 4H, $2CH_2$), 3.41–3.75 (m, 2H, CH_2), 3.85 (s, 6H, $2OCH_3$), 6.78 (s, 3H, aromatics), 6.95–7.32 (m, 4H, aromatics) ppm. Anal. ($C_{29}H_{43}N_5O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-isopropyl-*N*^{5}-[2-(*o*-azido-phenethyl)]-1,5-pentyl-diamine (13). 5 mL of HCl 6 N were added to a solution of 0.15 g (0.28 mmol) of **12** in 1 mL of ethyl acetate. The mixture was left for 12 h at room temperature, then treated with a 10% solution of NaOH, extracted with ethyl acetate and dried with Na_2SO_4. Removal of the solvent gave 0.11 g of **13** as an oil (92% yield). IR (neat) ν 3300–3450 (NH and NH_2), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.72 and 0.78 (2d, $J = 6.8$ Hz, 6H, $2CH_3$), 1.31–1.55 (m, H, CH), 1.74–1.95 (m, 6H, CH_2, NH_2 and CH_2), 2.59–2.73 (m, 2H, CH_2), 2.72–2.85 (m, 4H, $2CH_2$), 2.95 and 3.17 (2d, $J_{gem} = 12.4$ Hz, 2H, CH_2NH_2), 3.85 (s, 6H, $2OCH_3$), 6.79 (s, 3H, aromatics), 7.02-7.36 (m, 4H, aromatics) ppm. Anal. ($C_{24}H_{35}N_5O_2$) C, H, N.

***N*^{1},*N*^{5}-Dibiotinyl-2-(3,4-dimethoxyphenyl)-2-isopropyl-*N*^{5}-[2-(*o*-azidophenethyl)]-1,5-pentyldiamine (4a, 4b).** 0.11 g (0.26 mmol) of **13** and 0.18 g (0.52 mmol) of succinimido biotinate[34] were dissolved in 0.5 mL of anhydrous DMSO. After 12 h at room temperature $CHCl_3$ was added and the mixture was washed with water. After drying with Na_2SO_4 the solvent was removed under reduced pressure and the obtained solid was purified by column chromatography using $CHCl_3$:abs. EtOH: petroleum ether:NH_4OH 30% (340:65:60:8) as eluting system.

The first fraction (R_f 0.58) was **4a** (50 mg, 22% yield). Mp 170–172°C. IR (nujol) ν 3250 (NH), 2120 (N3), 1710 (CO), 1660 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.74

and 0.80 (2d, $J = 6.8$ Hz, 6H, $2CH_3$), 1.29–1.91 (m, 17H, $8CH_2$ and CH), 2.08–2.47 (m, 4H, $2CH_2$), 2.70–3.22 (m, 10H, $4CH_2 + 2CH$), 3.28–3.55 (m, 4H, $2CH_2$), 3.87 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 4.25–4.35 (m, 2H, 2CH), 4.38–4.53 (m, 2H, 2CH), 6.79 (s, 3H, aromatics), 7.02–7.36 (m, 4H, aromatics) ppm. ($M + H^+$) 878. Anal. ($C_{44}H_{63}N_9O_6S_2$) C, H, N.

The second fraction (R_f 0.47) was **4b** (60 mg, 26% yield). Mp 178–180°C. IR (nujol) ν 3250 (NH), 2120 (N_3), 1710 (CO) 1660 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.70 and 0.78 (2d, $J = 6.8$ Hz, 6H, $2CH_3$), 1.28–1.89 (m, 17H, $8CH_2$ and CH), 2.05–2.39 (m, 4H, $2CH_2$), 2.61–3.18 (m, 10H, $4CH_2 + 2CH$), 3.21–3.53 (m, 4H, $2CH_2$), 3.85 (s, 3H, OCH_3), 3.87 (s, 3H, OCH_3), 4.19–4.35 (m, 2H, 2CH), 4.38–4.51 (m, 2H, 2CH), 6.79 (s, 3H, aromatics), 7.02–7.36 (m, 4H, aromatics) ppm. ($M + H^+$) 878. Anal. ($C_{44}H_{63}N_9O_6S_2$) C, H, N.

N^1-*t*-Butoxycarbonyl-2-(3,4-dimethoxyphenyl)-2-isopropyl-N^5-[2-(*o*-azido-phenethyl)]-N^5-methyl-1,5-pentyldiamine (14). Following the procedure described for **12**, starting from **11** (0.32 g, 0.70 mmol) and 0.25 g (1.42 mmol) of **22**, title compound **14** (0.17 g, 45% yield) was obtained as an oil. IR (neat) ν 2120 (N_3), 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.78 and 0.81 (2d, $J = 7.2$ Hz, 6H, $2CH_3$), 1.40 (s, 9H, $3CH_3$), 1.40–1.58 (m, 2H, CH_2), 1.71–1.89 (m, 3H, CH_2 and CH), 2.31 (s, 3H, NCH_3), 2.35–2.85 (m, 6H, $3CH_2$), 3.51–3.82 (m, 2H, CH_2), 3.85 (s, 6H, $2OCH_3$), 6.78 (s, 3H, aromatics), 6.98–7.30 (m, 4H, aromatics) ppm. Anal. ($C_{30}H_{45}N_5O_4$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-isopropyl-N^5-[2-(*o*-azido-phenethyl)]-N^5-methyl-1,5-pentyldiamine (15). Following the procedure described for **13**, starting from **14** (0.15 g, 0.28 mmol). Title compound **15** (0.12 g, 97% yield) was obtained as a thick oil. IR (neat) ν 3450 (NH_2), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.72 and 0.78 (2d, $J = 6.8$ Hz, 6H, $2CH_3$), 1.25–1.65 (m, 3H, CH and NH_2), 1.74–1.98 (m, 4H, $2CH_2$), 2.29 (s, 3H, NCH_3), 2.38–2.69 (m, 4H, $2CH_2$), 2.65–2.78 (m, 2H, CH_2), 2.99 and 3.09 (2d, $J_{gem} = 12.4$ Hz, 2H, CH_2NH_2), 3.85 (s, 6H, $2OCH_3$), 6.80 (s, 3H, aromatics), 6.98–7.28 (m, 4H, aromatics) ppm. Anal. ($C_{25}H_{37}N_5O_2$) C, H, N.

N^1-Biotinyl-2-(3,4-dimethoxyphenyl)-2-isopropyl-N^5-[2-(*o*-azidophenethyl)]-N^5-methyl-1,5-pentyldiamine (5). Following the procedure described for **4**, starting from **15** (0.12 g, 0.27 mmol). Title compound **5** (0.08 g, 45% yield) was obtained as a thick oil. IR (neat) ν 3250 (NH), 2120 (N_3), 1710 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 0.74 and 0.78 (2d, $J = 6.8$ Hz, 6H, $2CH_3$), 1.28–1.89 (m, 11H, $5CH_2$ and CH), 2.06–2.18 (m, 2H, CH_2), 2.30 (s, 3H, NCH_3), 2.35–2.85 (m, 10H, $5CH_2$), 2.98–3.10 (m, 1H, CH), 3.85 (s, 6H, $2OCH_3$), 4.15–4.28 (m, 1H, CH), 4.39–4.48 (m, 1H, CH), 6.80 (s, 3H, aromatics), 7.02–7.28 (m, 4H, aromatics) ppm. ($M + H^+$) 666. Anal. ($C_{35}H_{51}N_7O_4S$) C, H, N. The oily product was transformed into the hydrochloride that was recrystallized from abs ethanol and anhyd ether. Mp 125–127°C.

2-(3,4-Dimethoxyphenyl)-5-hydroxyvaleronitrile (16). Following the procedure described for **5**, starting from (3,4-dimethoxyphenyl)acetonitrile (0.50 g, 2.82 mmol), title compound **16** (0.47 g, 70% yield) was obtained as a thick oil. IR (neat) ν 3500 (OH), 2240 (CN) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.64 (bs, 1H, OH), 1.66–1.82 (m, 2H, CH_2), 1.83–2.08 (m, 2H, CH_2), 3.68 (t, $J = 6.8$ Hz, 2H, CH_2), 3.80 (t, $J = 7.2$ Hz, 1H, CH), 3.87 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.81–6.88 (m, 3H, aromatics) ppm. Anal. ($C_{13}H_{17}NO_3$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-(3-chloropropyl)-5-hydroxy-valeronitrile (17). 0.5 g (2.12 mmol) of **16** were added to a solution of 2.5 mL (4.25 mmol) of BuLi (1.6 M in hexane) and 0.5 g (4.25 mmol) of potassium *tert*-butoxide in 10 mL of dry THF at −70°C. After 1 h, 0.5 mL (2.28 mmol) of 1-bromo-3-chloropropane were added and the mixture was allowed to return to room temperature. The mixture was then treated with NH_4Cl and extracted with ether. After drying with Na_2SO_4, the solvent was removed under reduced pressure and the residue purified by flash cromatography using ethylacetate:cyclohexane (80:20) as eluting system. Title compound **17** (0.29 g, 44% yield) was obtained as a thick oil. IR (neat) ν 3500 (OH), 2240 (CN) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.28–1.80 (m, 4H, $2CH_2$), 1.85 (bs, 1H, OH), 1.87–2.18 (m, 4H, $2CH_2$), 3.45 (t, $J = 5.6$ Hz, 2H, CH_2Cl), 3.58 (t, $J = 5.6$ Hz, 2H, CH_2O), 3.87 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 6.78–6.97 (m, 3H, aromatics) ppm. Anal. ($C_{16}H_{22}ClNO_3$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-[3-(1-hydroxy)propyl]-5-[2-(*o*-azidophenethyl) methylamino]valeronitrile (18). Following the procedure described for **12**, starting from **17** (0.29 g, 0.93 mmol) and **22** (0.17 g, 0.96 mmol), title compound **18** (0.14 g, 33% yield) was obtained as a thick oil. IR (neat) ν 3500 (OH), 2240 (CN), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.21–1.48 (m, 2H, CH_2), 1.56–1.78 (m, 2H, CH_2), 1.88–2.08 (m, 4H, $2CH_2$), 2.22 (s, 3H, NCH_3), 2.35–2.58 (m, 4H, $2CH_2$), 2.63–2.76 (m, 2H, CH_2), 3.58 (t, $J = 5.6$ Hz, 2H, CH_2O), 3.86 and 3.87 (2s, 6H, $2OCH_3$), 6.78–6.99 (m, 3H, aromatics), 7.00–7.28 (m, 4H, aromatics) ppm. Anal. ($C_{25}H_{33}N_5O_3$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-[3-(1-biotin)propylester]-5-[2-(*o*-azidophenethyl) methylamino]valeronitrile (6). Following the procedure described for **4**, starting from **18** (0.07 g, 0.15 mmol), title compound **6** (0.06 g, 60% yield) was obtained as a thick oil. IR (neat) ν 3250 (NH), 2240 (CN), 2120 (N_3), 1740 (CO), 1680 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.21–1.51 (m, 4H, $2CH_2$), 1.53–1.78 (m, 6H, $3CH_2$), 1.81–2.08 (m, 4H, $2CH_2$), 2.22 (s, 3H, NCH_3), 2.35–2.58 (m, 5H, $2CH_2 + CH$), 2.63–2.76 (m, 4H, $2CH_2$), 2.84–2.96 (m, 1H, CH), 3.08–3.19 (m, 1H, CH), 3.87 (s, 6H, $2OCH_3$), 3.94–4.18 (m, 2H, CH_2O), 4.21–4.32 (m, 1H, CH), 4.45–4.51 (m, 1H, CH), 5.42 (bs, 1H, NH), 5.82 (bs, 1H, NH), 6.78–6.99 (m, 3H, aromatics), 7.00–7.28 (m, 4H, aromatics) ppm. Anal. ($C_{35}H_{47}N_7O_5S$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-[3-(1-hydroxy)propyl]-5-[2-(*p*-azidophenethyl) methylamino]valeronitrile (19). Following the procedure described for **12**, starting from **17** (0.33 g, 1.06 mmol) and **23** (0.20 g, 1.13 mmol), title compound **19** (0.18 g, 37% yield) was obtained as a

thick oil. IR (neat) ν 3500 (OH), 2240 (CN), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.21–1.48 (m, 2H, CH_2), 1.52–1.74 (m, 2H, CH_2), 1.83–2.08 (m, 4H, $2CH_2$), 2.18 (s, 3H, NCH_3), 2.35–2.48 (m, 2H, CH_2), 2.43–2.55 (m, 2H, CH_2), 2.60–2.72 (m, 2H, CH_2), 3.58 (t, $J = 5.6$ Hz, 2H, CH_2O), 3.86 and 3.87 (2s, 6H, $2OCH_3$), 6.79–6.98 (m, 5H, aromatics), 7.08–7.17 (m, 2H, aromatics) ppm. Anal. ($C_{25}H_{33}N_5O_3$) C, H, N.

2-(3,4-Dimethoxyphenyl)-2-[3-(1-biotin)propylester]-5-[2-(*p*-azidophenethyl) methylamino]valeronitrile (7). Following the procedure described for **4**, starting from **19** (0.18 g, 0.40 mmol), title compound **7** (0.06 g, 22% yield) was obtained as a thick oil. IR (neat) ν 3250 (NH), 2240 (CN), 2120 (N_3), 1740 (CO), 1680 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 1.28–1.48 (m, 4H, $2CH_2$), 1.53–1.78 (m, 6H, $3CH_2$), 1.88–2.08 (m, 4H, $2CH_2$), 2.19 (s, 3H, NCH_3), 2.22–2.38 (m, 5H, $2CH_2 + CH$), 2.58–2.76 (m, 4H, $2CH_2$), 2.79–2.96 (m, 1H, CH), 3.08–3.19 (m, 1H, CH), 3.88 (s, 6H, $2OCH_3$), 3.94–4.18 (m, 2H, CH_2O), 4.21–4.32 (m, 1H, CH), 4.48–4.53 (m, 1H, CH), 5.47 (bs, 1H, NH), 5.78 (bs, 1H, NH); 6.75–6.98 (m, 5H, aromatics), 7.03–7.17 (m, 2H, aromatics) ppm. Anal. ($C_{35}H_{47}N_7O_5S$) C, H, N.

Trifluoroacetyl-2-(*o*-azidophenethyl)amide (20). Trifluoroacetic anhydride (0.5 mL, 3.8 mmol) was added to a solution of 2-(*o*-azidophenyl)ethylamine[40] (0.4 g, 2.5 mmol) in anhyd ethyl ether. After 30 min the solvent was removed and the crude material (0.65 mg) was used without purification for the following reaction. IR (neat) ν 3300 (NH), 2120 (N_3), 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.86–2.92 (m, 2H, CH_2), 3.56–3.63 (m, 2H, CH_2), 6.58 (bs, 1H, NH), 7.12–7.41 (m, 4H, aromatics) ppm.

Trifluoroacetyl-2-(*p*-azidophenethyl)amide (21). Following the procedure described for **20**, starting from 2-(*p*-azidophenyl)ethylamine, title compound **21** was obtained. IR (neat) ν 3300 (NH), 2120 (N_3), 1700 (CO) cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.83–2.90 (m, 2H, CH_2), 3.56–3.63 (m, 2H, CH_2), 6.55 (bs, 1H, NH), 7.00 (d, $J = 8.0$ Hz, 2H, aromatics), 7.17 (d, 2H, aromatics) ppm.

2-(*o*-Azidophenethyl)methylamine (22). A mixture of methyl iodide (5.7 g, 0.04 mol), **20** (1.89 g, 0.01 mol), and dry acetone (50 mL) was heated nearly to reflux and powdered potassium hydroxide (2.24 g, 0.04 mol) was then added. The reaction mixture was heated under reflux for 5 min, excess of methyl iodide and acetone was removed at reduced pressure, water (50 mL) was added, and then the mixture was heated under reflux for 10 min to give *N*-methylamine **22**. IR (neat) ν 3300 (NH), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.45 (s, 3H, CH_3), 2.79 (s, 4H, $2CH_2$), 6.98–7.35 (m, 4H, aromatics) ppm. Anal. ($C_9H_{12}N_4$) C, H, N.

2-(*p*-Azidophenethyl)methylamine (23). Following the procedure described for **22**, starting from **21**, title compound **23** was obtained. IR (neat) ν 3300 (NH), 2120 (N_3) cm^{-1}. ^1H NMR ($CDCl_3$) δ 2.43 (s, 3H, CH_3), 2.80 (s, 4H, $2CH_2$), 6.98 (d, 2H, aromatics), 7.18 (d, $J =$ 8.0 Hz, 2H, aromatics) ppm. Anal. ($C_9H_{12}N_4$) C, H, N.

Biology

Drugs and chemicals. Purified pirarubicin was provided by Laboratoire Roger Bellon (France). Concentrations were determined by diluting stock solutions to approximately 10^{-5} M and using $\varepsilon_{480} = 11500\ M^{-1}\ cm^{-1}$. Stock solutions were prepared just before use. Unless otherwise stated, buffer solutions were HEPES buffer containing 5 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 5 mM glucose, at pH 7.25.

Cell lines and cultures. K562 is a human leukemia cell line, established from a patient with a chronic myelogeneous leukemia in blast transformation.[41] K562 cells resistant to doxorubicin were obtained by continuous exposure to increasing doxorubicin concentrations, and were maintained in medium containing doxorubicin (400 nM) until 1–4 weeks before experiments. This subline expresses a unique membrane glycoprotein with a molecular weight of 180,000 daltons.[42] Doxorubicin-sensitive and -resistant erythroleukemia K562 cells were grown in suspension, in RPMI 1640 (Sigma) medium supplemented with L-glutamine and 10% FCS at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cultures, initiated at a density of 10^5 cells/mL grew exponentially to $8–10\times10^5$ cells/mL in 3 days. For the spectrofluorometric assays, in order to have cells in the exponential growth phase, culture was initiated at 5×10^5 cells/mL, and cells were used 24 h later, when the culture had grown to about $8–10\times10^5$ cells/mL. Cell viability was assessed by trypan blue exclusion. The cell number was determined by Coulter counter analysis.

Cellular drug accumulation. The uptake of THP-adriamycin in cells was followed by monitoring the decrease in the fluorescence signal at 590 nm ($\lambda_{ex} = 480$ nM) following the method previously described.[43] Using this method it is possible to accurately quantify the kinetics of the drug uptake by the cells and the amount of anthracycline intercalated between the base pairs in the nucleus at the steady state, as incubation of the cells with the drug proceeds without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 mL of buffer at 37°C. We checked that tested compounds did not affect the fluorescence of THP-adriamycin.

Photolabeling. Ultraviolet irradiation, in the presence of the photolabeler at concentration of 10 μM, was carried out using a 125-W lamp (HPW 125 W-TS, Philips) as a light source. Cells, 10^6/mL in 2 mL, were placed in a 25 mL cultured flask, and irradiation was carried out at a distance of 7 cm from the cells for 20 min at 25°C. The plastic flask allowed transmittance of UV light in the 300–400 nM range, and no additional filter was necessary. The intensity, I_o^i, of the light beam incident just within the flask was measured using the potassium ferrioxalate system as actinometer.[44] I_o^i was equal to $2.5 \pm 0.5\times10^{14}$ quanta/s. To eliminate the unbound molecules, cells were washed twice in a 25 mL volume of buffer.

References

1. Kane, S. E. In *Advances in Drug Research*; Academic Press, 1996; Vol. 24, pp 181–252.
2. Bosch, I.; Croop, J. *Biochim. Biophys. Acta* **1996**, *1288*, F37.
3. Nooter, K.; Stoter, G. *Path. Res. Pract.* **1996**, *192*, 768.
4. Higgins, C. F. *Ann. Rev. Cell Biol.* **1992**, *8*, 67.
5. Muller, M.; de Vries, E. G. E.; Jansen, P. L. M. *J. Hepat.* **1996**, *24* (Supp. 1), 100.
6. Jenkinson, H. F. *J. Dental Res.* **1996**, *75*, 736.
7. Bray, P. G.; Hawley, S. R.; Mungthin, M.; Ward, S. A. *Mol. Pharmacol.* **1996**, *50*, 1559.
8. van Veen, H. W.; Konings, W. N. *Biol. Chem.* **1997**, *376*, 769.
9. van Asperen, J.; Mayer, U.; van Tellingen, O.; Beijnen, J. H. *J. Pharm. Sci.* **1997**, *86*, 881.
10. Hunter, J.; Hirst, B. H. *Adv. Drug Deliv. Rev.* **1997**, *25*, 129.
11. Meijer, D. K. F.; Smit, I. W.; Muller, M. *Adv. Drug Deliv. Rev.* **1997**, *25*, 159.
12. German, U. A. *Eur. J. Cancer* **1996**, *32A*, 927.
13. Rosenberg, M. F.; Callaghan, R.; Ford, R. C.; Higgins, C. F. *J. Biol. Chem.* **1997**, *272*, 10685.
14. Bourhis, J.; Riou, G.; Bénard, J. *Bull. Cancer* **1990**, *77*, 957.
15. Gualtieri, F. *La Chimica e l'Industria* **1996**, *78*, 1233.
16. Robert, J. *Drug of the Future* **1997**, *22*, 149.
17. Ecker, G.; Chiba, P. *Exp. Opin. Ther. Patents* **1997**, *7*, 589.
18. Scala, S.; Akhmed, N.; Rao, U. S.; Paull, K.; Lan, L.; Dickstein, B.; Lee, J.; Elgemeie, G. H.; Stein, W. D.; Bates, S. E. *Mol. Pharmacol.* **1997**, *51*, 1024.
19. Pascaud, C.; Garrigos, M.; Orlowski, S. *Biochem. J.* **1998**, *333*, 351.
20. Qian, X.-D.; Beck, W. T. *Cancer Res.* **1990**, *50*, 1132.
21. Beck, T.; Qian, X.-D. *Biochem. Pharmacol.* **1992**, *43*, 89.
22. Safa, A. R.; Agresti, M.; Bryk, D.; Tamai, I. *Biochem.* **1994**, *33*, 256.
23. Borchers, C.; Ulrich, W.-R.; Klem, K.; Ise, W.; Gekeler, V.; Haas, S.; Schodl, A.; Conrad, J.; Przybyliski, M.; Boer, R. *Mol. Pharmacol.* **1995**, *48*, 21.
24. Ojima, I.; Duclos, O.; Dorman, G.; Simonot, B.; Prestwich, G. D. *J. Med. Chem.* **1995**, *38*, 3892.
25. Boer, R.; Dichtl, M.; Borchers, C.; Ulrich, W. R.; Marecek, J. F.; Prestwich, G. D.; Glossmann, H.; Striessnig, J. *Biochem.* **1996**, *35*, 1387.
26. Mankhetkorn, S.; Teodori, E.; Scapecchi, S.; Garnier-Suillerot, A. *Biochem. Pharmacol.* **1996**, *52*, 213.
27. Yang, C. P.; Mellado, W.; Horwitz, S. B. *Biochem. Pharmacol.* **1988**, *37*, 1417.
28. Wilchek, M.; Bayer, E. A. *Anal. Biochem.* **1988**, *171*, 1.
29. Auer, M.; Moore, K. J.; Meyer-Almes, F. J.; Guenter, R.; Pope, A. J.; Stoekly, K. A. *Drug Disc. Tod.* **1998**, *3*, 457.
30. Mannhold, R. In *Recent Advances in Receptor Chemistry*; Melchiorre, C.; Giannella, M., Eds.; Elsevier Science: Amsterdam, 1988, pp 147–171.
31. Safa, A. R. *Proc. Natl. Acad. Sci. USA* **1988**, *85*, 7187.
32. Pereira, E.; Teodori, E.; Dei, S.; Gualtieri, F.; Garnier-Suillerot, A. *Biochem. Pharmacol.* **1995**, *50*, 451.
33. Mitani, K.; Yoshida, T.; Sakurai, S.; Morikawa, K.; Iwanaga, Y.; Koshinaka, E.; Kato, H.; Ito, Y. *Chem. Pharm. Bull.* **1988**, *36*, 373.
34. Chaturvedi, D. N.; Knittel, J. J.; Hruby, V. J.; Castrucci, A. M. D. L.; Hadley, M. *J. Med. Chem.* **1984**, *27*, 1406.
35. Frézard, F.; Garnier-Suillerot, A. *Biochim. Biophys. Acta* **1991**, *1091*, 29.
36. Frézard, F.; Garnier-Suillerot, A. *Eur. J. Biochem.* **1991**, *196*, 483.
37. Pereira, E.; Borrel, M.-N.; Fiallo, M.; Garnier-Suillerot, A. *Biochem. Biophys. Acta* **1994**, *1225*, 209.
38. Pereira, E.; Garnier-Suillerot, A. *Biochem. Pharmacol.* **1994**, *47*, 1851.
39. Mankhetkorn, S.; Dubru, F.; Hesschenbrouck, J.; Fiallo, M.; Garnier-Suillerot, A. *Mol. Pharmacol.* **1996**, *49*, 532.
40. Molina, P.; Alajarin, M.; Vidal, A. *J. Org. Chem.* **1993**, *58*, 1687.
41. Lozio, C. B.; Lozzio, B. B. *Blood* **1975**, *45*, 321.
42. Tsuruo, T.; Iida, H.; Kawataba, H.; Oh-hara, T.; Hamada, H.; Utakoji, T. *Jpn. J. Cancer Res.* **1986**, *77*, 682.
43. Mankhetkorn, S.; Garnier-Suillerot, A. *Eur. J. Pharmacol.* **1998**, *343*, 313.
44. Calvert, J. C.; Pitts, J. N. *Photochemistry*. Wiley and Sons: New York, 1996.