ELSEVIER

European Journal of Pharmacology 389 (2000) 141–146

ejp

www.elsevier.nl/locate/ejphar

Cellular uptake and interaction with purified membranes of rebeccamycin derivatives

Jean-François Goossens [a], Jean-Pierre Hénichart [b], Fabrice Anizon [c], Michelle Prudhomme [c], Christophe Dugave [d], Jean-François Riou [e], Christian Bailly [f,*]

[a] *Laboratoire de Chimie Analytique, Faculté de Pharmacie, 59006 Lille, France*
[b] *Institut de Chimie Pharmaceutique, Faculté de Pharmacie, 59006 Lille, France*
[c] *SEESIB, UMR 6504 CNRS, Université Blaise Pascal, 63177 Aubière, France*
[d] *Département d'Ingénierie et d'Etudes des Protéines, CEA / Saclay, bâtiment 152, 91191 Gif-sur-Yvette, France*
[e] *Rhône-Poulenc Rorer, Centre de Recherche de Vitry-Alfortville, 94403 Vitry-sur-Seine, France*
[f] *INSERM U-524 et Laboratoire de Pharmacologie Antitumorale du Centre Oscar Lambret, IRCL, Place de Verdun, 59045 Lille, France*

Received 29 July 1999; received in revised form 3 December 1999; accepted 9 December 1999

Abstract

Rebeccamycin is an antitumor antibiotic possessing a DNA-intercalating indolocarbazole chromophore linked to a glycosyl residue. The carbohydrate moiety of rebeccamycin and related synthetic analogues, such as the potent antitumor drug NB-506 (6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]carbazole-5,7-(6*H*)-dione), is a key element for both DNA-binding and inhibition of DNA topoisomerase I. In this study, we have investigated the cellular uptake of rebeccamycin derivatives and their interaction with purified membranes. The transport of radiolabeled [^{3}H]dechlorinated rebeccamycin was studied using the human leukemia HL60 and melanoma B16 cell lines as well as two murine leukemia cell lines sensitive (P388) or resistant (P388CPT5) to camptothecin. In all cases, the uptake is rapid but limited to about 6% of the drug molecules. In HL60 cells, the uptake entered a steady-state phase of intracellular accumulation of about 0.26 ± 0.05 pmol/10^6 cells, which persisted to at least 90 min. The efflux of exchangeable radiolabeled molecules was relatively weak. Fluorescence studies were performed to compare the interaction of a rebeccamycin derivative and its aglycone with membranes purified from HL60 cells. The glycosylated drug molecules bound to the cell membranes can be extracted upon washing with buffer or by adding an excess of DNA. In contrast, the indolocarbazole drug lacking the carbohydrate domain remains tightly bound to the membranes with very little or no exchange upon the addition of DNA. The membrane transport and binding properties of indolocarbazole drugs related to rebeccamycin are reminiscent to those of other DNA-intercalating antitumor agents. The uptake most likely occurs via a passive diffusion through the plasma membranes and the glycosyl residue of the drug plays an essential role for the translocation of the drug from the membranes to the internal cell components, such as DNA.

Keywords: Indolocarbazole; Cell membrane; Cellular uptake; Carbohydrate; Rebeccamycin

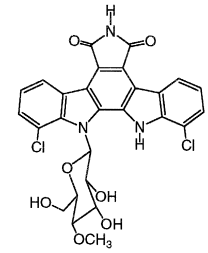
1. Introduction

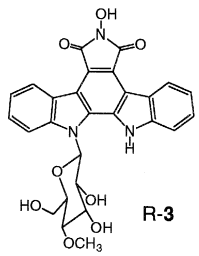
Indolocarbazoles related to the antibiotics BE-13793C and rebeccamycin (Fig. 1) exhibit a useful spectrum of antitumor activity(Prudhomme, 1997; Pindur et al., 1999). The synthetic analogue NB-506 (6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]carbazole-5,7-(6*H*)-dione) has shown a remarkable activity against colon and lung cancer xenografts and displays a low toxicity profile (Arakawa et al., 1995; Yoshinari et al., 1995). This compound is now undergoing clinical trials (Ohe et al. 1997; Saijo, 1998).

The anticancer activity of NB-506 and related compounds has been attributed to their capacity to stabilize DNA-topoisomerase I complexes (Yamashita et al., 1992; Yoshinari et al., 1993). Our structure–activity relationship

* Corresponding author. Tel.: +33-320-16-92-18; fax: +33-320-16-92-29.
E-mail address: bailly@lille.inserm.fr (C. Bailly).

0014-2999/00/$ - see front matter
PII: S0014-2999(99)00895-X





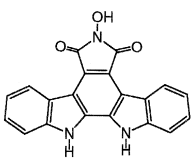


Fig. 1. Structure of indolocarbazole drugs mentioned in this study.

studies provided clear evidences that the sugar moiety attached to the indolocarbazole chromophore is a key element for both DNA-binding and topoisomerase I inhibition and, as a consequence, for the cytotoxicity (Rodrigues-Pereira et al., 1996; Anizon et al., 1997; Bailly et al., 1997, 1998, 1999a; Moreau et al. 1998, 1999a,b). Nevertheless, althought we may begin to understand the role of the sugar substituent in the interaction with DNA nothing is known about its influence on cell- and membrane-ligand interactions. There is no information concerning the capacity of the indolocarbazole drugs to penetrate into cells and to reach the nuclear compartment where the main target is located. For this reason, we have investigated the uptake of an indolocarbazole derivative by four tumour cell lines (HL60, B16, P388 and P388CPT5) and its interaction with purified membranes from HL60 leukemia cells.

For this study, we selected the compound R-**3** shown in Fig. 1, which is one of the most potent topoisomerase I inhibitor among the indolocarbazole derivatives known to date. In previous studies, the interaction of this indolocarbazole derivative with DNA and topoisomerase I–DNA complexes has been reported (Bailly et al., 1997, 1998, 1999b, Labourier et al., 1999). In addition, we used the aglycone derivative R-**4** to obtain information concerning the contribution of the sugar residue to the interaction with cell membranes.

2. Materials and methods

2.1. Drugs

The synthesis of the indolocarbazole drugs R-**3** and R-**4** has been reported (Rodrigues-Pereira et al., 1996). Drugs were dissolved in dimethylsulfoxide (DMSO) at 3 mg/ml and then further diluted with water. Fresh dilutions were prepared immediately prior to use. [^{3}H]dechlorinated rebeccamycin (10 Ci/mmol) was obtained by hydrogenolysis of the chlorine atoms of rebeccamycin (isolated from *Saccharotrix aerocolonigenes*) in the presence of $^3H_2$. Briefly, rebeccamycin (2.3 mg, 4.0 μmol) was dissolved in dry methanol (0.5 ml). Triethylamine (15 μl) and palladium on activated carbon (10.5 mg) were added. The mixture was stirred under an atmosphere of 99.9% tritium (1.65 bars, 58 Ci/mmol) for 4.5 h at room temperature. After removal of the residual tritium under reduced pressure, the mixture was filtrated over celite and the product was eluted with tetrahydrofuran. Elimination of the exangeable tritium was performed by repeated washing of the filtrate with methanol and evaporation of the solvent under reduced pressure. Purification of the crude product using a silica gel $60F_{254}$ plate (10 × 20 cm; eluent: ethyl acetate–cyclohexane 70–30) yielded tritiated compound R-**3** (18.6 mCi, 10 Ci/mmol; yield: 23%); Rf: rebeccamycin, 0.53; R-**3**, 0.27.

2.2. Cell cultures

Human HL60 promyelocytic leukemia cells were obtain from the European Collection of Cell Cultures (Sophia-Antipolis, France). Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2 in RPMI 1640 medium, supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU/ml) and streptomycin (100 μg/ml). B16 melanoma cells were obtained from subcutaneous primitive tumors implanted into C57 Black 6J mouse. Isolated cells were first cultured as monolayers in T75 flasks containing 25 ml Minimum Eagle's Medium (MEM — GIBCO) supplemented with 10% foetal bovine serum and 100 U/ml penicillin, in a humidified atmosphere (5% CO_2), at 37°C. After reaching confluence, the cells were exposed to 0.1% trypsin–0.02% EDTA for 5 min; ''fresh'' cells were diluted 5 times for further culture in the same medium. P388 murine leukaemia cell line was obtained from the tumor bank of the National Cancer Institute (Bethesda, MD, USA). P388CPT5 cell line resistant to campthotecin was derived from a stable clone of the P388CPT0.3 cell line obtained at the 42nd passage (Madelaine et al., 1993). Both cell lines were grown in RPMI 1640 medium containing 0.01 mM 2-mercaptoethanol, 10 mM L-glutamine, 10% (v/v) fetal calf serum, 100 IU/ml penicillin, 2 μg/ml streptomycin, 50 μg/ml

gentamycin and 50 μg/ml nystatin at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Cellular uptake studies

Each sample (5×10^5 cells in 0.5 mL) was treated with the [^{3}H]-drug in a 50 mM Tris buffer adjusted to pH 7.4, containing 2 mM $MgCl_2$. After incubation at 37°C, the cells were collected by vacuum filtration on glass fiber filters (GF/C Whatman preincubated for 2 h in 0.1% polyethyleneimine). The filters were washed four times with 2 ml of buffer and then placed in scintillation vials containing 2 ml Packard Ultima Gold MV. The radioactivity trapped onto the filters was counted using a EG&G Wallac 1900 scintillation spectrometer.

2.4. Fluorescence observations

Each sample (2 ml at 5×10^5 cells/ml) was treated with increasing concentrations of the drug R-**3** (5–100 μM) for 10 min at 37°C in complete culture medium. Half of the cell suspension was removed and washed once with drug-free phosphate buffered saline (PBS). After a brief centrifugation, the cell pellet was resuspended in 2 ml of PBS and 100 μl (25,000 cells) were used for the centrifugation (500 rpm, 5 min) on a slide. A drop of anti-fade solution was added and the treated portion of the slide was covered with a glass coverslip with the edges sealed with clear nail polish. The morphological aspect of the cell was observed with a fluorescence microscope (Zeiss Axiophot 2) using a UV filter and a ×100 objective. Images were captured using the software Quips Smart Capture™ (Vysis).

2.5. Fluorescence measurements

Purified membranes from HL60 cells were prepared using a described procedure (Casieri et al., 1992) and stored at −80°C in aliquots until use. The same membrane preparation was used throughout the study. Membranes were incubated with the test drug (usually at 50 μM) in 0.2 ml of Hank's buffer saline solution (HBSS). After 30 min incubation at 37°C, the fluorescence was determined with an excitation of 320 nm (R-**3**) or 340 nm (R-**4**) and an emission of 530 nm (R-**3**) or 605 nm (R-**4**) with a 8 nm band pass filter. The membrane samples (containing 70 μg protein/ml) were then pelleted by centrifugation and the residual fluorescence of the supernatant and the pellet (resuspended in 0.2 ml fresh HBSS buffer) was measured. After a 20-min incubation period, the membranes were again centrifuged and the washing procedure was repeated twice. The fluorescence intensity associated with the different pellet (P1-3) and supernatant (T1-3) fractions was measured. For the membranes-DNA competition experiments, each sample contained purified membranes (14 μg protein/mL) in 0.2 ml of buffer and the drug at 20 μM. A few μl of a concentrated solution of calf thymus DNA were added to each tube and the samples were left to equilibrate for 20 min under gentle mixing prior to centrifugation. The fluorescence emission in the supernatant fraction containing DNA and in the pellet were measured. All spectrum were recorded with a SPEX spectrofluorimeter Fluorolog.

3. Results

3.1. Time-course of drug uptake

We took advantage of a ^{3}H-labeled compound structurally similar to R-**3** to facilitate the tracing of the drug molecules in cells. Four cell lines were employed. A time course of uptake of 4.4 nM [^{3}H]dechlorinated rebeccamycin (4.4 pmol/10^6 cells) by HL60 leukemia cells and B16 melanoma cells is shown in Fig. 2. In both cases, the binding is very rapid but weak. A plateau level is reached after about 4 min incubation. Uptake entered a steady-state phase of intracellular accumulation of about 0.26 ± 0.05 pmol/10^6 cells which persisted to at least 90 min. Only about 6% of the tritiated drugs molecules were recovered in the cells. Competition experiments showed that only a limited amount of the labeled drug can be removed from the cells. The ^{3}H-labeled compound was first incubated with the HL60 cells prior to adding graded amounts of cold R-**3** drug. As shown in Fig. 3, a vast excess of R-**3** (1 μM, i.e. 10,000 times the concentration of ^{3}H-drug) is needed to removed 25% of the labeled compound from the cells. The efflux of exchangeable indolocarbazole molecules is relatively weak, suggesting that a major component of intracellular drug was tightly bound.

HL60 cells treated with R-**3** were directly observed by fluorescence microscopy. The typical image presented in Fig. 4 shows that the intracellular distribution is not uniform. The nucleus is much more fluorescent that the

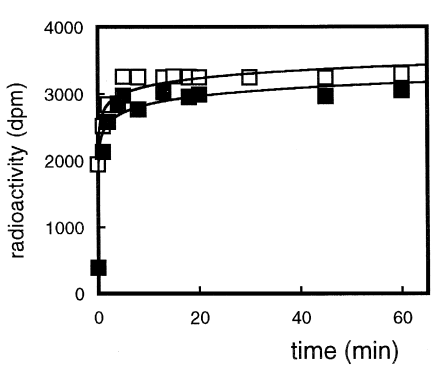


Fig. 2. Time-course of drug uptake by (open squares) HL60 leukemia cells and (filled squares) B16 melanoma cells. Cells (5×10^5 in 500 μl) were incubated with [^{3}H]-dechlorinated rebeccamycin (50,000 dpm, 4.4 nM) at 37°C in balanced salt solution. At the termination of incubation, cells were rapidly separated by filtration (GF/C Whatman filters), washed with buffer (4×2 ml) and radioactivity of the cell material was determined. Each point represents the mean of three experiments.

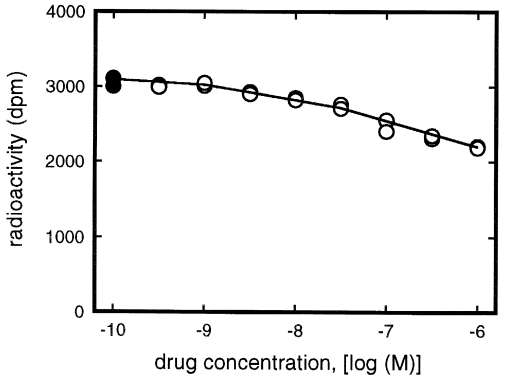


Fig. 3. Efflux of [^{3}H]-dechlorinated rebeccamycin. HL60 cells were treated with 0.1 pM [^{3}H]-dechlorinated rebeccamycin for 1 h at 37°C prior to adding the cold drug R-**3** (up to 1 μM). The cell samples were incubated for a further 30 min period and efflux was measured by determining the residual radioactivity in the cell after filtration.

cytoplasm suggesting that the drug accumulates into the nuclear compartment were its main targets, DNA and topoisomerase I, are located.

The association of the drug to the P388 murine leukemia cell line is about the same as determined with HL60 human leukemia cell line (Fig. 5A). Here again, only about 6% of the drug molecules were found in the cell fraction. The kinetic analysis of drug uptake were performed in parallel with P388 cells and P388CPT5 cells resistant to camptothecin. The resistance of the P388CPT5 cells has been attributed to the expression of a deficient form of topoisomerase I as a result of a mutation in the *top1* gene of these cells (Madelaine et al., 1993). In a recent study, we showed that P388CPT5 cells, which exhibit marked resistance to camptothecin (83-fold), are cross-resistant to R-**3** (Labourier et al., 1999). Interestingly, the level of drug binding to the resistant cell line is significantly weaker than with the sensitive cell line (Fig. 5). Therefore, the resistance of the P388CPT5 to indolocarbazole drugs such as R-**3** may be attributable to a reduced drug uptake capacity, in addition to the expression of a deficient topoisomerase I.

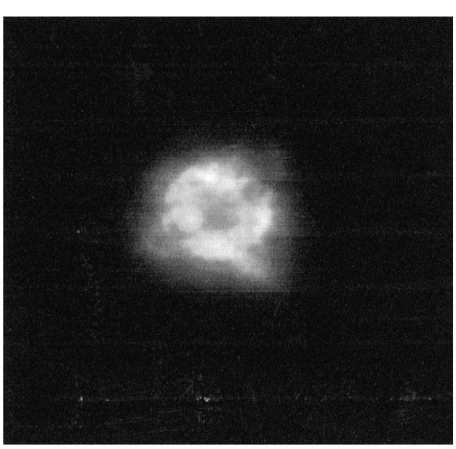


Fig. 4. Fluorescence microscopy of R-**3**-treated HL60 cells showing the intense fluorescence of the nucleus. Cells were incubated with 10 μM R-**3** for 10 min at 37°C prior to washing with buffer and cytocentrifugation. Magnification: ×100.

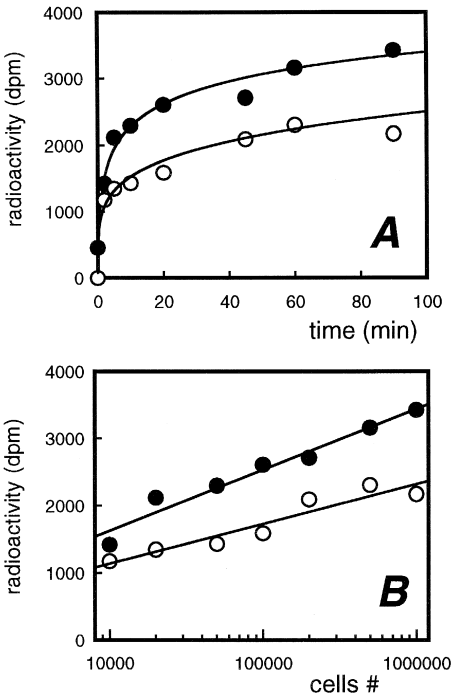


Fig. 5. (A) Uptake of [^{3}H]-dechlorinated rebeccamycin by (filled circles) P388 cells and (open circles) camptothecin-resistant P388CPT5 cells, as a function of (A) the incubation time and (B) the cell number. In (A), the leukemia cells were treated as described in the legend to Fig. 2. In (B), cells were incubated with the drug (50,000 dpm, 4.4 nM) for 30 min prior to filtration. Data are mean values of three experiments.

3.2. Drug binding to purified membranes

The study was done by fluorescence spectroscopy not to avoid the use of radioactive materials but to compare the binding of the glycosylated drug R-**3** and its aglycone

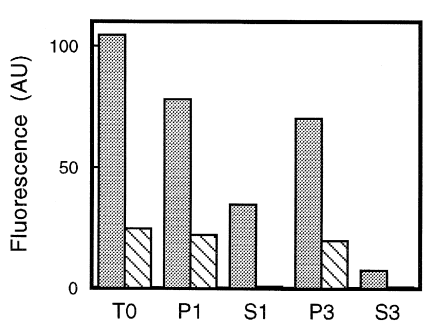


Fig. 6. Drug binding to membranes from HL60 cells. The histograms show the fluorescence recorded with (solid bars) R-**3** and (hatched bars) R-**4** in the membrane samples after 30 min incubation (T0). After the initial incubation period, the membranes were collected by centrifugation and the fluorescence recovered in the resuspended pellet (P1) and supernatant (S1) was measured. The pellet was washed twice with 200 μl saline buffer and the fluorescence found in the residual pellet (P3) and the final supernatant (S3) was measured.

derivative R-**4** to intact HL60 cells and purified membranes. The intrinsic fluorescence of the indolocarbazole chromophore is very useful to evaluate the association of the drugs with the cell membranes. With both R-**3** and R-**4**, the fluorescence emission is weak when the drug is free in solution but it is enhanced when the drug is located in a hydrophobic environment such as within the cell membranes or when the drug intercalates into DNA.

To investigate the interaction of the drugs with membranes from HL60 cells, a fixed amount of purified cytoplasmic membranes (14 μg protein/ml) in saline buffer was incubated with the indolocarbazole drugs. After a 30 min incubation period at 37°C, the fluorescence intensity of the whole sample (T0) was measured (Fig. 6). The fluorescence intensity is much higher with R-**3** than with R-**4**. A similar behavior was observed with intact cells (not shown). The samples were then centrifugated to pellet the membranes which were washed three times successively with 200 μl of saline buffer to try to remove drug molecules bound to the membranes. The fluorescence in the membrane fractions (pellets P1-3) and the supernatants (S1-3) was determined. Samples were left to incubate for 20 min at 37°C between each wash. As shown in Fig. 6, the washing procedure has little effect on the binding of R-**4** to the membranes. There was no fluorescence in fractions S1-3. In contrast, the drug R-**3** can be dissociated from the membranes, as judged from the fluorescence found in fractions S1-3.

Another procedure was tested to remove the drug molecules anchored into the membranes. The drugs were first incubated for 30 min with purified membranes and after removal of the unbound molecules, caf thymus DNA was added to the membrane samples. The mixture was incubated for another 30 min under gentle shaking and the samples were centrifugated to separate the membranes from the DNA. The transfer of the drug molecules from the membranes to the DNA double helix can be followed by fluorescence. The emission spectrum of R-**3** bound to the membranes presents a maximum at 535 nm. This maximum is shifted to 550 nm when DNA was added (Fig. 7) indicating that a large fraction of the drug molecules have been transferred from the membranes to the DNA. No such effect was detected with R-**4**. The addition of DNA does not permit to remove the aglycone molecules anchored into the lipidic bilayer. More than 98% of the fluorescence of R-**4** remained associated with the membranes even when using a vast excess of DNA to try to extract the drug. In contrast, R-**3** molecules containing the carbohydrate residue can be easily extracted from the membranes by the DNA.

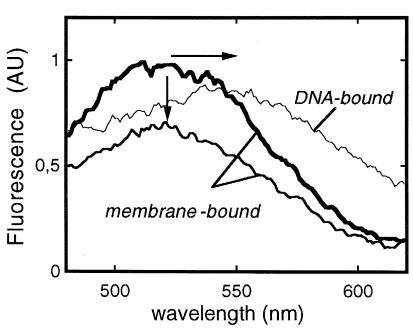


Fig. 7. Effect of the addition of DNA on the fluorescence of R-**3** bound to membranes from HL60 leukemia cells. The graph shows the fluorescence spectra of (thick line) 20 μM R-**3** bound to the membranes (200 μl of membranes at 14 μg protein/ml) and the same sample to which 0.4 mM calf thymus DNA was added. The DNA and the membranes were separated by centrifugation. The spectra represented with a dashed line and a solid line show the fluorescence of the drug molecules bound to DNA and the membranes, respectively. In each case, the fluorescence emission intensities were measured with an excitation set up at 320 nm. Horizontal and vertical arrows outline the bathochromic and hypochromic effects, respectively.

4. Discussion

This is the first time the cellular uptake and interaction of indolocarbazole rebeccamycin derivatives with purified cell membranes was investigated. Such a study is important for the design of novel compounds. The limited cellular uptake capacity of R-**3** needs to be taken into account for the development of tumor active compounds. The membrane transport and binding properties of the indolocarbazole are reminiscent to those described with other DNA-intercalating antitumor agents such as the anthraquinone drug mitoxantrone (Burns et al., 1987). The uptake is fast but limited to a few percent of the initial dose. This suggests that the mechanism of uptake was passive diffusion, as is the case with a variety of anticancer drugs including busulfan, procarbazine and hydroxyurea for example (Morgan et al., 1986).

The fluorescence measurements performed with the drug R-**3** and the corresponding aglycone R-**4** provide useful information on the role of the drug carbohydrate moiety. In previous studies, we showed that the sugar residue promotes the interaction of the drug with DNA and contributes to a more potent inhibition of topoisomerase I (Bailly et al., 1997, 1998, 1999b). Here, we show that the carbohydrate domain also contributes positively to the transfer of the drug molecules from the membranes to other cellular components such as DNA. The aglycone drug, which is highly lipophilic, remains tightly bound to the membranes whereas the glycosyl derivative R-**3** can be extracted from the membranes. It is therefore likely that the sugar residue helps to transfer the drug from the outside of the cell to the nucleus to reach the DNA–topoisomerase I complexes.

Acknowledgements

This work was supported by research grants from the Ligue Nationale Française Contre le Cancer and the Asso-

ciation pour la Recherche sur le Cancer. L.D. is currently supported by a fellowship from the Association pour la Recherche sur le Cancer. The authors thank the *Service Commun d'Imagerie Cellulaire de l'IFR22* for access to the fluorescence microscope.

References

Anizon, F., Belin, L., Moreau, P., Sancelme, M., Voldoire, A., Prudhomme, M., Ollier, M., Sevère, D., Riou, J.F., Bailly, C., Fabro, D., Meyer, T., 1997. Syntheses and biological activity (topoisomerases inhibition, antitumoral and antimicrobial properties) of rebeccamycin analogues bearing modified sugar moieties and substituted on the imide nitrogen with a methyl group. J. Med. Chem. 40, 3456–3465.

Arakawa, H., Iguchi, T., Morita, M., Yoshinari, T., Kojiri, K., Suda, H., Okura, A., Nishimura, S., 1995. Novel indolocarbazole compound 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]carbazole-5,7-(6*H*)-dione (NB-506): its potent antitumor activities in mice. Cancer Res. 55, 1316–1320.

Bailly, C., Carrasco, C., Hamy, F., Vezin, H., Prudhomme, M., Saleem, A., Rubin, E., 1999b. The camptothecin-resistant topoisomerase I mutant F361S is cross-resistant to antitumor rebeccamycin derivatives. Biochemistry 38, 8605–8611.

Bailly, C., Colson, P., Houssier, C., Rodrigues-Pereira, E., Prudhomme, M., Waring, M.J., 1998. Recognition of specific sequences in DNA by a topoisomerase I inhibitor derived from the antitumor drug rebeccamycin. Mol. Pharmacol. 53, 77–87.

Bailly, C., Qu, X., Graves, D.E., Chaires, J.B., 1999a. Calories from carbohydrates. Energetic contribution of the carbohydrate moiety of rebeccamycin to DNA binding and the effect of its orientation on topoisomerase I inhibition. Chem. Biol. 6, 277–286.

Bailly, C., Riou, J.F., Colson, P., Houssier, C., Rodrigues-Pereira, E., Prudhomme, M., 1997. DNA cleavage by topoisomerase I in the presence of indolocarbazole derivatives of rebeccamycin. Biochemistry 36, 3917–3929.

Burns, C.P., Haugstad, B.N., North, J.A., 1987. Membrane transport of mitoxantrone by L1210 leukemia cells. Biochem. Pharmacol. 36, 857–860.

Casieri, M.A., Ber, E., Fong, T.M., Sadowski, S., Bansal, A., Swain, C., Saward, E., Frances, B., Burns, D., Strader, C.D., 1992. Characterization of the binding of a potent, selective, radioiodinated antagonist to the human neurokinin-1 receptor. Mol. Pharmacol. 42, 458–463.

Labourier, E., Riou, J.F., Prudhomme, M., Carrasco, C., Bailly, C., Tazi, J., 1999. Poisoning of topoisomerase I by an antitumor indolocarbazole drug: stabilization of topoisomerase I-DNA covalent complexes and specific inhibition of the protein kinase activity. Cancer Res. 59, 52–55.

Madelaine, J., Prost, S., Naudin, A., Riou, G., Lavelle, F., Riou, J.F., 1993. Sequential modifications of topoisomerase I activity in a camptothecin resistant cell line established by progressive adaptation. Biochem. Pharmacol. 45, 339–348.

Moreau, P., Anizon, F., Sancelme, M., Prudhomme, M., Bailly, C., Carrasco, C., Ollier, M., Sevère, D., Riou, J.F., Fabro, D., Meyer, T., Aubertin, A.M., 1998. Syntheses and biological evaluation of indolocarbazoles, analogues of rebeccamycin, modified at the imide heterocycle. J. Med. Chem. 41, 1631–1640.

Moreau, P., Anizon, F., Sancelme, M., Prudhomme, M., Bailly, C., Ollier, M., Sevère, D., Riou, J.F., Fabbro, D., Meyer, T., Aubertin, A.M., 1999a. Syntheses and biological activities of rebeccamycin analogues. Introduction of a halogenoacetyl substituent. J. Med. Chem. 42, 584–592.

Moreau, P., Anizon, F., Sancelme, M., Prudhomme, M., Sevère, D., Riou, J.F., Goossens, J.F., Hénichart, J.P., Bailly, C., Labourier, E., Tazi, J., Fabbro, D., Meyer, T., Aubertin, A.M., 1999b. Synthesis, mode of action and biological activities of rebeccamycin bromo-derivatives. J. Med. Chem. 42, 1816–1822.

Morgan, J.S., Creasey, D.C., Wright, J.A., 1986. Evidence that the antitumor agent hydroxyurea enters mammalian cells by a diffusion mechanism. Biochem. Biophys. Res. Commun. 134, 1254–1259.

Ohe, Y., Tanigawara, Y., Fujii, H., Ohtsu, T., Wakita, H., Igarashi, T., Minami, H., Eguchi, K., Shinkai, T., Tamura, T., Kunotoh, H., Saijo, N., Okada, K., Ogino, H., Sasaki, Y., 1997. Phase I and pharmacology study of 5-day infusion of NB-506. Proc. ACSO 16, 199a.

Pindur, U., Kim, Y.-S., Mehrabani, F., 1999. Advances in indolo[2,3-*a*]carbazole chemistry: design synthesis of protein kinase C and topoisomerase I inhibitors. Curr. Med. Chem. 6, 29–69.

Prudhomme, M., 1997. Indolocarbazoles as anti-cancer agents. Curr. Pharm. Des. 3, 265–290.

Rodrigues-Pereira, E., Belin, L., Sancelme, M., Prudhomme, M., Ollier, M., Rapp, M., Servère, D., Riou, J.F., Fabbro, D., 1996. Structure-activity relationships in a series of substituted indolocarbazoles: topoisomerase I and protein kinase C inhibition and antitumoral and antimicrobial properties. J. Med. Chem. 39, 4471–4477.

Saijo, N., 1998. New chemotherapeutic agents for the treatment of non-small cell lung cancer. Chest 113, 17S–23S.

Yamashita, Y., Fujii, N., Murakata, C., Ashizawa, T., Okabe, M., Nakano, H., 1992. Induction of mammalian DNA topoisomerase I mediated DNA cleavage by antitumor indolocarbazole derivatives. Biochemistry 31, 12069–12075.

Yoshinari, T., Matsumoto, M., Arakawa, H., Okada, H., Noguchi, K., Suda, H., Okura, A., Nishimura, S., 1995. Novel antitumor indolocarbazole compound 6-*N*-formylamino-12,13-dihydro-1,11-dihydroxy-13-(β-D-glucopyranosyl)-5*H*-indolo[2,3-*a*]pyrrolo-[3,4-*c*]carbazole-5,7-(6*H*)-dione (NB-506): induction of topoisomerase I-mediated DNA cleavage and mechanisms of cell line-selective cytotoxicity. Cancer Res. 55, 1310–1315.

Yoshinari, T., Yamada, A., Uemura, D., Nomura, K., Arakawa, H., Kojiri, K., Yoshida, E., Suda, H., Okura, A., 1993. Induction of topoisomerase I-mediated DNA cleavage by a new indolocarbazole, ED-110. Cancer Res. 53, 490–494.