Journal of Inclusion Phenomena and Macrocyclic Chemistry **39:** 13–18, 2001.

Taxol Inclusion Complexes with a Cyclodextrin Dimer: Possibilities to Detoxify Chemotherapeutics and to Target Drugs Specifically to Tumors? *

JOERG G. MOSER†, IRENE ROSE, BIRGIT WAGNER, TIM WIENEKE and ANJA VERVOORTS
Institute of Laser Medicine, Heinrich-Heine University, Universitaetsstr 1, D-4 0225 Duesseldorf, Germany

(Received: 12 June 1998; in final form: 22 November 1999)

Key words: cyclodextrin dimers, inclusion complexation, taxol, tumor chemotherapy

Abstract

The natural drug, paclitaxel (taxol), is highly effective as a tumor chemotherapeutic with a low probability of inducing chemoresistance, but shows severe toxic side effects at the therapeutic dose. How can this toxicity be overcome? Here we report the synthesis of cyclodextrin dimers connected at the secondary face by amide-bonded aliphatic spacers. The spacer length of one of the dimers referred to as diβCD(2N-A4C5A4) or dimer **7c** matches the distance between the two benzoic acid residues of paclitaxel. We investigated the physical inclusion of taxol into this dimer using the TNS-label competition method. Affinity constants with the dimer in comparison to free β-cyclodextrin are found to be of the order of 10^7 l/mole. When included into the cyclodextrin dimer, the drug shows a considerable time delay of incorporation into human tumor cell cultures (OAT SCLC cells) or a total exclusion from the cells. This is the prerequisite to avoid intoxication of other organs of a patient. Possibilities are discussed to detoxify chemotherapeutics and to target their inclusion complexes specifically to tumors using specific biological signals.

Introduction

The natural chemotherapeutic drug, paclitaxel (taxol) exerts its cytotoxic action by hyperstabilizing the subcellular microtubules and stopping cell proliferation in the early mitosis phase (G_2/M) of the cell cycle [1, 2]. Its chemical structure (Figure 1a) indicates a nearly insoluble drug containing one phenyl and two benzoic acid residues attached to side chains of the taxane skeleton. Its analogue, docetaxel (Taxotere^{TM}) (Figure 1b) is somewhat more soluble due to a t-butoxycarbonyl residue instead of one of the benzoic acid residues. Most efforts to solubilize these drugs for chemotherapeutic purposes include incorporation into oil emulsions and dissolution in detergents (e.g., Cremophor^{TM}). Recently, several attempts have been made to solubilize taxol by inclusion into monomeric substituted cyclodextrins [3, 4]. The result of these investigations was that the cyclodextrins used as a pharmaceutical adjuvant are toxic by themselves and have to be detoxified first in order to allow the application of the tumor therapeutic dose of 10–25 mg of taxol/kg body weight. This is in line with toxicological investigations [5] claiming a maximum dose of β-cyclodextrin of no more than 10 mg/day as non-toxic for human patients.

An alternative possibility that remains to be discussed is a drastic reduction of the therapeutic dose while targeting the drug specifically to the tumor. The lethal cytotoxic concentration of taxol is of the order of 10^{-7} M or about 85 μg/kg tumor (Figure 2) if applied specifically to the tumor and not via a generalized intoxication of the patient at high dose as done during medical tumor chemotherapy today. This is the source of the aggravating side effects of this life-saving drug which otherwise produces only rare chemoresistance and is highly effective.

In order to avoid this toxicity the drug should be included into a complexing agent which does not allow for uptake of the drug by any living cell. Such a complex may then be targeted to the tumor using a biological signal structure. Prodrug constructs and concepts of this kind were successfully developed by several groups [6–9] over the last few years. Our group has published another concept [10, 11] using cyclodextrin dimers (for review see [12]) as the complexing agent which could be first realized with porphyrinoid photosensitizers for photodynamic tumor therapy and fluorescent tumor diagnosis [13]. A similar approach is described here for the taxane derivatives mentioned above including biological tests.

Experimental

Taxol complexation

A stock solution (2 mM) of taxol was prepared by dissolving 5 mg of paclitaxel (Sigma St. Louis, MO USA, #T1912, MW 853) in 2.93 mL DMSO. Aliquots of this solution were added to cyclodextrin (CD) solutions of concentrations between 0.1 and 20 mM (see the respective figure legends). Concentrations of CD solutions were determined

* Patent application #PCT/EP98/07229 at the European Patent Office.

† Author for correspondence.

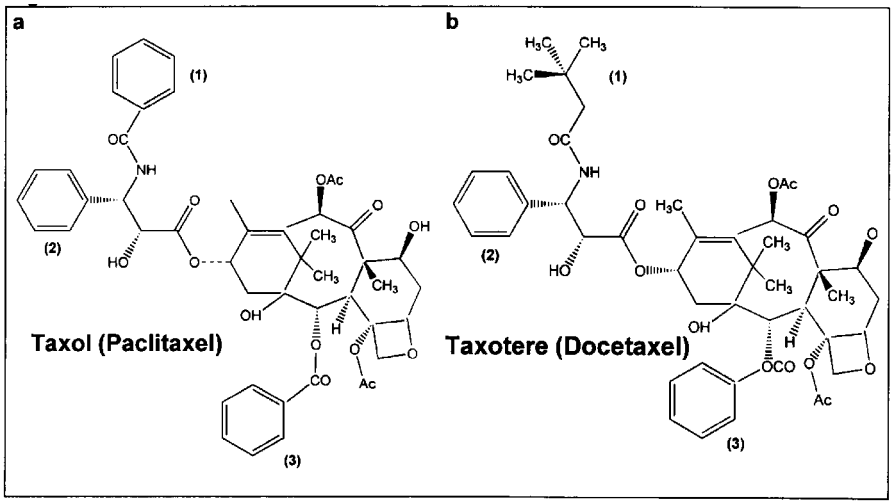

Figure 1. Drugs of the taxane family: Paclitaxel (left) and Docetaxel (right). The structures show the benzoic acid(1,3) and phenyl(2) residues in taxol and its sub tert.butoxy carbonyl residue in the Docetaxel (Taxotere) structure.

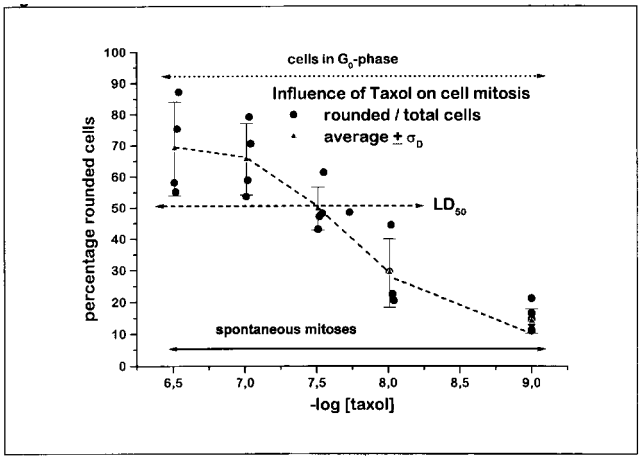

Figure 2. Cytotoxicity of taxol on human cancer cells (A375 amelanotic melanoma) *in vitro.* Number of cells per growth area in culture vessel. The number of cells surviving 24 hours treatment at different concentrations of taxol were counted in several culture vessels. Error bars represent 95% confidence level of median.

with the anthron-sulfuric acid reaction using glucose and β-cyclodextrin as the standards. Various cyclodextrins (Wacker Chemie, Burghausen, Germany) were used including α-CD, β-CD, γ-CD, methyl β-CD, hydroxypropyl β-CD and hydroxypropyl γ-CD, of which only β-CD was chosen for more extended evaluation. The spacered dimer, diβCD(2N-A4C5A4) or dimer **7c** (Figure 3) was synthesized according to [13] and extensively purified by ion exchange chromatography on CM-Sepharose and QAE-Sepharose (Pharmacia, Uppsala, Sweden) before use. MALDI mass spectrometry was performed on a matrix of dihydroxy benzoic acid in a linear TOF laser mass spectrometer. The stock solution of the dimer in HEPES buffer (pH 7.4) was adjusted to 100 μM.

Binding constants

The fluorescent competitor, 6-(4-toluidino)-2naphthalene sulfonic acid (TNS; FLUKA Switzerland, #89653, MW 314) [14] was dissolved as a 1 mM stock solution in DMSO and further diluted in water. Fluorescence measurements were performed on a Shimadzu RF-1501 spectrofluorometer with a constant temperature cuvette holder connected by water jackets to two water baths of 20 °C and 70 °C. TNS fluorescence emission was routinely excited at 308 nm and the spectrum taken between 350 and 550 nm. The fluorescence maximum depends strongly on the substances incorporated into the sample. Negligible fluorescence occurs in pure aqueous solutions. Fluorescence spectra of taxol in CD containing solutions were excited at 277 nm, and the emission spectra were recorded from 300 nm upward. Binding constants, K, and competition constants, K_i, were calculated from Benesi–Hildebrand plots [15] of the fluorescence maximum values [16]. Error bars refer to 95% confidence levels [17, 18].

Tumor cell growth

Human tumor cell lines (OAT SCLC, A375) were grown in DMEM culture medium containing 5% fetal calf serum (FCS), 5 mM glucose, and antibiotics in 75 mL culture flasks. Cells were harvested at confluence by trypsin-EDTA treatment and seeded at 3–5 mio. cells per flask for the experiments. After 1–2 days of growth taxol solutions of concentrations between 10^{-7} and 10^{-9} M with or without inclusion into the CD-dimer were added, and the growth and rounding-up of cells were observed by counting the cells on the TV screen of a Leitz inverted microscope at about 100 fold magnification. Slides and TV records were taken with a microscope camera [Wild-Leitz MPS 45] and a TV recorder [Hitachi M235E VHS] respectively.

Results

Formation of inclusion complexes

The insertion of taxol competes with the K_{11} binding constant of the monomer or dimer which was explicitly determined at low concentrations of monomeric β-cyclodextrin

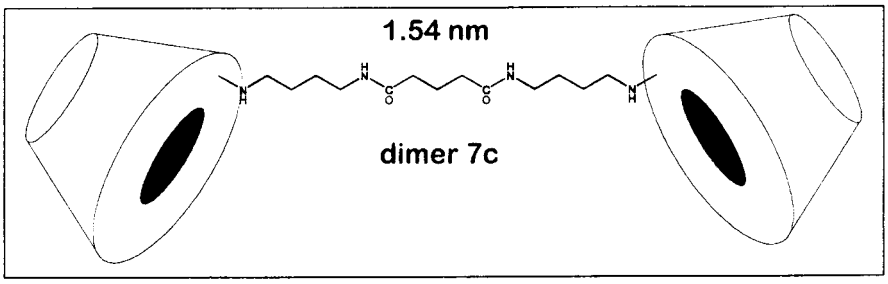

Figure 3. Structure of the purified CD-dimer diβCD(2N-A4C5A4) [13].

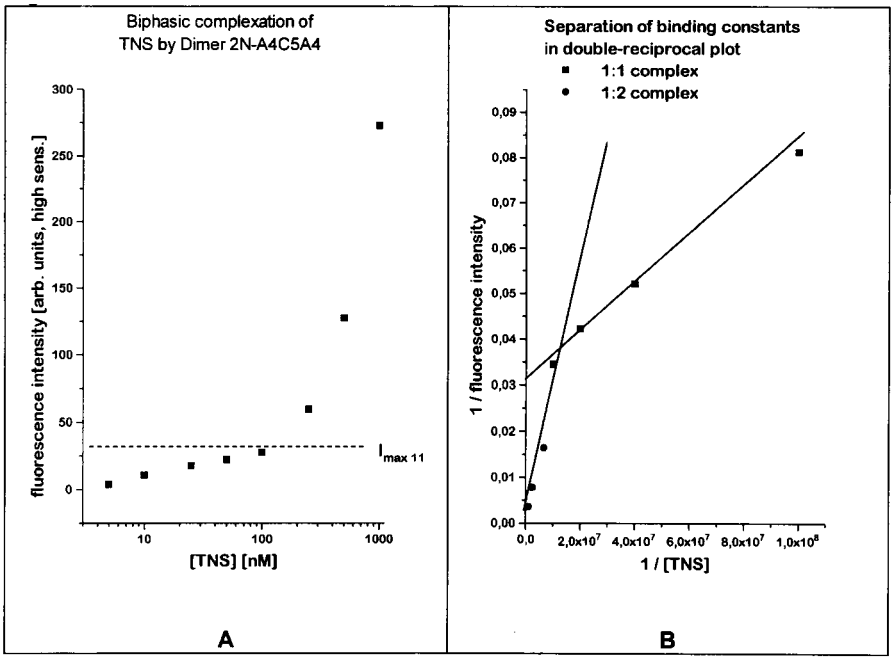

Figure 4. Biphasic fluorescence emission of TNS in solutions of the dimer (A), estimation of the binding constants (B) by a Benesi-Hildebrandt plot.

(Figure 4b). For this purpose the aggregation behaviour of β-CD has to be taken into account. It is well known from laser scattering measurements that β-CD is solvated [19] and aggregated in solutions at room temperature. Higher temperatures are thought to be necessary to break these aggregates into monomers which can then react with the guest to be included. A temperature of 70 °C for 1 min was chosen to build up the complex and the solution cooled down to 20 °C before fluorescence measurements. For taxol complex formation the solution was heated to 50 °C overnight and cooled to room temperature or lower for several hours before fluorescence measurements. The fluorescence excitation spectrum showed constant behaviour.

The molecular distances between the benzoic acid groups in taxol [substituents (1) and (3) in Figure 1a] and the length of the spacer of the dimer were measured using the software Alchemy III of Tripos Associates, Moscow (Russia). In taxol, the distance was estimated as 15.4 Å while the distance between the terminal amino groups of the spacer in the CD-dimer shows a maximum of 16.1 Å. The spacer is not expected to be totally stretched but somewhat twisted which hinders complex formation to a certain extent and requires prolonged time for the 1:1 complex to form.

Table 1. Binding constants of TNS to cyclodextrin monomers and the dimer

Substance	K_{11} [μM]	A_{11} [$\times 10^6$ l/mol]
Monomers		
β-cyclodextrin	37	0.027
methyl β-cyclodextrin	55.5	0.018
hydroxypropyl γ-CD	330	0.003
diβCD(2NA-4C5A4)	0.017*	59

*Cf. Figure 6.
K = dissociation constant [M/I], A = affinity constant [I/M] = 1/K.

Monomeric β-CD and the CD-dimer were incubated with taxol. While 1 min. heating to 70 °C was enough for the monomer to form a stable and reproducible complex, the dimer needed a 50 °C incubation overnight for stable complex formation. Higher temperatures lead to destruction of the included drug as shown by decay of the 236 nm fluorescence. After cooling to 4 °C for several hours the affinity constants could be determined by the competition reaction with TNS.

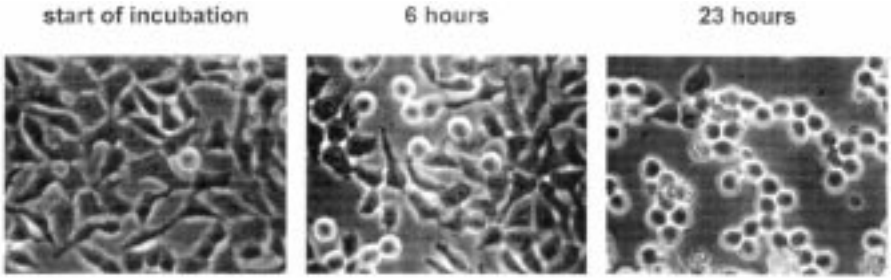

Figure 5. Increasing numbers of rounded-up cells indicate the mitotic arrest by free taxol. Registrations with a video camera and video recorder were elaborated by a frame grabber program, Screen Machine II, and further processed with Corel PhotoPaint.

Table 2. Apparent affinity constants between β-cyclodextrin monomer, the CD dimer and taxol [5–10 μM]

Substance	[host]/[guest]	K_{taxol} [μM]	A_{taxol} [$\times 10^6$ l/mol]
β-CD monomer	5	69	14,500
methyl-β-CD	10	21	47,400
diβD(2N-A4C5A4)	1	0.19	5,200,000

20–100 μM taxol in 100 μM CD-dimer can lead to crystal formation. This was avoided by lower concentrations of taxol (5–10 μM in 10–100 μM of the dimer). Similar experimental results were given by Sharma *et al.* [4] with CD-monomers. For the dimer additional experiments were performed at various dimer concentration.

Influence on cell mitosis

The high affinity constant of the complex of the order of 10^7 l/mole begs the question whether this complex is stable enough to prevent the transfer of the included drug into living cells. A certain affinity to cell membranes may be assumed since only two of the three phenyl groups can be covered by a dimeric cyclodextrin. As seen in Figure 2, a concentration of 40 nM of free taxol is highly poisonous to OAT SCLC cells. When incorporated in the complex the concentration of free taxol should be of the order of <2 nM. At this concentration no more than 20% mitotic arrest in our cell line should be expected. Similarly, the concentration of a CD-dimer solution of 400 nM is below the toxicity level in these cells which was experimentally confirmed. The crucial experiment with cell cultures was performed using a very simple method of counting the percentage of rounded-up vs. total cells at 40 nM taxol with and without including the drug in the CD-dimer. The complex was prepared in the usual way by heating a stock solution of 10 μM taxol in 100 μM of CD-dimer overnight to 50 °C and diluting 1 mL of this solution to 250 mL of culture medium. Every two to three hours after the beginning of the experiment the rounded and total cells were counted at 9 different locations of the culture vessel and the time course of development of mitotic arrest was monitored. The results are shown in Figures 5 and 6. Solubilization in monomeric β-cyclodextrin shows the same mitostatic effects as free taxol (not shown).

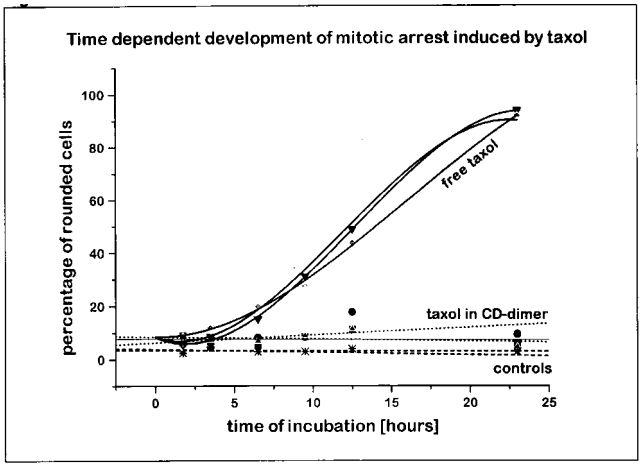

Figure 6. Development of mitotic arrest comparing free taxol with the drug complexed with the CD-dimer. $[taxol]_{total}$ was 40 nM in both cases.

After 48 hours no change was observed in the samples containing the dimer-complexed drug which indicates full stability of the complex over this time period. The fluorescence spectra indicate only the monomerization of the drug inside the complex but not any general change indicating that the drug is intact inside the complex.

Proof of the intactness of the complexed drug

In order to prove the intactness of the included drug, the dicyclodextrin shell was digested with cyclodextrinase from *Klebsiella oxytoca.* As shown in Figure 7, the drug could be released by digesting the cyclodextrin dimer shell which again leads to mitotic arrest of the cells while the undigested preparation does not differ from control values. The Michaelis–Menten constant of the enzyme is of the order of 1–3 mM against β-cyclodextrin (pH 7, 23 °C) [20]. The concentration of the complex used is no more than 10 μM before further dilution. The complex was incubated at room temperature for a prolonged period (18 hours) to show the effect of complex digestion. The enzyme by itself and the untreated cyclodextrin dimer have no effect on mitotic arrest.

Discussion

Our work on taxol complexation proves that inclusion of taxol into cyclodextrin dimers results in a nearly perfect pro-

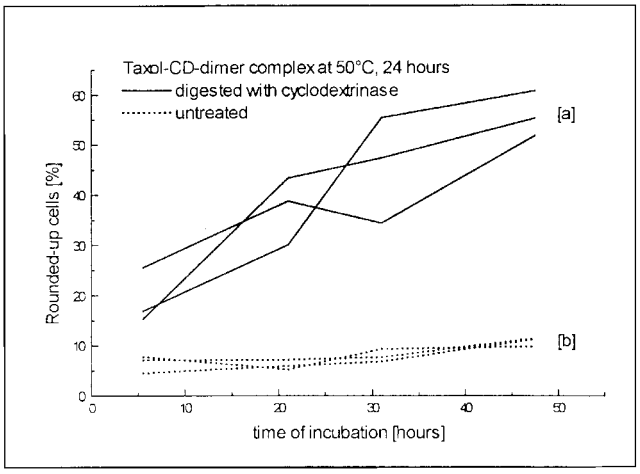

Figure 7. Digestion of the taxol-CD-dimer complex with cyclodextrinase shows again the recovery of mitotic arrest. The complex formed at [taxol] = 10 μM at 50 °C was separated into two samples: sample (a) was treated for 18 hours with cyclodextrinase of *Klebsiella oxytoca* at room temperature (b) was stored at 4 °C. Thereafter, both samples were diluted to 40 nM with culture added to pregrown OAT SCLC cells.

tection against poisoning of cells by this chemotherapeutic drug.

Our processing of the complexation reaction also sheds light on the extended time needed to produce stable complexes. There is no indication in the literature for how long and at what temperatures the complexes with cyclodextrins are formed. In our experiments, complex formation with taxol and the cyclodextrin dimer is still incomplete after 8 hours at 50 ° C while the complex formed over 24 hours is stable as shown in Figure 6. Equally, for the determination of the binding constants the sequence of substances added to a fluorescent sample is important: establishing an equilibrium in such systems is highly time consuming despite the validity of the mass equilibrium law at infinity. In systems acquiring their high molecular order only slowly an independence on time cannot be expected.

Previous experiments of complexation of taxol with cyclodextrin monomers were performed in order to find alternatives for the solubilization of the drug in oil emulsions or detergents. These ingredients are responsible for such side effects as heart failure and hyperallergic reactions in treated patients which should be avoided by the cyclodextrins [3, 4]. Unfortunately, cyclodextrins by themselves show considerable side effects, e.g., on the functionality of the kidney epithelium, at doses to be applied together with the chemotherapeutically effective dose of taxol: the cyclodextrin toxicity has to be reduced before any application with taxol becomes reasonable [5, 3].

By contrast, our investigation was undertaken in order to examine an alternative concept which allows a drastic reduction of the therapeutic dose as combined with the effect of solubilization. The experiments shown in Figures 5 and 6 realize a long expected, but until now yet to be realized innovation in pharmacotherapy. Four years ago, we postulated theoretically that an improvement of photochemotherapy and general chemotherapy can be expected only when the drugs employed are encapsulated into a hydrophilic or electronegative shell which prevents the uptake of such drugs by other living cells [10]. This general intoxication produces the dangerous side effects as intrinsic to present day general tumor chemotherapy. Molecular encapsulation of these drugs is the *first prerequisite* of a successful polyphasic chemotherapy with reduced or excluded side effects. The structure of the complex has to be investigated by NMR [21] in the future.

In order to enhance the dose at the tumor site the shell should be provided with a biological signal which connects the complex to the tumor. Polyphasic administration of such labeled complexes is the subject of ongoing studies in our group. A specific hormone- or antibody-directed chemotherapy should also drastically reduce the dose to be applied for tumor destruction. The lethal dose for destruction of tumor cells (see Figure 2) is of the order of 10^{-7} M or about 85 μg taxol/kg tumor. The present therapeutic dose of taxol amounts to 10–25 mg/kg body weight or about 0.7–2 g per patient. Using a specific route focused on the tumor, the dose remnant in the patient may be reduced to $<$1/10,000 of the presently used dose. This dose reduction alone is likely to reduce undesired side effects and should enhance the therapeutic effect.

Acknowledgements

The authors gratefully acknowledge the generosity of Dr. H. Reuscher (WackerChemie, Burghausen/Munich, Germany) for several donations of cyclodextrin samples.

Prof. A. Boeck PhD (Munich) allowed us to use his cyclodextrinase preparation provided by Dr. M. Pajatsch. The authors are also indebted to Dr. Anja Ruebner for her engaged help to provide CD-dimers. She, Dr. Inke Bahr and Dieter Kirsch helped with mass spectrometric proof of the cyclodextrin dimers and with valuable discussions during preparation of the manuscript. This work has been performed in part fulfilling Research Contracts of the Ministry of Education and Research of the Federal Republic of Germany (BMBF #13N6291) and was supported by a research grant of the Faculty of Medicine of the Heinrich-Heine University (Duesseldorf, Germany). Most of the work performed by one of us (I.R.) fulfilled the requirements for the State's Examination for Public High School employment.

References

1. S.B. Horwitz: *Annals Oncology* **5**(6), S3 (1994).
2. Y. Toyama, S. Forry-Schaudies, B. Hoffman, and H. Holtzer: *Proc. Natl. Acad. Sci. USA* **79**, 6556 (1982).
3. J. Szente, M. Viukmon, J. Szemán, and J. Szeijtli: *Pharmaceutical Applications Conference.* Kansas City, USA. Poster Abstr. **32** (1997).
4. U.S. Sharma, S.V. Balasubramanian, and R.M. Straubinger: *J. Pharmaceutic. Sci.* **84**, 1223 (1995).
5. G. Antlsperger and G. Schmid: *8th Int. Symp. on Cyclodextrins [Budapest]* (1996). pp. 149–156.
6. K. D. Bagshawe: *Br. J. Cancer* **56**, 531 (1987).
7. K. Bosslet, A. Steinstraesser, A. Schwarz, H.P. Harthus, G. Lueben, L. Kuhlmann, and H.H. Sedlacek: *Eur J. Nucl. Med.* **14**, 523 (1988).
8. K. Bosslet, J. Czech, P. Lorenz, H.H. Sedlacek, M. Schuermann, and G. Seemann: *Br J. Cancer* **65**, 234 (1992).

9. F. Fazio and G. Paganelli: *Eur J. Nucl. Med.* **20**, 1138 (1993).
10. J.G. Moser, A. Heuermann, P. Oehr, H. Scheer, A. Vervoorts and S. Andrees: *SPIE Biomed. Optics* **2523**, 92 (1994).
11. J.G. Moser, A. Ruebner-Heuermann, A. Weitemeyer, U. Michelsen, D. Woehrle, A. Rueck, W.S.L. Strauss, D. Kirsch, S. Andrees, and C. Schroers: *SPIE Biomed. Optics* **2625**, 138 (1995).
12. R. Breslow, S. Halfon, and B. Zhang: *Tetrahedron* **51**, 377 (1995).
13. A. Ruebner, D. Kirsch, S. Andrees, W. Decker, B. Roeder, B. Spengler, R. Kaufmann, and J.G. Moser: *J. Incl. Phenom. Mol. Recogn.* **37**, 69 (1997).
14. M.D. Johnson and V.C. Reinsborough: *Austr. J. Chem.* **45**, 1961 (1992).
15. H. Benesi and J.H. Hildebrand: *J. Am. Chem. Soc.* **71**, 2703 (1949).
16. K. A. Connors: *Binding Constants. The Measurement of Molecular Complex Stability*, New York: J. Wiley & Sons (1987), pp. 175–187.
17. R.C. Campbell: *Statistics for Biologists*, London: Cambridge University Press (1974).
18. A. Linder: *Handliche Sammlung mathematisch-statistischer Tafeln*, Basel: Birkhaeuser (1961).
19. B. Manunza, S. Deiana, M. Pintore, and C. Geesa: *Http://antas.agraria.uniss.it/electronic_papers/eccc3/bcd/welcome.htm* (1998), 12 pp.
20. R. Feederle, M. Pajatsch, E. Kremmer, and A. Boeck: *Arch. Microbiol.* **165**, 206 (1996).
21. H.J. Schneider, T. Blatter, and S. Simova: *J. Am. Chem. Soc.* **113**, 1996 (1991).
22. A.P. Savitzki, I.G. Meerovich, J.G. Moser, R.I. Yakubovskaya, O.L. Kaliya, and E.A. Luk'yanets: *SPIEBiomed. Optics* **3191**, 34 (1998).