Differential inhibition of cytosolic and membrane-derived protein kinase C activity by staurosporine and other kinase inhibitors

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Abstract The hypothesis was tested that 9 kinase inhibitors with diverse specificities for protein kinase C (PKC), including staurosporine and four of its analogues, interfere differently with PKC derived from either the cytosolic or particulate fractions of MCF-7 breast carcinoma cells. GF 109203X inhibited the enzyme identically in either preparation. CGP 41251 and calphostin C inhibited cytosolic PKC more effectively than membrane-derived PKC with ratios of IC_{50} (cytosolic PKC) over IC_{50} (membrane-derived PKC) of 0.07 and 0.04, respectively. The other six agents inhibited membrane-derived PKC more potently than cytosolic enzyme. Staurosporine and RO 31 8220 exhibited IC_{50} ratios of 12.3 and 21.6, respectively. The results suggest that there are dramatic differences between kinase inhibitors in their divergent effects on cytosolic and membrane-derived PKC which should be borne in mind in the interpretation of their pharmacological properties.

Key words: Protein kinase C inhibition; Staurosporine analogue; MCF-7 breast carcinoma cell

1. Introduction

Protein kinase C (PKC) is a family of enzymes which are pivotal constituents of signalling pathways and thus regulate cellular growth, differentiation and a variety of processes involved with homeostasis [1]. In its inactive state a major proportion of PKC can be recovered as a soluble enzyme in most cell types. However the activated apoenzyme is thought to be positioned at the surface of intracellular membranes to permit efficient phosphorylation of membrane proteins [2]. In most cells PKC activition elicits the rapid redistribution of enzyme from the cytosol to the membrane and nucleus [3]. Inhibitors of PKC have been frequently used as probes to clarify the role which the enzyme plays in regulating cell function [4]. They are also under consideration as potential therapeutic agents in the treatment of diseases such as asthma, dermatological disorders, rheumatoid athritis, cancer and AIDS [5]. Many of the agents which possess PKC-inhibitory activity, like the indolocarbazole staurosporine, isolated from *Streptomyces staurosporeus* [6], inhibit a variety of kinases indiscriminately and thus lack selectivity for PKC. However during the last few years analogues of staurosporine have been synthesised which retain, at least in part, the high kinase-inhibitory potency of the parent molecule, but possess much better selectivity for PKC [7–10]. Two such PKC-specific analogues, UCN-01 and CGP 41251, have been shown to possess antineoplastic activity in human tumour models grown in rodents [9, 11]. In most investigations of the ability of compounds to inhibit PKC, cytosolic enzyme preparations have been used. In the light of the fact that it is predominantly PKC in the membrane which is of functional importance, we tested the hypothesis that kinase inhibitors display different potencies against membrane-derived as compared to cytosolic enzyme. To that end nine well-characterised PKC inhibitors, including staurosporine and four analogues, were investigated with respect to their ability to inhibit PKC prepared from the cytosol or membrane of human-derived MCF-7 breast cancer cells. The susceptibility of these cells towards the growth-arresting ability of PKC inhibitors has recently been described [12].

2. Materials and methods

2.1. Chemicals and reagents

Calphostin C, H-7 and GF 109203X were purchased from Calbiochem-Novabiochem Co. (Nottingham, UK). UCN-01, RO 31 8220, CGP 41251, NPC 15–437 and miltefosine were gifts from Kyowa Hakko Kogyo Co. (Tokyo, Japan), Roche Research Centre (Welwyn Garden City, UK), Ciba Geigy (Basel, Switzerland), Nova Pharmaceutical Co. (Baltimore, MD, USA) and Asta Pharma (Frankfurt, Germany), respectively. All other chemicals and reagents including staurosporine were obtained from Sigma Chemical Co. (Poole, UK). Cell culture medium, and serum were purchased from Gibco BRL (Paisley, UK). Stock solutions of drugs were prepared in DMSO, except in the case of NPC 15–437 which was dissolved in water, and stored at −20°C. Monoclonal antibodies against PKC-α and -ε were purchased from Tissue Culture Services Ltd. (Botolph Claydon, UK) and Affiniti Ltd. (Nottingham, UK), respectively, and a polyclonal antibody against PKC-ζ was obtained from Gibco BRL (Paisley, UK).

2.2. Cell growth and preparation of cellular fractions

MCF-7 breast carcinoma cells from the European Collection of Animal Cell Cultures (Salisbury, UK) were maintained routinely in an atmosphere of O_2:CO_2 (95:5) in minimum essential medium (Eagles modified) with fetal calf serum (10%), pyruvate (1 mM), L-glutamine (2 mM), non-essential amino acids, penicillin (100 iu/ml) and streptomycin (100 μg/ml). Cells were cultured in petri dishes (140 mm diameter) until they reached confluency. Cell cytosol was prepared according to Dale et al. [13]. The particulate (membrane) fraction was obtained from cells which had been exposed to phorbol dibutyrate (10 μM) for 30 min prior to fractionation to achieve redistribution of PKC to the membrane. Several methods of membrane preparation were evaluated. The procedure ultimately chosen was essentially that described by Greif et al. [14], except that the membrane pellet, once collected, was suspended by sonication and solubilised in buffer containing Brij 58 (0.1%), as described by Rush et al. [15]. The suspension was left on ice for 1 h and recentrifuged. The resultant supernatant was investigated. In comparison to the other protocols tested this method was optimal in that it furnished a preparation which (i) allowed endogenous inhibitors to be diluted out effectively, (ii) contained only low 'non-specific' radioactivity (10% of total), which means activity not associated with PKC-specific substrate, and (iii) retained PKC activity associated with the membrane. Membrane-derived PKC activity was between 185 and

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Abbreviations: IC_{50}, concentration which inhibited enzyme activity by 50%; PKC, protein kinase C; aPKC atypical PKC; cPKC, conventional PKC; nPKC, novel PKC;

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198% of that seen in cells which had not been exposed to phorbol dibutyrate.

2.3. PKC activity assay

Cytosolic and membrane preparations were diluted by a factor of between 5 and 20, depending on preparation, with H8 buffer (20 mM Tris-HCl, 2 mM of both EDTA and EGTA, 6 mM β-mercaptoethanol, 2 μg/ml of both leupeptin and aprotinin, at pH 7.5) until endogenous inhibitors, which would have masked effects of the drugs under investigation, were diluted out. Aliquots (25 μl) of the diluted cytosol or particulate fraction were placed in microplate wells and PKC activity was determined using a kit from Amersham International plc (Aylesbury, UK). PKC was activated with 12-*O*-tetradecanoylphorbol-13- acetate and the incorporation of the γ-phosphate moiety of [^{32}P]ATP into a PKC-specific peptide was measured. Radioactivity was counted on a Packard Tricarb 1500 scintillation counter. Drugs were added to the incubate and degree of inhibition of PKC activity was expressed as percentage of drug-free control. Whereas cytosolic PKC did not phosphorylate the PKC-specific substrate significantly in the absence of TPA, $Ca^{2+}$and phospholipid, membrane-derived enzyme exhibited already 85% of maximal achievable activity in the absence of TPA, and 90% without added Ca^{2+} and phospholipid.

2.4. Western blot analysis

Immunoblot analysis was performed as described previously [16]. The amount of protein loaded per lane was 20 μg. Detection was by enhanced chemiluminescence generated by oxidation of luminol in the presence of hydrogen peroxide using an ECL kit from Amersham International.

3. Results

In order to compare the effect of kinase inhibitors on PKC activity associated with cellular cytosol or membrane, cytosol was obtained from naive cells and membrane fraction from cells in which PKC had been redistributed to the membrane by exposure to phorbol dibutyrate for 30 min. Immunoblot analysis showed that cytosolic PKC consisted mainly of PKC-ε and -ζ with little PKC-α, which afforded a faint band not detectable by densitometry or on a photograph. PKC-ζ was also found in the membrane of untreated cells (Fig. 1). On treatment with phorbol dibutyrate nPKC-ε underwent translocation to the particulate fraction, whereas the phorbol ester-insensitive aPKC-ζ did not (Fig. 1). Fig. 2 shows that there were remarkable differences between the PKC preparations in their susceptibility towards the inhibitory potential of the nine compounds tested. Staurosporine was the most and miltefosine the least potent PKC inhibitor. Only one compound, GF 109203X, furnished the same IC_{50} value irrespective of enzyme source (Table 1). UCN-01, H-7, NPC 15-437 and miltefosine were all stronger inhibitors of membrane-derived than of cytosolic PKC. For these agents differences in IC_{50} values amounted to between 2.4- to 5.5-fold (Table 1). The most dramatic discrepancies in inhibitory potency between cytosolic and membrane-derived PKC were seen with RO 31 8220, staurosporine, CGP 41251 and calphostin C. RO 31 8220 and staurosporine inhibited membrane-derived enzyme 22 and 12 times, respectively, more effectively than cytosolic PKC, as judged by differences in IC_{50} values. In contrast, CGP 41521 and calphostin C inhibited cytosolic PKC 14 and 28 times, respectively, better than membrane-derived enzyme.

4. Discussion

The nine kinase inhibitors tested in this study are widely used as probes for the involvement of PKC in cellular processes, and

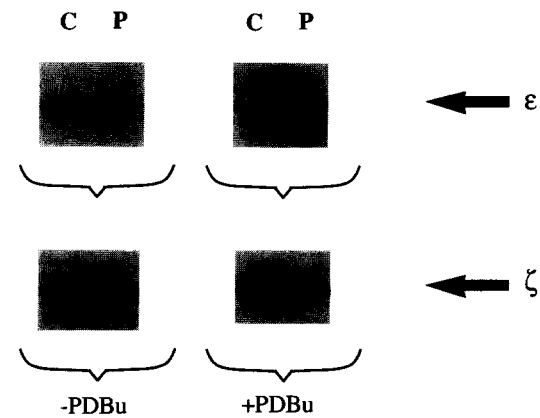

Fig. 1. Effect of exposure of MCF-7 cells to phorbol dibutyrate (PDBu) (10 μM) on localisation of PKC-ε and -ζ. Cytosolic (C) and particulate fractions (P) were prepared and analysed by Western blotting as described in section 2. Cells did not immunoreact with antibodies against PKC-β, -γ, -δ, or -θ, and cytosol reacted only faintly with a monoclonal anti-PKC-α antibody (results not shown).

two of them, UCN-01 and CGP 41251 are experimental antitumour agents [9,11]. The results presented above show, for the first time, that all but one differ markedly in their ability to modulate PKC activity depending on whether the enzyme was derived from the cytosol or the membrane subsequent to its redistribution there. PKC in the membrane is arguably more important as a functional kinase than cytosolic enzyme. The results presented above imply that potent kinase modulators can display as much as a twentyfold difference in inhibitory efficacy between cytosolic and membrane-derived PKC. This discrepancy may explain the frequently observed lack of correlation between pharmacological response and PKC inhibition. Particularly noteworthy is the increased inhibitory potency of RO 31 8220 and staurosporine against membrane-derived as compared to cytosolic enzyme, and the markedly decreased ability of CGP 41251 and calphostin C to inhibit membrane-derived in comparison to cytosolic PKC. Calphostin C does not only inhibit PKC, but also inactivates it irreversibly in a light-dependent fashion [17]. Gopalakrishna et al. showed that calphostin C exhibited higher potency against membrane-derived than cytosolic PKC in experiments in which cells were treated with the drug preceeding preparation of cellular subfractions and subsequent determination of enzyme activity [18]. This finding contrasts starkly with the result described above according to which calphostin C, when incubated with cellular subfractions rather than intact cells, inhibited cytosolic PKC much more effectively than membrane-derived enzyme. The disparity suggests that intact cells are able to provide cofactors and/or to catalyse calphostin C metabolism necessary for maximal inactivation of the enzyme, whereas isolated homogenate fractions lack this ability. Few investigations have addressed differences in susceptibility towards pharmacological inhibitors between cytosolic and membrane-derived PKC. The ether lipid 1-octadecyl-2-*O*-methyl-*sn*-glycerol-3-phosphocholine, an experimental antitumour drug, was found to inhibit PKC when enzyme was derived from cellular cytosol, but paradoxically, to behave like a PKC activator in preparations in which the enzyme was obtained after its redistribution to the membrane [19].

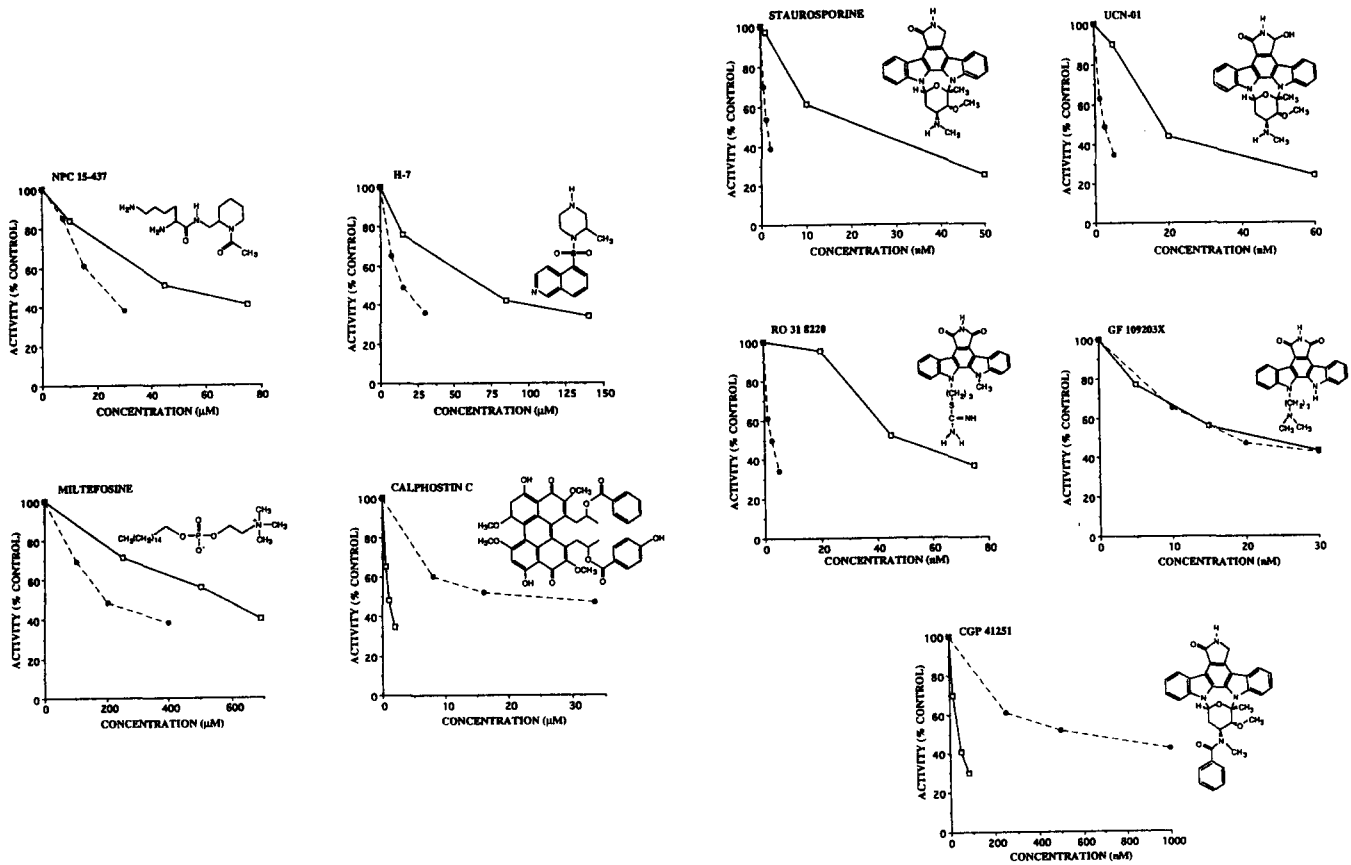

Fig. 2. Inhibition of cytosolic (open squares) and membrane-associated PKC (closed circles) from MCF-7 cells by staurosporine, UCN-01, RO 31 8220, GF 109203X, CGP 41251, NPC 15–437, H-7, miltefosine and calphostin C. In preliminary experiments a suitable concentration range was established for each compound and 3 appropriately spaced concentrations were chosen. The graphs show the result of one experiment representative of three.

In the light of this finding and the results described above for calphostin C, CGP 41251, staurosporine and RO 31 8220, it is conceivable that the selectivity of some kinase inhibitors for PKC, when it is derived from the membrane, over other kinases is very different from that based on values reported in the literature which were derived using cytosolic PKC.

Table 1
Inhibition of cytosolic and membrane PKC from MCF-7 cells by kinase inhibitors

Compound	IC_{50}[a] (μM)		
	Cytosolic PKC	Membrane PKC	Ratio[b]
Staurosporine	0.0163 ± 0.0045	0.0013 ± 0.0001	12.3
UCN-01	0.0178 ± 0.0020	0.0032 ± 0.0009	5.5
GF 109203X	0.021 ± 0.004	0.021 ± 0.004	1.0
CGP 41251	0.044 ± 0.006	0.61 ± 0.14	0.07
RO 31 8220	0.048 ± 0.006	0.0022 ± 0.0002	21.6
Calphostin C	1.09 ± 0.18	30.0 ± 6.2	0.04
NPC 15-437	43.3 ± 6.9	17.9 ± 2.8	2.4
H-7	54.3 ± 7.5	15.2 ± 0.6	3.5
Miltefosine	577 ± 29	184 ± 17	3.1

[a]Concentration at which enzyme activity is inhibited by 50%. Values are the mean ± S.D. of 3 experiments. PKC was measured using a PKC-specific peptide substrate as described in section 2.
[b]IC_{50} (cytosolic PKC)/IC_{50} (membrane-associated PKC).

Six of the agents studied here, H-7 [20], staurosporine [6] and its cogeners UCN-01 [7], RO 31 8220 [8], CGP 41251 [9] and GF 109203X [10], are thought to inhibit PKC at the catalytic site. Three of them, calphostin C [21], NPC 15–437 [22] and miltefosine [23] act at the regulatory domain of the enzyme. UCN-01, RO 31 8220, CGP 41251, GF 109203X, calphostin C and NPC 15–437 possess specificity for PKC, whereas H-7, staurosporine and miltefosine are non-selective kinase inhibitors. In the light of the results outlined above it is doubtful that the differences between the compounds are mechanistically linked to either PKC domain, at which the inhibitors interact with the enzyme, or degree of selectivity for PKC.

The MCF-7 cells used in this study express only PKC-ε and -ζ, apart form traces of PKC-α at the detection limit. Other PKC isoenzymes were not found in the cells [16]. On the assumption that there are no additional as yet undefined phorbol ester-responsive PKC isoenzymes in MCF-7 cells, the activity measured by the assay used in this study was due to PKC-ε in both the cytosolic and membrane preparations, in the latter after efficient redistribution by phorbol dibutyrate. In the absence of altered isoenzyme content, which difference between the two preparations might explain their differential sensitivity towards the kinase inhibitors? It is probably the fact that unlike cytosolic enzyme, membrane-derived PKC on isolation from phorbol ester-pretreated cells exists in a 'physiologically acti-

vated state'. It has been pointed out that membrane-derived PKC differs considerably in activation properties from cytosolic enzyme, even after the lipid environment has been similarly reconstituted in both [2]. Membrane-derived PKC seems to maintain its characteristic shape and properties in spite of the fact that its isolation procedure requires the use of detergent disrupting the intrinsic interactions between the protein and its lipid microenviroment.

It could be argued that the observations outlined above are applicable only to the particular cocktail and relative levels of PKC isoenzymes expressed in the MCF-7 cells used in this study. The results of a preliminary experiment conducted with human-derived A549 lung carcinoma cells render this possibility unlikely. These cells express predominantly PKC-α, together with PKC-ζ and -ε at lower levels [16]. The differences in potency of PKC inhibitors for cytosolic and membrane-derived PKC in these cells were very similar to those shown in Fig. 2 (results not shown).

Staurosporine at 10^{-9} M and its analogues UCN-01, RO 31 8220 and GF 109203X in the 10^{-7} to 10^{-6} M range cause the translocation of PKC-ε from the cytosol to the membrane and nucleus of A549 cells [12]. In contrast, CGP 41251 does not possess this property. It is intriguing that among the staurosporine analogues studied here CGP 41251 is the only one which inhibited membrane-derived PKC less potently than cytosolic enzyme. The two phenomena, ability to induce PKC-ε translocation on the one hand and high affinity for membrane PKC on the other, might be mechanistically related in this class of compound.

In summary, the results presented here show that kinase inhibitors can interfere with cytosolic and membrane-derived PKC to a markedly different degree. The high potency of staurosporine and RO 31 8220, and the weak efficacy of CGP 41251 and calphostin C against membrane-derived PKC should clearly be considered in the interpretation of their pharmacological effects.

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References

[1] Nishizuka, Y (1992) Science 258, 607–614.
[2] Lester, D.S. (1992) in: Protein Kinase C – Current Concepts and Future Perspectives (Lester D.S. and Epand, R.M. Eds.) pp. 80–101, Ellis Horwood, Chichester, UK.
[3] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
[4] Tamaoki, T. and Nakano, H. (1990) Biotechnology 8, 732–735.
[5] Nixon, J.S., Bishop, J., Bradshaw, D., Davis, P.D., Hill, C.H., Elliott, L.H., Kumar, H., Lawton, G., Lewis, E.J., Mulqueen, M., Westmacott, D., Wadsworth, J. and Wilkinson, S.E. (1992) Biochem. Soc. Transact. 20, 419–425.
[6] Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M. and Tomita, F. (1986) Biochem. Biophys. Res. Commun. 135, 397–402.
[7] Takahashi, I., Kobayashi, E., Asano, K., Yoshida, M. and Nakano, H. (1987) J. Antibiot. 40, 1782–1784.
[8] Davis, P.D., Elliott, L., Harris, W., Hurst, S.A., Keech, E., Kumar, H., Lawton, G., Nixon, J.S. and Wilkinson, S.E. (1992) J. Med. Chem. 35, 994–1001.
[9] Meyer, T., Regenass, U., Fabbro, D., Alteri, E. Rösel, J., Müller, M., Caravatti, G. and Matter, A. (1989) Int. J. Cancer 43, 851–856.
[10] Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Loriolle, F., Duhamel, L., Charon, D. and Kirilovsky, J. (1991) J. Biol. Chem. 266, 15771–15781.
[11] Akinaga, S., Gomi, K., Morimoto, M., Tamaoki, T. and Okabe, M. (1991) Cancer Res. 51, 4888–4892.
[12] Courage, C., Budworth, J. and Gescher, A. (1995) Br. J. Cancer, in press.
[13] Dale, I.L., Bradshaw, T.D., Gescher, A. and Pettit, G.R. (1989) Cancer Res. 49, 3242–3245.
[14] Greif, H., Ben-Chaim, J., Shimon, T., Bechor, E., Eldar, H. and Livneh, E. (1992) Mol. Cell. Biol. 12, 1304–1311.
[15] Rush, J.S., Klein, J., Fanti, P., Bhat, N.R. and Waechter, C.J. (1992) Anal. Biochem. 58, 304–310.
[16] Stanwell, C., Gescher, A., Bradshaw, T.D. and Pettit, G.R. (1994) Int. J. Cancer 56, 585–592.
[17] Bruns, R.F., Miller, F.D., Mirriman, R.L., Howbert, J.J., Heat, W.F., Kobayashi, E., Takahashi, I., Tamaoki, T. and Nakano, H. (1991) Biochem. Biophys. Res. Commun. 176, 288–293.
[18] Gopalakrishna, R., Chen, Z.H. and Gundimeda, U. (1992) FEBS Lett. 314, 149–154.
[19] Heesbeen, E.C., Verdonck, L.F., Hermans, S.W.G., van Heugten, H.G., Staal, G.E.J. and Rijksen, G. (1991) FEBS Lett. 290, 231–234.
[20] Hidaka, H., Inagaki, M., Kawamoto, S. and Sasaki, Y. (1984) Biochemistry 23, 5036–5041.
[21] Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. 159, 548–553.
[22] Sullivan, J.P., Connor, J.R., Shearer, B.G. and Burch, R.M. (1991) Agents and Actions 34, 142–144.
[23] Überall, F., Oberhuber, H., Maly, K., Zaknun, J., Demuth, L. and Grunicke, H.H. (1991) Cancer Res. 51, 807–812.