Polyethylene glycol induces apoptosis in HT-29 cells: potential mechanism for chemoprevention of colon cancer

Hemant K. Roy*, John K. DiBaise, John Black, William J. Karolski, Anne Ratashak, Sajid Ansari

Section of Gastroenterology/Hepatology, Department of Internal Medicine, University of Nebraska Medical Center/Eppley Institute for Research into Cancer and Allied Diseases, 982000 Nebraska Medical Center, Omaha, NE 68198-2000, USA

Received 12 April 2001; accepted 19 April 2001

First published online 24 April 2001

Edited by Veli-Pekka Lehto

Abstract Recent experimental evidence suggests that polyethylene glycol (PEG) is a highly effective chemopreventive agent against colon cancer; however, the mechanism(s) remain largely unexplored. To further elucidate this issue, we evaluated the effect of PEG on two human colon cancer cell lines. PEG treatment resulted in a dose- and time-dependent reduction in cell number without alteration in markers of cell proliferation. However, there was a dramatic and specific, concentration-dependent induction of apoptosis, with 50 mM PEG rendering approximately half the cells apoptotic. This corresponded with a 17-fold induction in the expression of the pro-apoptotic protein, prostate apoptosis response-4. Our data suggest that induction of apoptosis may be responsible, at least in part, for the ability of PEG to prevent experimental colon cancer.

Key words: Polyethylene glycol; Chemoprevention; Colon cancer; Apoptosis

1. Introduction

Experimental and clinical studies have demonstrated that a variety of structurally unrelated agents protect against colon cancer; however, limited efficacy and/or potential toxicity of most of these agents have impeded their clinical implementation [1]. Recent data from experimental models of colon cancer suggests that polyethylene glycol (PEG) may be the most effective chemopreventive agent studied to date, surpassing even the non-steroidal anti-inflammatory drugs (NSAIDs) [2–6]. Furthermore, oral PEG solutions have been used safely in humans to treat chronic constipation [7]. While the mechanism of action of PEG has not been explored, chemopreventive agents against colon cancer typically induce epithelial apoptosis and/or inhibit cellular proliferation in colonic epithelial cells [1]. It was our aim, therefore, to investigate the effect of PEG on apoptosis and cell proliferation in human colon cancer cell lines. In order to investigate potential molecular mechanisms involved, we also evaluated the expression of prostate apoptosis response (Par)-4, a protein recently implicated in NSAID-induced apoptosis in colon cancer cells [8].

*Corresponding author. Fax: (1)-402-559 9004.
E-mail: hroy@unmc.edu

2. Materials and methods

2.1. Cell culture

HT-29 and CaCo-2 cells (American Type Tissue Culture, Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium and minimal essential medium, respectively, with 10% fetal calf serum (all from Gibco Lifesciences, Grand Island, NY, USA). Cells were treated with PEG (molecular weight 800), sterile sorbitol or sodium chloride (Sigma, St. Louis, MO, USA).

2.2. Cell viability assay

Cell viability was assessed by measurement of the ability to metabolize 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) performed according to standard protocols [9]. Briefly, cells were grown in 96 well plates, washed and then incubated with MTT for 4 h at 37°C. Production of the tetrazolium salt was measured spectroscopically at absorbance of 560 nm (Titertek Microskan, Flow Laboratories, McLean, VA, USA).

2.3. Apoptosis assays

Apoptosis was quantitated using two distinct assays that we have previously described [10]. Our primary method was through determination of the fraction of cells recognized by the M30 CYTODEATH antibody (Boehringer-Mannheim, Indianapolis, IN, USA) using flow cytometry. This antibody detects a neo-epitope generated by caspase 3 degradation of cytokeratin 18 and, thus, is specific for an early event in apoptosis [11]. We confirmed this by evaluating the subdiploid fraction of propidium iodide-treated cells also by flow cytometry. This method evaluates DNA degradation and, hence, is a later event in apoptosis. We utilized the FACScalibur (Becton-Dickinson, San Jose, CA, USA) flow cytometer for these studies and CellQuest Software (Becton-Dickinson, San Jose, CA, USA) for DNA content analysis while Modfit LT (Verity Software House, Chula Vista, CA, USA) was used for M30 analysis.

2.4. Western blot analysis

Western blot analysis was performed as previously described [12]. Briefly, cellular lysates (50 μg protein) were subjected to SDS–PAGE, transferred to polyvinylidene fluoride membranes, blocked with 5% non-fat milk, and probed with polyclonal antibody to Par-4 and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Xerograms were developed with enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and quantitated with laser densitometry. Equal protein loading was confirmed by India ink staining of membranes and β-actin expression (Sigma, St. Louis, MO, USA).

3. Results

Incubation of the HT-29 and CaCo-2 colon cancer cells with PEG did not result in immediate toxicity, as indicated by trypan blue exclusion (data not shown). However, a dose-dependent reduction in cell number was evident at all time

0014-5793/01/$20.00 © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
PII: S0014-5793(01)02420-6

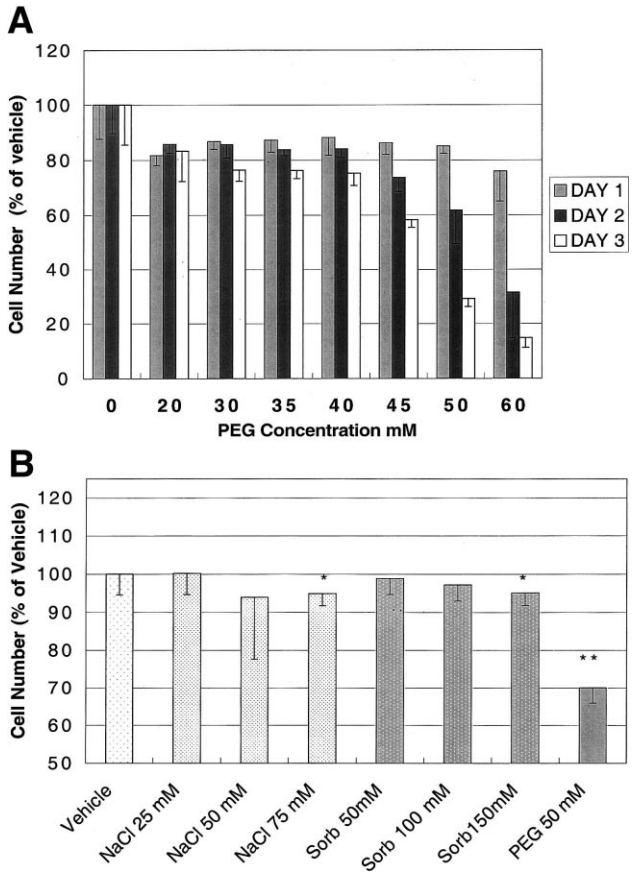

Fig. 1. PEG, but not osmotically equivalent doses of sorbitol or sodium chloride, dramatically decreased HT-29 cell viability as assessed by MTT assay. A: PEG caused a dose-dependent inhibition in cell numbers. All PEG-treated data points were significantly lower ($P < 0.05$) than the corresponding vehicle-treated group. When compared to day 1, PEG concentrations ≥ 45 mM and ≥ 30 mM gave statistically significant cell number reduction at days 2 and 3, respectively. B: Treatment for 3 days with osmotic controls, sorbitol and sodium chloride. $P < 0.05$ versus vehicle.

points assessed (Fig. 1A). There was also a duration-dependent effect detectable at the higher PEG concentrations. Data from the MTT assay were confirmed by manual cell counts (data not shown). In order to exclude the possibility of a non-specific effect from the PEG, we utilized both sorbitol and sodium chloride as osmotic control agents. At concentrations of up to 100 mosm, neither agent decreased cell numbers. However, at 150 mosm, both sorbitol and sodium chloride caused a small but significant decrease in cell numbers (by 5.0% and 5.2%, respectively; $P < 0.05$) (Fig. 1B).

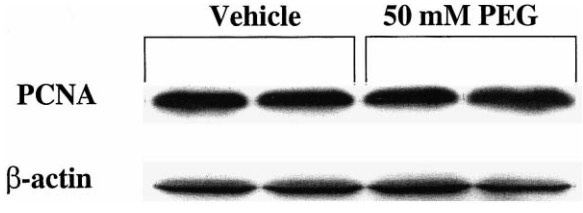

Fig. 2. PEG did not alter expression of PCNA. Representative Western blot assay of two observations of HT-29 treated with vehicle or PEG 50 mM for 3 days. Densitometric analysis of 10 separate experiments did not reveal any significant differences in PCNA expression in the vehicle- versus PEG-treated cells.

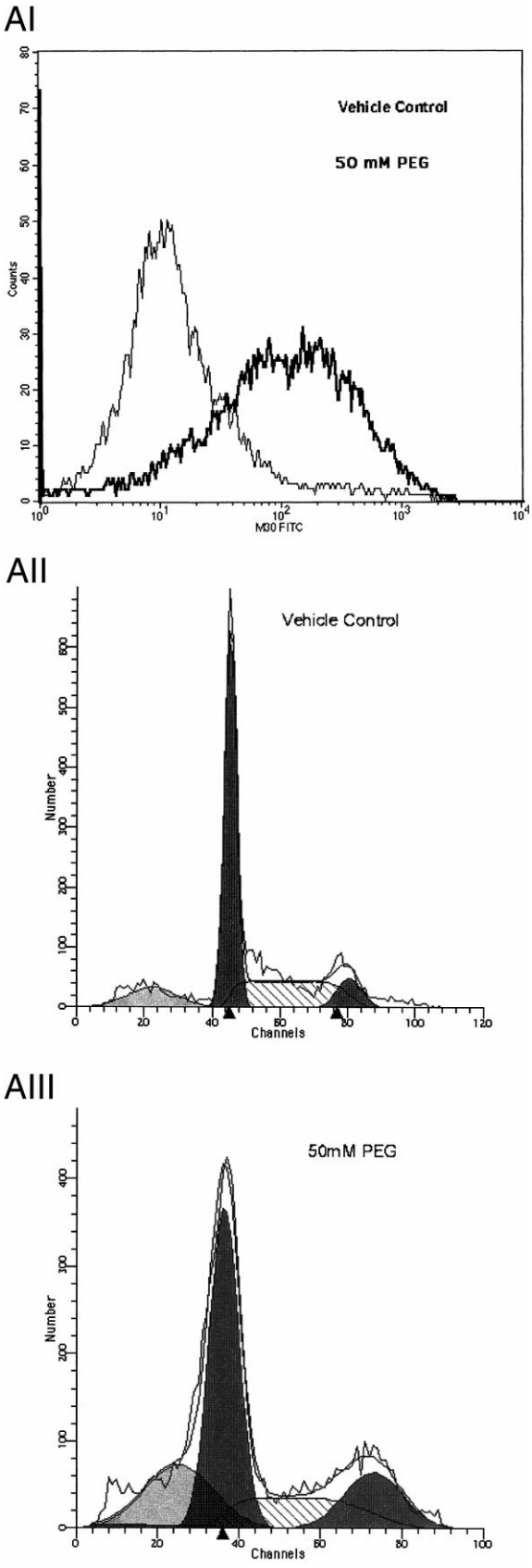

Fig. 3.

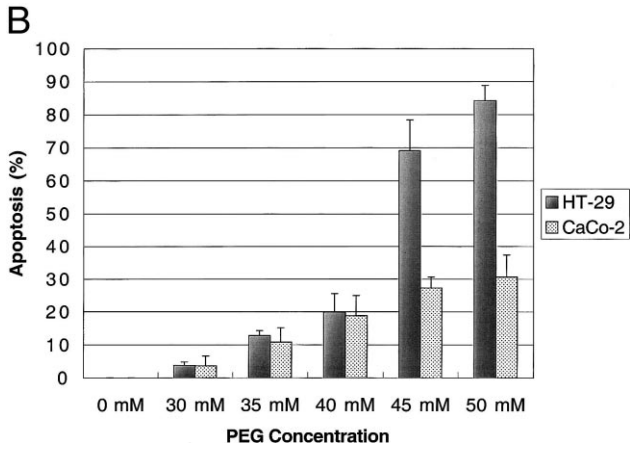

Fig. 3. PEG induced apoptosis in colon cancer cell lines in a dose-dependent fashion. Cells were treated for 3 days with PEG or vehicle. A: Representative assays for apoptosis, all conducted with 50 mM PEG for 3 days. (I) M30 by FACS analysis. The light line represents control cells while the dark line is the PEG-treated cells. PEG causes a marked increase in cells recognized by the M30 antibody. (II) Flow cytometric analysis of DNA content in vehicle-treated cells. The arrowheads represent cells with 2N and 4N DNA content. The subdiploid (apoptotic) fraction is clearly identified. (III) Flow cytometric analysis of DNA content in the PEG-treated group. The arrowhead represents 2N DNA content. There is a marked increase in the subdiploid fraction with PEG treatment. B: Dose-dependent induction of apoptosis by M30-FACS analysis in HT-29 cells and CaCo-2 cells treated for 3 days.

To better understand the mechanism by which PEG decreased cell numbers, we assessed both cell proliferation and apoptosis. Cell proliferation was assessed through PCNA expression, a well-validated marker of proliferative status [13]. Treatment with 50 mM PEG for 5 days had no significant effect on PCNA expression (Fig. 2). Moreover, flow cytometric analysis did not demonstrate any alterations in cell cycle distribution (data not shown). Next, we focused on apoptosis using two distinct methodologies. Our primary method was via assessment of reactivity to the M30 antibody, a well-validated early marker of apoptosis in colon carcinogenesis [14]. PEG resulted in a dose-dependent increase in apoptosis in HT-29 cells (Fig. 3A). Immunocytochemical detection of M30 yielded comparable results to FACS analysis (data not shown). We confirmed the apoptosis fraction by analyzing the subdiploid fraction detected through flow cytometry (Fig. 3A). The similarity of results obtained from M30 and DNA degradation-based methods of apoptosis quantitation is in agreement with our previous work in HT-29 cells [10] and a recent report in human colon carcinogenesis [14]. To ascertain that these effects were not cell line specific, we demonstrated similar although somewhat less dramatic results in the other human adenocarcinoma cell line, CaCo-2 (Fig. 3B).

Finally, in order to investigate the potential mechanisms involved in PEG-mediated apoptosis, we evaluated the expression of the pro-apoptotic protein, Par-4, which has previously been implicated in NSAID-mediated apoptosis in human colon cancer cell lines [8]. Par-4 was faintly detectable in the vehicle-treated cells, but there was a striking 17-fold induction of Par-4 expression after treatment with 50 mM PEG (Fig. 4).

4. Discussion

Some recent reports, utilizing the azoxymethane (AOM) rodent model, have demonstrated a dramatic inhibition of colon carcinogenesis by PEG [2–6]. This established model recapitulates many of the genetic, cellular and pathological events in human colorectal cancer [15]. PEG inhibited AOM-induced aberrant crypt foci, a well-validated intermediate biomarker of colon cancer, by 80%, and suppressed AOM-induced colon tumors by 95%. Importantly, the doses of PEG used did not induce significant alterations in bowel habits or other adverse events [2,3]. In contrast to these reports, Naigamwalla and colleagues failed to demonstrate a protective effect of PEG in a genetic model of intestinal tumorigenesis [6].

Our report is the first to demonstrate a biologically plausible mechanism for PEG, thus, underscoring PEG's role as a chemopreventive agent against colon cancer. We demonstrated, in vitro, that PEG reduced cell number in a time- and dose-dependent manner. While PEG did not inhibit cellular proliferation, it did result in a marked, concentration-dependent induction in apoptosis. Inhibition of apoptosis is a critical early event in neoplasia, allowing the otherwise short-lived colonocyte to acquire the requisite mutations in tumor suppressor genes and proto-oncogenes [16]. Moreover, induction of apoptosis has been a central theme in a variety of chemopreventive agents against colon cancer such as NSAIDs [1]. The molecular mechanisms behind the induction of apoptosis are still not completely understood; however, an alteration in the balance between pro- and anti-apoptotic proteins appears to be of importance [17]. Emerging data, from a variety of tissue types, suggest that Par-4, a zinc-finger-containing transcription factor, may be important in the control of apoptosis [18]. Indeed, a recent study using a human colon cancer cell line demonstrated a marked induction in Par-4 by NSAIDs [8]. Par-4 appears to be important in the control of apoptosis in cells transformed by Ras [19], a proto-oncogene frequently activated in colon carcinogenesis [16]. Par-4 may act at least partly through inhibition of NF-κB and Bcl-2 [20]. Additionally, Par-4 interacts with atypical protein kinase C isoforms [21], such as PKC ζ, which we have previously shown to be an important mechanism in the chemoprevention of colon cancer by NSAIDs [22]. Our data demonstrated that PEG induced a 17-fold induction in Par-4 expression, a finding suggesting a potential mechanism for PEG-mediated apoptosis and chemoprevention. However, because we did not assess other putative modulators of NSAID-induced apoptosis, such as 15-lipoxygenase 1 [23] or BAX/BCL-X_L [24], we cannot conclude that Par-4 upregulation is the sole mechanism for PEG-induced apoptosis.

There is biological precedence for compounds with structural similarity to PEG-inducing apoptosis in cell culture. Treatment of HT-29 and a hepatocellular cancer cell line

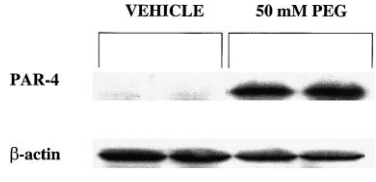

Fig. 4. PEG treatment dramatically induced Par-4. Representative Western blot assay of two observations of HT-29 treated with vehicle or PEG 50 mM for 3 days. Densitometric analysis of ten separate experiments revealed a 17-fold induction in Par-4 expression with PEG treatment ($P < 0.001$).

with Triton X-100 resulted in apoptosis [25,26]. Similar data have been reported with two other non-ionic detergents, Tween 20 and NP-40 [27]. Induction of apoptosis with these agents may be related to caspase 3 activation [27], potentially through alterations in dimerization or in subcellular distribution of proteins in the Bcl-2 family [28]. While PEG shares many chemical similarities with these agents, particularly side-chain composition, it lacks much of their detergent ability. Many of these detergent studies do not exclude the possibility that their osmotic properties alone may lead to apoptosis, as has been previously demonstrated in cell culture [29]. In our report, the lack of effect on cell numbers from osmotic controls strongly supports a specific role of PEG. Another potential confounding factor is PEG-induced cell fusion which has been described in HT-29 cells, but at very high PEG concentrations (27% v/v) [30]. We were unable to detect any evidence of this either by microscopy or flow cytometric analysis of DNA content, again, arguing for a specific effect of PEG.

The moieties on the PEG molecule that determine its apoptotic and, hence, chemopreventive effect have not been explored; however, the molecular weight of PEG appears to be important. While a wide range of PEG species (molecular weights 400–35 000) have been shown to inhibit AOM-induced intermediate biomarkers for colon cancer, the peak efficacy was at a molecular weight of 8000 [3]. Our study cannot address this issue due to the limited solubility of PEG in cell culture. However, the potential for designing superior PEG derivatives is underscored by the recent demonstration that pluronic F68 block polymer is five times more potent than the parent PEG molecule [31].

In conclusion, we demonstrate for the first time that PEG induces apoptosis in a time- and dose-dependent fashion in human cancer cell lines. Our data suggest that induction of Par-4 may be responsible, at least in part, for this decrease in cell survival. These observations provide another compelling line of evidence that reinforces PEG's potential as a chemopreventive agent against colorectal cancer. Further studies are necessary to completely understand the mechanisms involved.

References

[1] Janne, P.A. and Mayer, R.J. (2000) New Engl. J. Med. 342, 1960–1968.
[2] Corpet, D.E. and Parnaud, G. (1999) Carcinogenesis 20, 915–918.
[3] Parnaud, G., Tache, S., Peiffer, G. and Corpet, D.E. (1999) Cancer Res. 59, 5143–5147.
[4] Wali, R.K., Skarosi, S., Hart, J., Zhang, Y., Dolan, M.E., Moschel, R.C., Nguyen, L., Mustafi, R., Brasitus, T.A. and Bissonnette, M. (1999) Carcinogenesis 20, 2355–2360.
[5] Corpet, D.E., Parnaud, G., Delverdier, M., Peiffer, G. and Tache, S. (2000) Cancer Res. 60, 3160–3164.
[6] Naigamwalla, D., Chia, M.C., Tran, T.T., Medline, A., Hay, K., Gallinger, S. and Bruce, W.R. (2000) Cancer Res. 60, 6856–6858.
[7] DiPalma, J.A., DeRidder, P.H., Orlando, R.C., Kolts, B.E. and Cleveland, M.B. (2000) Am. J. Gastroenterol. 95, 446–450.
[8] Zhang, Z. and DuBois, R.N. (2000) Gastroenterology 118, 1012–1017.
[9] Rahman, M.A., Dhar, D.K., Masunaga, R., Yamanoi, A., Kohno, H. and Nagasue, N. (2000) Cancer Res. 60, 2085–2089.
[10] Roy, H.K., Karolski, W.J., Ratashak, A. and Smyrk, T.C. (2001) Br. J. Cancer, in press.
[11] Vermes, I., Haanen, C. and Reutelingsperger, C. (2000) J. Immunol. Methods. 243, 167–190.
[12] Roy, H.K., Karolski, W.J. and Ratashak, A. (2001) Int. J. Cancer, in press.
[13] Lohr, F., Wenz, F., Haas, S. and Flentje, M. (1995) Cell Prolif. 28, 93–104.
[14] Carr, N.J. (2000) Arch. Pathol. Lab. Med. 124, 1768–1772.
[15] Banerjee, A. and Quirke, P. (1998) Dis. Colon Rectum 41, 490–505.
[16] Chung, D.C. (2000) Gastroenterology 119, 854–865.
[17] Hengartner, M.O. (2000) Nature 407, 770–776.
[18] Sells, S.F., Han, S.S., Muthukkumar, S., Maddiwar, N., Johnstone, R., Boghaert, E., Gillis, D., Liu, G., Nair, P., Monnig, S., Collini, P., Mattson, M.P., Sukhatme, V.P., Zimmer, S.G., Wood Jr., D.P., McRoberts, J.W., Shi, Y. and Rangnekar, V.M. (1997) Mol. Cell. Biol. 17, 3823–3832.
[19] Barradas, M., Monjas, A., Diaz-Meco, M.T., Serrano, M. and Moscat, J. (1999) EMBO J. 18, 6362–6369.
[20] Camandola, S. and Mattson, M.P. (2000) J. Neurosci. Res. 61, 134–139.
[21] Diaz-Meco, M.T., Municio, M.M., Frutos, S., Sanchez, P., Lozano, J., Sanz, L. and Moscat, J. (1996) Cell 86, 777–786.
[22] Roy, H.K., Bissonnette, M., Frawley, B.P., Wali, R.K., Niedziela, S.M., Earnest, D. and Brasitus, T.A. (1995) FEBS Lett. 366, 143–145.
[23] Shureiqi, I., Chen, D., Lee, J.J., Yang, P., Newman, R.A., Brenner, D.E., Lotan, R., Fischer, S.M. and Lippman, S.M. (2000) J. Natl. Cancer Inst. 92, 1136–1142.
[24] Zhang, L., Yu, J., Park, B.H., Kinzler, K.W. and Vogelstein, B. (2000) Science 290, 989–992.
[25] Borner, M.M., Schneider, E., Pirnia, F., Sartor, O., Trepel, J.B. and Myers, C.E. (1994) FEBS Lett. 353, 129–132.
[26] Ahn, J.M., Kim, S.J., Kim, H., Park, C., Kim, W.H. and Park, J.H. (1997) Yonsei Med. J. 38, 52–59.
[27] Strupp, W., Weidinger, G., Scheller, C., Ehret, R., Ohnimus, H., Girschick, H., Tas, P., Flory, E., Heinkelein, M. and Jassoy, C. (2000) J. Membr. Biol. 175, 181–189.
[28] Hsu, Y.T. and Youle, R.J. (1997) J. Biol. Chem. 272, 13829–13834.
[29] Mockridge, J.W., Benton, E.C., Andreeva, L.V., Latchman, D.S., Marber, M.S. and Heads, R.J. (2000) Biochem. Biophys. Res. Commun. 273, 322–327.
[30] Laboisse, C.L., Maoret, J.J., Triadou, N. and Augeron, C. (1988) Cancer Res. 48, 2498–2504.
[31] Parnaud, G., Tache, S., Peiffer, G. and Corpet, D.E. (2001) Br. J. Cancer 84, 90–93.