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Effects of parabens on apoptosis induced by serum-free medium

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ABSTRACT

Alkyl esters of $p$-hydroxybenzoic acids (parabens), an endocrine disrupter, are used as preservatives in cosmetics and foods. In this study, to
understand the relationship between parabens and differentiation in infants, the effects of parabens on apoptosis induced by serum deprivation in PC12 cells were investigated. In addition, apoptosis-related factors were assayed. As results, a tendency toward enhancement of apoptosis was observed in the cells cultured in the serum-free medium with methylparaben, and this tendency was suggested to be related to the contents of Bad. Butylparaben did not show any tendency to enhance apoptosis.

**Keywords:** PC12 cell, endocrine disrupter, food additives, apoptotic factors, methylparaben, butylparaben

**INTRODUCTION**

Parabens, alkyl esters of \( p \)-hydroxybenzoic acid, are most commonly used as preservatives in foods, cosmetics, toiletries and pharmaceuticals because of their relatively low toxicity in humans and their effective antimicrobial activity. \( p \)-Hydroxy substitution on the aromatic ring has been recognized as an
important requirement for the estrogenic activity of some alkylphenols.\textsuperscript{[1]}

Parabens are structurally similar to alkylphenols. These compounds exert weak estrogenic activity as described by Lemini et al.\textsuperscript{[2]}, Routledge et al.\textsuperscript{[3]}, Hossaini et al.\textsuperscript{[4]} and Byford et al.\textsuperscript{[5]} The magnitude of the estrogenic response increases with the alkyl group size, as shown by the fact that ethylparaben, propylparaben, and butylparaben are approximately 150,000-, 30,000- and 10,000-fold less potent than 17\(\beta\)-estradiol.\textsuperscript{[6]}

Some studies have examined the effects of early exposure to environmental toxicants using embryos.\textsuperscript{[7-8]} They reported that exposure to some chemicals increased mortality, induced morphologic deformations, increased apoptosis, and accelerated embryogenesis in the embryo stage. White\textsuperscript{[9]} reported growth effects of methylparaben, propylparaben and EDTA on a femur weight in organ cultures of embryonic chicks. The methylparaben and propylparaben concentrations found to stimulate growth were 0.25 to 2.5 \(\mu\)g/mL and 0.025 to 2.5 \(\mu\)g/mL, respectively. These results suggested that parabens might have some effects on infants and embryos. From this viewpoint, there were some interesting reports that endocrine disrupters affected apoptosis in cells.\textsuperscript{[10-13]}
Apoptosis is a morphological and biochemical description of a physiological cell death mechanism that is commonly associated with programmed events necessary for the differentiation and development of individuals and organs.\textsuperscript{[14-15]} Maroto and Perez-Polo \textsuperscript{[15]} wrote that although apoptosis is often referred to as programmed cell death, the two terms are not synonymous. Apoptotic events are not programmed but rather occur in response to environmental cellular signals in many instances.\textsuperscript{[16]} The activation of apoptosis by the signals and its role in organisms need to be studied. Apoptotic cell death is characterized by chromatin condensation, DNA fragmentation and membrane blebbing, resulting in the formation of apoptotic bodies.\textsuperscript{[14]} Apoptosis is regulated and executed by two major protein families, the Bcl-2 and caspase families, which are highly conserved from worms to humans.\textsuperscript{[17-18]} Each caspase is synthesized as an inactive precursor, which is activated only after a cell has received a death signal.\textsuperscript{[19]} The Bcl-2 family, which is involved in the regulation of caspase activity, is subdivided into pro-apoptotic (for example, Bax and Bad) and anti-apoptotic (for example, Bcl-2) members, which share one or more similar regions.\textsuperscript{[20-21]} However, the influence of parabens on apoptosis in cultured cells has not been
In rat pheochromocytoma PC12 cells, apoptosis is induced when the cells are cultured in serum-free medium. Apoptotic PC12 cells exhibit DNA fragmentation.\textsuperscript{[22-23]} Thus, these cells have proven to be a useful model for studying the mechanism of induction and inhibition of apoptosis.\textsuperscript{[15, 24]}

In this study, to determine the effects of parabens in differentiation of embryos and infants, their effects on apoptosis induced by serum deprivation were investigated using the PC12 cell system. In addition, apoptotic factors such as caspase-3 and the Bcl-2 family in PC12 cells exposed to parabens were measured by enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase (RT)-PCR.

\textbf{MATERIALS AND METHODS}

\textbf{Materials}

PC12, a rat pheochromocytoma cell line, was purchased from the American Type Culture Collection (USA and Canada). Dulbecco’s
modified Eagle’s medium (DMEM), streptavidin-conjugated peroxidase,
phosphate-citrate buffer with sodium perborate capsules and
o-phenylenediamine dihydrochloride (OPD) were from Sigma (St. Louis,
MO, USA). Fetal bovine serum (FBS) was from HyClone (Rockville, MD,
USA). The caspase assay kit, SV total RNA isolation kit, RT-PCR kit and
access RT-PCR introductory kit were from Promega (Madison, WI, USA).
Methyl p-hydroxybenzoate (methylparaben) and butyl p-hydroxybenzoate
(butylparaben) were obtained from Nacalai Tesque (Kyoto, Japan). Biotin
16-dUTP, proteinase K and the blocking reagent were from Roche
Diagnostics (Mannheim, Germany). Polyclonal antibodies against Bax,
Bad and Bcl-2 were from Oncogene Research Products (Boston, MA,
USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA). The
secondary antibody, biotinylated donkey anti-rabbit immunoglobulin, was
from Amersham Pharmacia Biotech (Buckinghamshire, UK). The DNA
7500 assay kit and RNA 6000 Nano Assay kit were from Agilent
Technologies (Waldbonn, Germany). Other chemicals were of analytical
reagent grade. Methylparaben and butylparaben were dissolved in ethanol
and used as the vehicle.
Cell Culture and Exposure Conditions

PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO₂. Inactivation of FBS was carried out at 56°C for 30 min. The cells were preincubated in 25 cm² flasks (Nunc, USA) for two or three days, and then the medium was replaced with serum/serum-free DMEM with or without methylparaben or butylparaben as follows.

Cells were incubated in serum-free DMEM, 0, 0.001, 0.01, 0.1 and 10 mg/mL (final concentration) of methylparaben or butylparaben for one, two and three days. Five µL of a 1000-fold concentration of methylparaben or butylparaben in ethanol was added to the 5 mL cell medium. Five microliters of ethanol was added to the serum-free DMEM and DMEM supplemented with 10% FBS as positive and negative control of apoptosis, respectively. When the medium was changed to serum-deprived medium, cells in the flask were washed twice with serum-free DMEM.

Oberdoerster et al. [25] reported that 150mM ethanol-induced enhancement of cell death by serum deprivation increased the extent of apoptosis in
PC12 cells. Furthermore, Neuman et al. \cite{26} observed that 100 mM ethanol induced apoptosis in cultured skin cells, through 40 mM ethanol rarely affected apoptosis in the cells. Based on these reports, it was presumed that using ethanol at a concentration of 1 µL/mL (approximately 17 mM) in this study did not affect apoptosis in PC12 cells.

**Nuclear DNA Isolation**

After that the PC12 cells were treated with 0 to 10 µg/mL methylparaben and butylparaben, and 1 µL/mL ethanol as a control for three days. The cells were then harvested using a scraper, and centrifuged at 1,500 rpm for 10 min. Then the supernatants were removed and the cells were washed twice at 1,500 rpm with 10 mM Tris-5 mM HCl containing 150 mM NaCl for 5 min. The obtained cells were incubated with 10 mM Tris-5 mM HCl, 0.1 M EDTA, 0.5% SDS and 20 µg/mL pancreatic RNAase for 1 hr at 37°C. Proteinase K was added to the suspension up to 100 µg/mL, and the enzyme reaction was carried out for 3 hr at 50°C. After cooling, the nuclear DNA was serially extracted with phenol and chloroform.
160 Quantification of DNA Fragmentation in PC12 Cells by the
161 TdT-mediated dUTP-biotin Nick End Labeling (TUNEL) Method

164 The DNA obtained from PC12 cells treated with methylparaben and
165 butylparaben was resuspended in 100 mM Tris-HCl, pH 8.0, containing 1
166 mM EDTA, and equal amounts of DNA were put into 96-well plates.

167 Quantification of DNA fragmentation in PC12 cells was done by the
168 method of Kurasaki et al. [27]

170 ELISA of Apoptotic Factors in PC12 Cells

172 The PC12 cells were incubated with 0 to 10 µg/mL methylparaben or
173 butylparaben, or 1 µL/mL ethanol as a control for one, two, or three days.
174 After the cultivation, the cells were harvested using a scraper. The
175 harvested cells were washed twice at 1,500 rpm with 10 mM Tris-5 mM
176 HCl buffer containing 150 mM NaCl for 5 min. The cells were then
177 resuspended in 10 mM Tris-5 mM HCl buffer containing 150 mM NaCl
178 and disrupted by sonication for 30 sec with a Sonifier 250 (Branson). The
cell debris and unbroken cells were removed by centrifugation. The
contents of Bad, Bax and Bcl-2 in the cells were measured by the method
of Yamanoshita et al.\textsuperscript{[12]} The cell extracts after incubation with parabens
were adjusted to equal protein concentrations with 40 mM Tris-5, mM
HCl containing 150 mM NaCl for ELISA. The primary antibody for Bad,
Bax or Bcl-2 (diluted 1:250 with 1% [v/v] blocking reagent in the same
buffer) was reacted with antigens for 1 hr. The contents of the three
factors were calculated from the absorbance measured at 405 nm with a
Microplate Reader model 450 (BIO-RAD).

\textbf{RNA Isolation}

The total RNA of PC-12 cells treated with methylparaben or butylparaben
was extracted using an SV total RNA isolation kit according to the
instruction manual after the cells were treated with parabens. The details
are described in the legend of Figure 5. The total RNA obtained was
verified using a RNA 6000 Nano Assay kit with an Agilent 2100 Bio
Analyzer. Total RNA was stored at -20°C until use for one or two days.
Expression of bad, bax and bcl-2 mRNAs, in the PC12 cells exposed to parabens was detected using the Access RT-PCR kit and Access RT-PCR introductory kit according to the manufacturer’s instructions. The PCR primers of bad, bax, bcl-2 and β-actin were designed from their DNA sequences. The samples were heated at 48°C for 45 min and subsequently at 94°C for 2 min for enzyme reaction. The time course of one cycle was 94°C for 30 s for denaturation, 60°C for β-actin and bax, 55°C for bad and 47°C for bcl-2 for 1 min for annealing, and 68°C for 2 min for extension. The number of cycles performed was 30 or 40. β-Actin was chosen as an internal control. PCR products were verified using a DNA 7500 Assay kit with a 2100 Bio Analyzer (Agilent Technologies, USA) according to the manufacturer’s instructions.

Measurement of Caspase-3 Like Activity

PC12 cells were incubated in the medium containing a caspase inhibitor, Z-VAD or Z-VAD-free medium with 0 to 10 µg/mL methylparaben or 1
\[ \text{µL/mL ethanol as a control for two days. The activity of caspase-3-like protease was detected using the Caspase Assay System kit. The activity of caspase-3 like protease in the PC12 cells was indirectly measured as absorbance at 405 nm depending on the pNA released from the cleaved substrate. After overnight incubation, absorbance at 405 nm was measured as the caspase-3 like activity.} \]

**Statistical Analysis**

Each value is expressed as mean ± SEM. Analysis of variance (ANOVA) followed by the Bonferroni/Dunn multicomparison test was employed. Statistical analyses were performed using StatView (HULINKS, Tokyo, Japan).

**RESULTS**

**Effects of Methylparaben and Butylparaben on Apoptosis**

To study the effects of methylparaben and butylparaben on apoptosis,
TUNEL signals from nuclear DNA in PC12 cells cultured in serum-free medium and treated with 0.001, 0.01, 0.1, 1 and 10 µg/mL methylparaben or butylparaben for three days were measured (Fig. 1).

The TUNEL signals in the cells cultured in the serum-deprived medium were significantly increased as compared with those in the control cells.

The signals in the DNA of the cells treated with methylparaben showed a tendency to increase (Fig. 1a). On the other hand, TUNEL signals in the cells treated with butylparaben at various concentrations showed no significant differences (Fig. 1b).

These changes did not show the dose-response effect. However these kinds of irregular responses were showed in similar experiments. [13, 29]

Contents of Apoptotic Factors Measured by ELISA

To understand the reason for the different tendencies of TUNEL signals between the PC12 cells treated with methylparaben and butylparaben, the contents of the apoptotic factors Bad, Bax and Bcl-2 in the PC12 cells cultured in serum-free medium containing 0.001, 0.01, 0.1, 1 or 10 µg/mL methylparaben or butylparaben for one, two or three days, were measured
After one-day exposure, the contents of Bad increased according to the increase in the concentration of methylparaben as expected (Fig. 2a). Bad contents in the cells exposed to 0.1 and 10 µg/mL methylparaben for one day increased significantly (P<0.05). After two days of exposure, the contents of Bad increased slightly in the cells treated with methylparaben (Fig. 2b). Bad contents increased significantly (P<0.05) in cells treated with 1 µg/mL methylparaben for two days. Unexpectedly, however, there was no significant difference among Bad contents in cells treated with the various concentrations of methylparaben for three days (Fig. 2c). There was no significant difference among Bad contents in the cells treated with butylparaben for one, two or three days at the various concentrations as expected (Figs. 2d-f).

In addition, there was no significant difference among Bax contents in the cells treated with methylparaben or butylparaben for one, two or three days at the various concentrations (Fig. 3).

Bcl-2 contents in the cells exposed to 0.1 µg/mL methylparaben for one day increased significantly (P<0.05; Fig. 4a). However, there was no significant difference among Bcl-2 contents in the cells treated with
methylparaben for two and three days at the various concentrations (Figs. 274b and c).

There was no significant difference among Bcl-2 contents in the cells treated with butylparaben for one, two or three days at the various concentrations (Figs. 4d-f).

Expression of Apoptotic Factor mRNAs Detected by RT-PCR

To verify changes of apoptotic factors in PC12 cells treated with methylparaben or butylparaben, the expression of bad, bax and bcl-2 mRNAs in PC12 cells cultured in the serum-free medium with 0.001, 0.01, 0.1, 1 or 10 µg/mL methylparaben or butylparaben added for one, two or three days, was measured by RT-PCR.

There was a tendency for the expression of bad mRNA to increase in the cells treated with methylparaben after one day similar to the changes of Bad contents detected by ELISA in the cells treated with methylparaben for one and two days (Figs. 5a and b). There was no significant difference of bad mRNA expression among cells treated with methylparaben for two and three days at the various concentrations (Fig. 5c), the same as seen in
Figure 2C. After one- and two-day exposures, there was a tendency for the bad mRNA in the cells treated with butylparaben to increase (Figs. 5d and e). There was no significant difference of bad mRNA expression among the cells treated with butylparaben for three days at 0.001 to 1 µg/mL (Fig. 5f). However the cells died as a result of treatment with 10 µg/mL butylparaben for two and three days (Figs. 5e and f). There was no significant difference among bax and bcl-2 mRNAs in the cells treated with methylparaben or butylparaben for one, two or three days at the various concentrations (data not shown).

**Measurement of Caspase-3 Like Activity**

To investigate whether apoptosis-related factors under the conditions of apoptosis induced by serum deprivation were changed by addition of methylparaben, caspase-3 activity was measured in PC12 cells cultured in serum-free medium containing methylparaben (Fig. 6). The caspase-3 like activity in the cells increased with increasing concentrations of methylparaben as expected. On the other hand the activity in the cells did not change with butylparaben (data not shown).
The aim of this experiment was to study effects of parabens on apoptosis induced in advance. PC12 cells are well known to be a useful model for studying the mechanisms of induction and inhibition of apoptosis under conditions with and without serum, because apoptosis is induced by serum deprivation in the cells. In our laboratory the influence of endocrine disrupters on apoptosis induced by serum deprivation has been investigated using PC12 cells.\cite{12-13, 30} When serum is present, apoptosis was not induced by addition of parabens (data not shown). This means that parabens cannot induce apoptosis itself. On the other hand, under serum deprivation, a tendency for apoptosis to be induced occurred after addition of methylparaben in the \textit{in vitro} model system described above (Fig. 1a). This means that there is a possibility for enhancement of apoptosis by addition of methylparaben. In this study serum was used as a regulatory factor for apoptosis. The any tendency for apoptosis to be induced by serum deprivation did not show in the cells treated with various concentrations of butylparaben (Fig. 1b).
It was reported that the binding of parabens to the estrogen α-receptor (ERα) of MCF7, human breast cancer cells, increased with increasing alkyl chain length. Moreover, the estrogenic activity of parabens has been demonstrated to increase with increasing length of the linear alkyl chain from methylparaben to butylparaben. In addition, $4 \times 10^{-4}$ M methylparaben and $3 \times 10^{-6}$ M butylparaben showed 10% of the activity of $10^{-7}$ M 17-β estradiol. These concentrations of parabens are more than ten mg/mL. However, in the present study, the TUNEL signals showed no relationship between alkyl group size and effects on apoptosis in PC12 cells treated with parabens (Figs. 1a and b). From the results, it was indicated that the effects of parabens on apoptosis in the cells were independent of the function of ERα, because PC12 is an ERα-negative cell line.

To study whether enhancement of apoptosis by treatment with methylparaben or butylparaben was regulated by the apoptotic factors Bax, Bad and Bcl-2, we measured the relative contents of these apoptotic factors in PC12 cells treated with methylparaben or butylparaben under serum-free conditions using ELISA and RT-PCR. The content of Bad, a pro-apoptotic protein, was slightly increased with increasing
concentrations of methylparaben for one day (Fig. 2a). However, no
significant effects were seen on the expression of bad mRNA, although
there was a tendency toward an increase after one-day exposure. No
changes were observed in the contents of Bax and Bcl-2 under the same
conditions. These results could indicate that the enhancement of
apoptosis in the PC12 cells cultured in serum-free medium containing
methylparaben could be associated with an increase of Bad content.
Moreover, caspase-3 activity was increased in the cells treated with 0.001
to 10 µg/mL methylparaben (Fig. 6). It was considered that the
enhancement of apoptosis in the PC12 cells treated with methylparaben
was mediated by the caspase-3 pathway.
Paraben-induced cytotoxicity has been speculated to depend not only on
the dose of paraben, but also on the length of the exposure period.
However, the contents of the apoptotic factors in the cells treated with
parabens differed markedly at one day as compared with those for two and
three days. Maroto and Peres-Polo[15] reported that expression of Bad and
Bax was decreased by serum deprivation for 3hr and 24hr in PC12 cells.
However, in this study, the tendency for Bad contents to increase in cells
treated with methylpareben was only observed on day one, although the
contents of Bax and Bcl-2 showed no changes under the same conditions for one to three days.

In this study, PC12 cells died after the treatment with butylparaben at 10 µg/mL (5.15 × 10^{-5} M) (Figs. 5e-f). Darbre et al. \cite{31} reported that benzylparaben was cytotoxic at a concentration of 10^{-4} M for ERα-negative MDA-MB-231 human breast cells. On the other hand, methylparaben showed cytotoxicity at about 400 µg/mL in human THP-1 cells.\cite{32} With the experimental concentration (maximum 10 µg/mL) in this study, methylparaben did not show any cytotoxicity.

Janjua et al. \cite{33} reported that butylparaben peaked in serum after a few hours, reaching 81 µg/L after daily whole-body topical application of a 2 mg/cm² basic cream formulation (2% [w/w]) for two weeks. In addition, five days after the treatment about 20 µg/L butylparaben remained in the serum. Gelb et al. \cite{34} investigated effects of intravenously administered methylparaben on cerebral blood flow in the human brain. They reported that the concentration of methylparaben in serum was more than 30 µg/L.

On the basis of these reports, the concentrations of methylparaben and butylparaben used in this study were in the expected range in human blood after uptake of the materials containing parabens.
In this study, to investigate the effects of parabens on differentiation and development, the relationship between apoptosis induced by serum deprivation in PC12 cells and parabens was studied. Apoptosis is a physiological cell death mechanism that is commonly associated with programmed events necessary for the differentiation and development of individuals and organs.\(^{[14-15]}\) We found that there was no significant effect of parabens on induced apoptosis in PC12 cells. However, methylparaben showed a tendency to enhance apoptosis (Fig. 1). In addition, methylparaben slightly increased the contents of Bad (Fig. 2) and caspase-3 activity (Fig. 6), thereby possibly further enhancing the apoptosis induced by the serum deprivation. As parabens are hydrophobic compounds, they can be transferred from the maternal body to infants.\(^{[7-8]}\)

**CONCLUSION**

From the results, a tendency toward enhancement of apoptosis was observed in the cells cultured in the serum-free medium with methylparaben, and this tendency was suggested to be related to the contents of Bad. Butylparaben did not show any tendency to enhance
apoptosis. Exposure to trace levels of some chemicals increases morphologic deformations and caused adverse effects on embryogenesis in the embryo stage. The human population is chronically exposed to parabens through many thousands of foods, drugs and cosmetic products. From this viewpoint, to clarify the detailed effects of long-term exposure to parabens, further investigation is needed.

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FIGURE CAPTIONS

Figure 1. Effects on DNA fragmentation in PC12 cells treated with methylparaben (a; n=6) or butylparaben (b; n=5) for three days. The TUNEL signals of DNA fragmentation are presented as relative percentages against those of cells cultured in serum-free medium containing 1 µL/mL ethanol. Asterisks denote values significantly different (**P<0.05) or having a tendency to be different (*P<0.1) from cells incubated with serum-free medium without parabens. (mean ± SEM)

Figure 2. Contents of Bad in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of Bad are presented as relative percentages against those of cells cultured in serum-free medium containing 1 µL/mL ethanol for one day. The mean ± SEM was calculated four (a, b, c) and five (d, e, f) independent experiments. Asterisks denote values significantly different (**P<0.05) from these of cells incubated in serum-free medium without paraben.

Figure 3. Contents of Bax in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of Bax are presented as relative percentages against those of cells cultured in serum-free medium containing 1 µL/mL ethanol for one day. The mean ± SEM was calculated four (a, b, c) and five (d, e, f) independent experiments.

Figure 4. Contents of Bcl-2 in PC12 cells treated with methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f)
days. The contents of Bcl-2 are presented as a relative percentage against those of cells cultured in serum-free medium containing 1 µL/mL of ethanol for one day. The mean ± SEM was calculated for four (a, b, c) and five (d, e, f) independent experiments. Asterisk denotes a value significantly different (**P<0.05) from cells incubated in serum-free medium without parabens.

**Figure 5.** Contents of bad mRNA in PC12 cells treated with 0 to 10 µg/mL methylparaben (a, b, c) or butylparaben (d, e, f) for one (a, d), two (b, e) and three (c, f) days. The contents of bad mRNA are presented as relative percentages against those of cells cultured in medium containing serum and 1 µL/mL ethanol. The mean ± SEM was calculated from three (one day) and five (two and three days) independent experiments.

**Figure 6.** The specific activity of caspase-3 in PC12 cells treated with 0.001 to 10 µg/mL methylparaben and serum deprivation for two days. The specific activity of caspase-3 is presented as a relative value against that of the cells cultured in medium containing serum and 1 µL/mL ethanol as a control. The values are means from two representative experiments.
Fig. 1

Relative index of DNA fragmentation (%)

- serum + ethanol
- serum-free + ethanol (Control)
- serum-free + parabens

(a) Serum

(b) Parabens

parabens: 0, 0.001, 0.01, 0.1, 1, 10 (µg/mL)
Fig. 4

(a) Relative contents of Bcl-2 (%) for serum + ethanol, serum-free + ethanol (Control), and serum-free + parabens.

(b) Effect of parabens on Bcl-2 content (mg/mL).

(c) Comparison of Bcl-2 content at different concentrations of parabens.

(d) Graph showing Bcl-2 content for varying paraben concentrations.

(e) Bcl-2 content for different paraben concentrations.

(f) Graph illustrating Bcl-2 content at various paraben concentrations.