

# The effect of chemically induced differentiation on caspase 8 and Caspase 10 mRNA levels in Neuroblastoma cells

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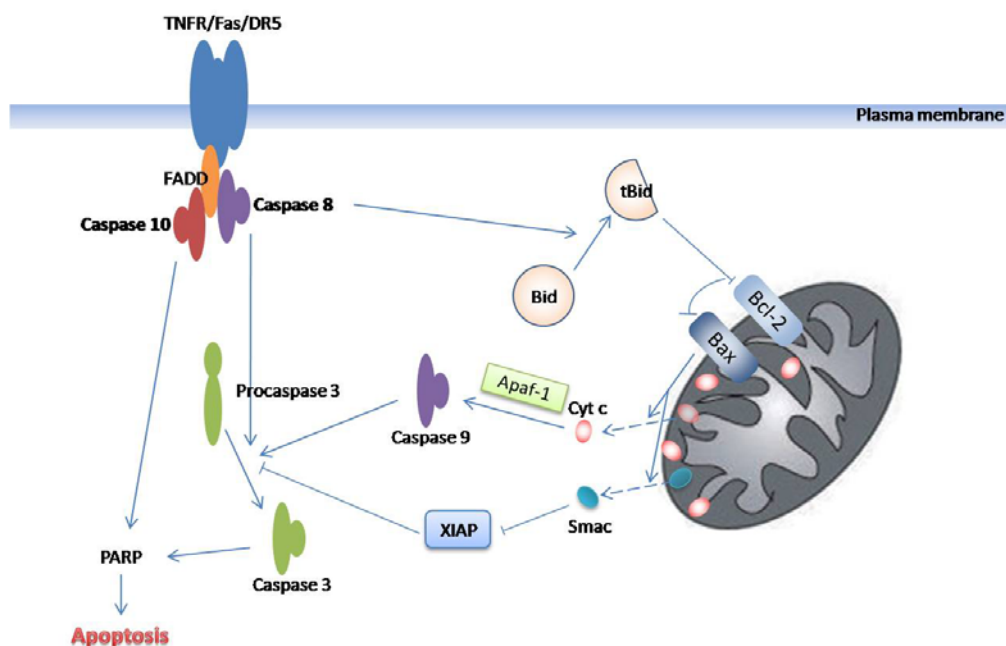
## Abstract

Neuroblastoma is a pediatric extracranial solid tumor. They can be categorized into three types based on morphological and biochemical properties: N-type (neuroblastic), S-type (Schwann/glial) and I-type (intermediate). Retinoic acid (RA) and BUdR are drugs that can induce I-type cell differentiation into N-type and S-type cells respectively, resulting in suppression of cell proliferation. In order to find out whether RA and BUdR can inhibit cancer growth by endowing the cells with the capacity to die when necessary, RT-PCR was carried out to detect the mRNA levels of caspase 8 and 10, two apoptosis initiators, in untreated, RA- and BUdR-treated I-type neuroblastoma cell line, BE-(2)-C. The result showed that caspase 8 transcripts in treated cells increased to more than two fold compared to the untreated cells, while there is no such effect on caspase 10 expression. These results suggest that the two differentiation agents may sensitize neuroblastoma cells to various apoptotic signals.

## Introduction

Caspase 8 and caspase 10 are aspartate-specific cysteine proteases involved in programmed cell death initiation. Caspase 8 can be activated through autocleavage of procaspase 8 recruited by

Fas/TNFR/DR5 (TRAIL receptor) activation mediated FADD (Fas-associating protein with death domain) binding, known as the extrinsic apoptotic pathway. The active form of caspase 8 in turn activates caspase 3 (death executor), which leads to subsequent DNA and protein degradation. Caspase 8 is also involved in the intrinsic pathway of apoptosis, where it truncates Bid to give rise to tBid, a Bcl-2 inhibitor. The inhibition of Bcl-2 will release ion-channel formed by Bax, which changes the membrane potential of mitochondrial outer membrane and leads to the release of cytochrome c and Smac/DIABLO (Cui, 2005). Cyt c associates Apaf-1 and cleaves the inactive procaspase 9, which in turn activate caspase 3. Smac/DIABLO is an inhibitor of IAP family proteins that inhibit the protease activity of caspase through binding (caspase 8/10-induced apoptosis pathway is illustrated in Figure 1).



**Figure 1** Caspase 8/10 in apoptotic signaling pathway

The deficiency of caspase 8 expression is observed in a majority (Jiang, 2008; Eggert, 2001) or at least more than half (Harada, 2002) of neuroblastoma (a pediatric solid tumor, arises from neural crest) cell lines and primary tumor tissues. Their resistance to FasL, TNF- $\alpha$  (Kim, 2004),

TRAIL (Eggert, 2001) or p53 mediated apoptosis (Ding, 2000) and other chemo- (Hannum, 1997)/immuno-/radiation therapy is correlated to this deficiency. Restoration of caspase 8 expression by demethylation reagents (Fulda, 2001; Casciano, 2004) or gene transfer (Fulda, 2001) increased the responsiveness of neuroblastomas to apoptotic signals, while treatment with caspase 8 inhibitor Z-IETD-FMK reduced the caspase-8-dependent anoikis induced by detachment (Bozzo, 2006), which determines the metastasis capacity of neuroblastoma (Stupack, 2006). The expression of caspase 8 also has a negative correlation with the aggressiveness of neuroblastomas (Teitz T, 2001).

Caspase 10 can also be recruited and activated by FADD in the same way as caspase 8. Although CASP10 collocates with CASP8 at 2q33, they are said to have different downstream cleavage substrates (Wang, J., et al, 2001). Vincenz, et al (1997) showed that caspase 10 can cleave poly (ADP-ribose) polymerase (PARP) directly, bypassing the traditional caspase 8 – caspase 3 – PARP pathway. Caspase 10 is also shown to be deficient in a majority of neuroblastomas (Eggert, 2001; Harada, 2002) and may be a crucial factor in influencing their apoptotic potential.

RA (or ATRA, all-trans retinoic acid), a derivative of vitamin A, is a chemotherapeutic agent that is shown to induce neuroblastoma apoptosis (Niizuma, 2006) or I-type to N-type cell differentiation (Walton, 2004). I-type cells are regarded as the neuroblastoma stem cells that have an intermediate morphology to N-type (neuroblastic) and S-type (substrate-adherent, non-neuronal or Schwann/glial) cells (Cui, 2006). RA was recently been shown to have an inductive effect on caspase 8 expression in primary tumors and neuroblastoma cell lines and increase the apoptotic responses of these cells to TNF- $\alpha$  and other cytotoxic agents (Jiang, 2008). This provides another aspect of RA's anti-tumor effect other than differentiation (Walton, 2004), cell

cycle arrest (Wainwright, 2001) and direct induction of apoptosis (Niizuma, 2006). To see whether the differentiated I-type cell line BE-(2)-C10 by RA and 5-bromo-2'-deoxyuridine (BUdR) exhibit an enhancement of caspase 8 and 10 expression level as compared to their I-type progenitor, the amount of these two transcript were semiquantified in the untreated cells and cells that are treated with the two differentiation agents, which induce N-type and S-type cell differentiation respectively.

## **Materials and Methods**

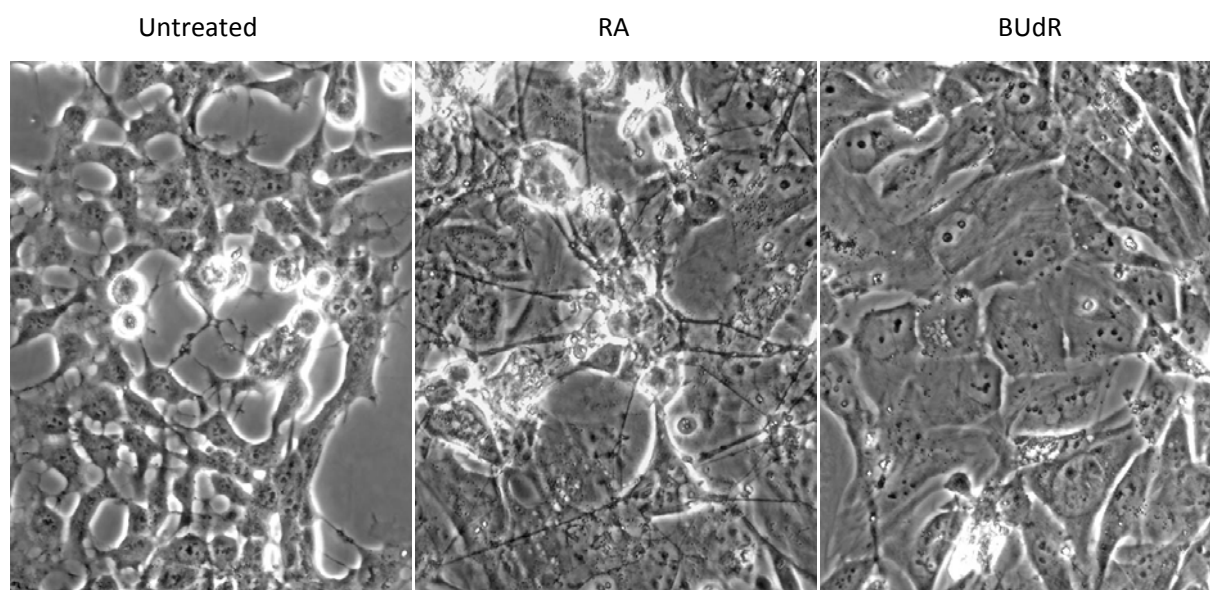
BE-(2)-C cell line and RA/BUdR-treated cells were kindly provided by the Laboratory of Neurobiology, Department of Biological Sciences, Fordham University. The total RNA was extracted using RNeasy® Plus Mini Kit (QIAGEN), according to the manufacturer's instructions. RT-PCR was performed using QIAGEN® OneStep RT-PCR Kit following the instructions. 20ng RNA was added to each RT-PCR reaction. The primers were designed to span at least one intron. Those used for caspase 8 (NCBI access number: NM\_033355.3) were: forward, 5'-TGTTGGAGGAAAGCAATCTG-3', and reverse, 5'-GCTGAATTCTTCATAGTCG-3'. Primers for caspase 10 (NCBI access number: NM\_032977.2): forward, 5'-CTCAAGGTCAACATTGGTATTC-3', and reverse, 5'-CAGAAGGAAGATCATGTCCT-3'. GAPDH was used as the loading control and amplified by primers reported earlier: forward, 5'-GAAGGTGAAGGTCGGAGT-3', and reverse 5'-GAAGATGGTGATGGGATTTTC-3'. The lengths of the PCR products are 388bp, 407bp and 226bp, respectively. Amplification conditions for caspase 8 and 10 reactions were as follows: 50°C for 30min; 95°C for 15min; 40 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s; 72°C for 10min. Those of GAPDH reactions were 50°C for 30min; 95°C for 15min; 30 cycles of 94°C for 30s, 55°C for 30s, and 72°C for 30s; 72°C for 10min.

The PCR products were separated on a 2% agarose gel and visualized by ethidium bromide in a UV trans-illuminator (BioRad). The intensities of the bands were quantified by SigmaGel software. The average mRNA levels are obtained by three replicated PCR experiments (n = 3).

The remaining PCR products were purified using the QIAquick® PCR Purification Kit (QIAGEN) following the manufacturer's instructions and subsequently sequenced in order to confirm the identity of the PCR products.

## Results

RA- and BUdR-treated BE-(2)-C10 cells showed N-type and S-type morphology (Figure 2) respectively as described earlier (Walton, 2004).



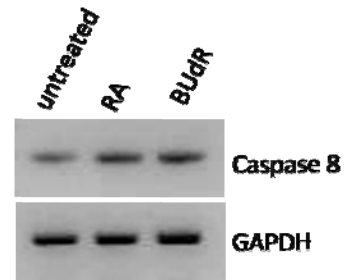
**Figure 2** Morphology of RA-, BUdR-treated and untreated BE-(2)-C10. They have I-type (untreated), N-type (RA-treated) and S-type (BUdR-treated) morphologies respectively.

Both RA and BUdR have inductive effect on caspase 8 mRNA level (Figure 3), while the increase of caspase 10 is not obvious (Figure 4). The enhancement of the transcript amount by BUdR is slightly higher than that of RA.

a)

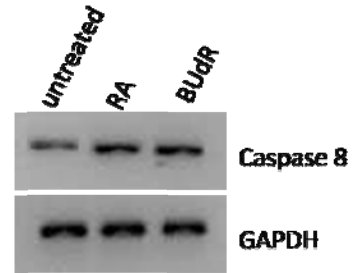
Experiment 1

	caspace 8	GAPDH	Normalized to GAPDH
untreated	542	1579	1
RA	1672	1727	2.82
BUdR	1547	1963	2.30



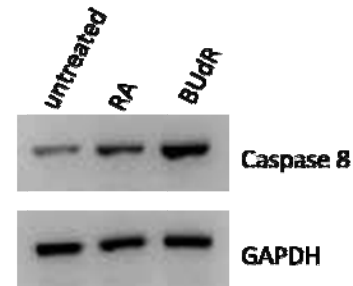
Experiment 2

	caspace 8	GAPDH	Normalized to GAPDH
untreated	739	2222	1
RA	1244	1688	2.22
BUdR	1885	1869	3.03



Experiment 3

	caspace 8	GAPDH	Normalized to GAPDH
untreated	795	2172	1
RA	1976	2534	2.13
BUdR	2892	2768	2.85



b)

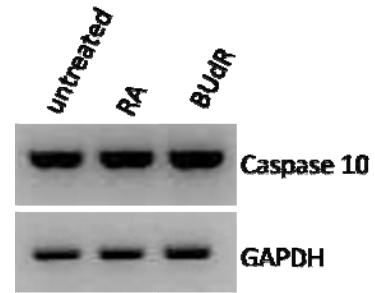
	Exp1	Exp2	Exp3	Mean
Untreated	1	1	1	
RA	2.82	2.22	2.13	<b>2.39</b>
BUdR	2.30	3.03	2.85	<b>2.73</b>

**Figure 3** a) Gel pictures and corresponding band intensities of RT-PCR products amplified by caspase8 primers in three separate experiments. All the intensities are first normalized to that of the loading control GAPDH, and by setting the untreated intensity at 1. b) The average increases (folds) of caspase 8 transcripts in RA and BUdR treated cells.

a)

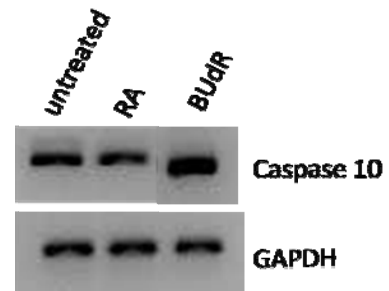
Experiment 1

	caspase 10	GAPDH	Normalized to GAPDH
<b>untreated</b>	1339	1579	1
<b>RA</b>	1967	1727	1.34
<b>BUdR</b>	1936	1963	1.16



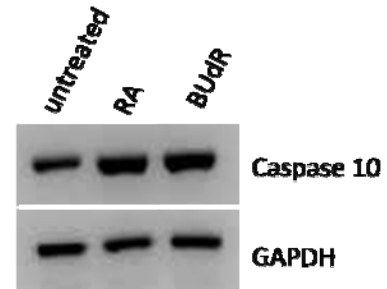
Experiment 2

	caspase 10	GAPDH	Normalized to GAPDH
<b>untreated</b>	1886	2222	1
<b>RA</b>	1893	1688	1.32
<b>BUdR</b>	2657	1869	1.67



Experiment 3

	caspase 10	GAPDH	Normalized to GAPDH
<b>untreated</b>	1372	2172	1
<b>RA</b>	2165	2534	1.35
<b>BUdR</b>	2976	2768	1.70



b)

	Exp1	Exp2	Exp3	Mean
<b>Untreated</b>	1	1	1	
<b>RA</b>	1.34	1.32	1.32	<b>1.33</b>
<b>BUdR</b>	1.16	1.67	1.67	<b>1.50</b>

**Figure 4** a) Gel pictures and corresponding band intensities of RT-PCR products amplified by caspase 10 primers in three independent experiments. All the intensities are first normalized to that of the loading control GAPDH, and by setting the untreated intensity at 1. b) The average increases (folds) of caspase 10 transcripts in RA and BUdR treated cells.

The identity of the PCR products is confirmed by BLASTing their sequencing results (shown in Figure 5). They match the sequences of NM\_033355.3 (caspase 8B mRNA) and NM\_032977.2 (caspase 10D mRNA) respectively.

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Caspase 8      1      TGTGGAGGAAAGCAATCTGTCCTTCCTGAAGGAGCTGCTCTCCGAATT-ATAGACTGG      59
NM_033355.3   367    TGTGGAGGAAAGCAATCTGTCCTTCCTGAAGGAGCTGCTCTCCGAATTAATAGACTGG      426

Caspase 8      60      ATTTGCTGATTACCTACCTAAACACTAGAAAGGAGGAGATGGAAAGGGAACCTCAGACAC      119
NM_033355.3   427    ATTTGCTGATTACCTACCTAAACACTAGAAAGGAGGAGATGGAAAGGGAACCTCAGACAC      486

Caspase 8      120     CAGGCAGGGCTCAAATTTCTGCCTACAGGGTCATGCTCTATCAGATTCAGAAGAAGTGA      179
NM_033355.3   487    CAGGCAGGGCTCAAATTTCTGCCTACAGGGTCATGCTCTATCAGATTCAGAAGAAGTGA      546

Caspase 8      180     GCAGATCAGAATTGAGGCTCTTTAAGTTTCTTTTGCAAGAGGAAATCTCCAAATGCAAAC      239
NM_033355.3   547    GCAGATCAGAATTGAGGCTCTTTAAGTTTCTTTTGCAAGAGGAAATCTCCAAATGCAAAC      606

Caspase 8      240     TGGATGATGACATGAACCTGCTGGATATTTTCATAGAGATGGAGAAGAGGGTCATCCTGG      299
NM_033355.3   607    TGGATGATGACATGAACCTGCTGGATATTTTCATAGAGATGGAGAAGAGGGTCATCCTGG      666

Caspase 8      300     GAGAAGGAAAGTTGGACATCCTGAAAAGAGTCTGTGCCCAAATCAACAAGAGCCTGCTGA      359
NM_033355.3   667    GAGAAGGAAAGTTGGACATCCTGAAAAGAGTCTGTGCCCAAATCAACAAGAGCCTGCTGA      726

Caspase 8      360     AGATAATCAACGACTAT      376
NM_033355.3   727    AGATAATCAACGACTAT      743

Caspase 10     1      CTCAAGGTCAACATTGGTATTCCAGTTCAGATAAAAACTGT-AAGTGAGCTTTCGTGAGA      59
NM_032977.2   426    CTCAAGGTCAACATTGGTATTCCAGTTCAGATAAAAACTGTAAAGTGAGCTTTCGTGAGA      485

Caspase 10     60      AGCTTCTGATTATTGATTCAAACCTGGGGGTCCAAGATGTGGAGAACCTCAAGTTTCTCT      119
NM_032977.2   486    AGCTTCTGATTATTGATTCAAACCTGGGGGTCCAAGATGTGGAGAACCTCAAGTTTCTCT      545

Caspase 10     120     GCATAGGATTGGTCCCCAACAAGAAGCTGGAGAAGTCCAGCTCAGCCTCGGATGTTTTTG      179
NM_032977.2   546    GCATAGGATTGGTCCCCAACAAGAAGCTGGAGAAGTCCAGCTCAGCCTCAGATGTTTTTG      605

Caspase 10     180     AACATCTCTTGGCAGAGGATCTGCTGAGTGAGGAAGACCCTTTCTTCTGGCAGAACTCC      239
NM_032977.2   606    AACATCTCTTGGCAGAGGATCTGCTGAGTGAGGAAGACCCTTTCTTCTGGCAGAACTCC      665

Caspase 10     240     TCTATATCATACGGCAGAAGAAGCTGCTGCAGCACCTCAACTGTACCAAAGAGGAAGTGG      299
NM_032977.2   666    TCTATATCATACGGCAGAAGAAGCTGCTGCAGCACCTCAACTGTACCAAAGAGGAAGTGG      725

Caspase 10     300     AGCGACTGCTGCCACCCGACAAAGGGTTTCTCTGTTTAGAAACCTGCTCTACGAAGTGT      359
NM_032977.2   726    AGCGACTGCTGCCACCCGACAAAGGGTTTCTCTGTTTAGAAACCTGCTCTACGAAGTGT      785

Caspase 10     360     CAGAAGGCATTGACTCAGAGAACTTAAAGGACATGATCTTCCTTCTG      406
NM_032977.2   786    CAGAAGGCATTGACTCAGAGAACTTAAAGGACATGATCTTCCTTCTG      832

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**Figure 5** Alignment of caspase 8 and 10 PCR products' sequences with the corresponding mRNA sequences in NCBI database.

## Discussion

Being able to escape death is one of the properties that lead to the malignance of cancer. This could be the result of function loss or mis-regulation in numerous genes involved in apoptosis, such as p53 (signals DNA damage, hypoxia) [Gottlieb, 1998], integrin (signals the detachment of cell to their matrix), apoptosis initiators – caspase 2/8/9/10, apoptosis executors – caspase 3/6/7, etc. In this study, the expression of two genetically related apoptosis initiators, caspase 8 and 10, was tested. The result shows that the mRNA amount of caspase 8 rose to more than two fold as compared to untreated cells, while the increase in caspase 10 did not necessarily happen. This is probably because the basal mRNA amount of caspase 10 in untreated cells is higher than that of caspase 8. To determine whether there is a rescuing effect of caspase 10 on caspase 8 deficiency, further study needs to be done in other neuroblastoma cell lines and tumor samples. The lack of correlation of caspase 8 and 10 mRNA level is consistent with previous findings (Harada, 2002), suggesting that even though the two genes are genetically related, their transcription might be regulated through different pathways or their transcripts might have distinct stability. After all, the differentiation induction of RA and BUdR did enhance BE-(2)-C cells' caspase 8 mRNA level. It implies the potentialities of both RA and BUdR being used to sensitize neuroblastoma cells to apoptotic signals. Whether this effect applies to every single cell in the neuroblastoma cell population needs to be elucidated by in situ studies, or else the two differentiation drugs are just selection stimuli promoting the dominance of death-resistant, more malignant cancer cells in the population. Furthermore, the detection of protein amount, especially the functional (cleaved) caspase 8 and 10, will also help with drawing a final conclusion about their influences on neuroblastoma cells' lifetime.

Retinoic acid can induce neuroblastoma cell differentiation through RAR-RXR nuclear receptor (Mangelsdorf, 1995), or PI-3 kinase – Akt/PKB pathway (Lo'pez-Carballo, 2002) and their subsequent regulation of expression of genes, such as TrkA/B, BDNF, and Ret receptor (Esposito, 2008), ID transcription factors (Lo'pez-Carballo, 2002), etc. The pro-apoptotic effect of RA is thought to be differentiation dependent because transcription factor CREB was shown to be involved in induction of caspase 8 transcription (Jiang, 2008), and its upstream effector GPCR (G-protein coupled receptor) can be trans-activated by TrkA/B (Esposito, 2008), involved in triggering neuroblastoma cell differentiation. However, the PI-3 kinase – Akt/PKB pathway has an anti-apoptotic effect, which is contradictory to what was discussed here. So it is necessary to distinguish the effect of RA at different concentrations applied to different types of neuroblastoma cells. In addition, figuring out the time these processes needed to respond to RA-treatment will also help strengthening its anti-tumoral effect.

BUdR, whose mechanism to induce differentiation is not clear, was said to be incorporated into the genome to change the expression of genes (Ashman, 1980). Further study on the genetic make-up and epigenetic modifications of caspase 8 gene is therefore required to determine the mechanism of its effect on the expression of this gene.

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