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Cyclopentenyl Cytosine and Neuroblastoma SK-N-BE(2)-C Cell Line Cells

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We studied the effect of cyclopentenyl cytosine (CPEC) on human neuroblastoma SK-N-BE(2)-C cell line cells. CPEC had an IC_{50} value of 100 nM for non-synchronised SK-N-BE(2)-C cells. These cells were arrested in G_0/G_1-phase or early S-phase of the cell cycle upon treatment with CPEC. After treatment of synchronised S-phase cells with 1 μM CPEC, the number of cells present after 3 days was less than 10% of that observed for the untreated cells. S-phase synchronised cells treated with CPEC and deoxycytidine showed an increased viability in comparison with cells treated with CPEC alone. Approximately 15% of the cells treated with CPEC and deoxycytidine traversed through one cell cycle. The amount of CTP declined to undetectable levels within 3 h after addition of 1 μM CPEC. The presence of cytidine prevented, to a large extent, the cytostatic effect of CPEC.

Key words: cyclopentenyl cytosine (CPEC), CTP synthetase, neuroblastoma, SK-N-BE(2)-C
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INTRODUCTION

CANCER CELLS have a high requirement for nucleotides as their metabolism is accelerated to facilitate cell proliferation. This increased metabolism of nucleotides has been associated with increased activities of "key" enzymes of the nucleotide pathways such as CTP synthetase and inosine monophosphate dehydrogenase (IMPDH) [1–4]. Manipulation of balances between ribonucleotide pools by inhibiting the increased activity of these enzymes in cancer cells resulted in temporary restoration of the pools to levels comparable with those observed in their normal counterparts. These manipulations induced growth arrest and terminal differentiation of human HL-60 cells [5, 6]. Similar results were obtained with the K562 human leukaemic cell line [6]. Tiazofurin, an inhibitor of IMPDH, induced trans-differentiation in neuroblastoma tumours [7].

The neural-crest derived pheochromocytoma PC-12 cells have an imbalance in their pyrimidine ribonucleotide content in comparison to adrenal medulla tissue [2]. Therefore, an inhibitor of CTP synthetase may be effective as a chemotherapeutic agent with a selectivity for tumour cells derived from the neural crest in comparison to mature neuronal tissues. Because mature neuronal cells do not proliferate and, therefore, have a limited need for nucleotides, the effect of CTP synthetase inhibitors might be less harmful for these cells.

A chemotherapeutic strategy based on reducing the increased activity of CTP synthetase will temporarily alter the balance between the various ribonucleotide and deoxyribonucleotide pools. This may lead to a concomitant reduction of the proliferative potential and trigger an adaptive response which engages either a programme of cell death (apoptosis) or of terminal differentiation [2, 8].

In order to inhibit CTP synthetase in neuroblastoma cells, we tested cyclopentenyl cytosine (CPEC) (NSC 375575), a carbocyclic analogue of cytidine, in which the ribofuranose moiety is replaced by a cyclopentenyl ring [9] (Figure 1). Figure 2 shows the pyrimidine pathway involved in the metabolism of cytidine and CPEC. CPEC acts as a substrate inhibitor for uridine-cytidine kinase, the enzyme catalysing the initial phosphorylation of CPEC to CPE-CMP. Subsequently, CPE-CMP is converted to the 5'-triphosphate form CPE-CTP. In addition to the inhibition of uridine-cytidine kinase by CPEC, CPE-CTP inhibits the conversion of UTP to CTP by CTP synthetase causing a depletion of the endogenous CTP pool [10].

CPEC has an antineoplastic activity against several murine leukaemias [11], human tumour xenografts and human colon-carcinoma cells cultured *in vitro* [12]. Recently, phase I clinical trials with this agent have been initiated. The effect of CPEC on neuroblastoma cells has not yet been studied.

MATERIALS AND METHODS

Cell line

The SK-N-BE(2)-C human neuroblastoma cell line cells were routinely cultured in Dulbecco's Modified Eagles medium (DMEM) (Gibco Laboratories, Paisley, Scotland) supplemented with 2 mM L-glutamine (Flow Laboratories, Irvine, U.K.), 50 I.U./ml penicillin, 50 μg/ml streptomycin (Imperial, U.K.) and 10% (v/v) fetal calf serum (FCS) (Gibco Laboratories). The cells were incubated in loosely capped culture flasks (Costar Corp., Cambridge, Massachusetts, U.S.A.) at 37°C, in humidified (96%) air with 5% CO_2. Cells were passaged once a week

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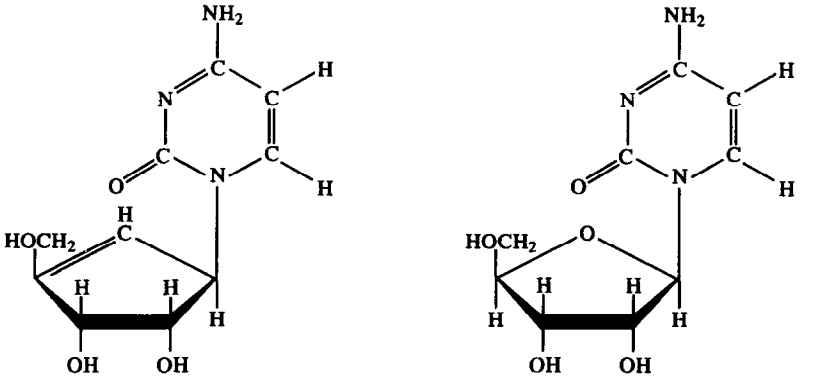

Cyclopentenylcytosine (CPEC)

Cytidine

Figure 1. Chemical structures of cyclopentenylcytosine (CPEC) and cytidine.

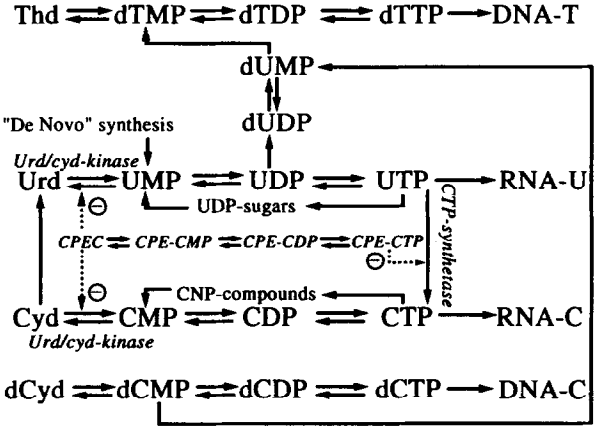

Figure 2. A simplified scheme of the pyrimidine pathway showing the metabolism of CPEC and its inhibiting modes on the "key" enzymes CTP-synthetase and uridine/cytidine kinase. Uridine (Urd) and cytidine (Cyd) are "salvaged" from the medium. Included is the *de novo* synthesis of UMP. Furthermore, the salvage of deoxycytidine (dCyd) and thymidine (dThd) into DNA-precursors is presented. A broken arrow marked (⊖) indicates inhibition of the respective reactions.

and maintained in logarithmic growth phase. Cultures were consistently free of mycoplasma (tested with Genprobe, ICN, U.K.). For specific experiments (see results), cells were cultured in a chemically defined medium (CDM) consisting of DMEM supplemented with 0.5% (w/w) highly purified human serum albumin (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany), 50 I.U./ml penicillin, 50 μg/ml streptomycin (Imperial, U.K.), 3 μM uridine, 4 μM hypoxanthine, 2 mM L-glutamine (Flow Laboratories), 1 mM Na pyruvate, 5 μg/ml insulin, 100 μg/ml human transferrin, 20 nM progesterone, 200 μM putrescine and the trace elements selenium, manganese and copper ($Na_2SeO_3 \cdot 5H_2O$ + $MnCl_2 \cdot 4H_2O$ + $CuSO_4 \cdot O$; 30 nM for each compound) [13]. Cells were synchronised by an overnight incubation in growth medium supplemented with 1.5 μM hydroxyurea (Sigma Chemical Company, St. Louis, U.S.A.) The viability of the cells prior to the start of the experiment was more than 95% as determined by the Trypan Blue exclusion method.

Determination of drug sensitivity

A modified (MTT) assay (Boehringer Mannheim GmbH Biochemica) was used to determine the sensitivity of SK-N-BE(2)-C neuroblastoma cells to CPEC (NSC 375575, Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, North Carolina, U.S.A.). Cells (2×10^3 cells/well) were plated into 96-well microtitre plates and incubated at 37°C. 24 h later, CPEC was added to each well (final volume 100 μl) at various concentrations. Cells were exposed to CPEC for 24 or 72 h and the number of surviving cells were quantitated using an MTT assay [14]. Additionally, cells were counted under a phase-contrast microscope and/or quantified by measuring protein concentrations [15].

Quantitative determination of apoptosis

Cells were plated in 96-well microtitre plates and cultured in the presence of CPEC as described above. At the end of the exposure period, the cells were gently washed with phosphate-buffered saline without Ca^{2+} or Mg^{2+}. To assess apoptosis, core histones and DNA fragments (mono- and oligonucleosomes) were detected with a sandwich enzyme-linked immuno-sorbent (ELISA) assay (Boehringer Mannheim GMBH Biochemical) [16]. The enrichment of the cytoplasm of cells treated with CPEC with mono- and oligonucleosomes was compared to untreated cells. Apoptosis was also morphologically confirmed by the presence of cells showing apoptotic bodies and zeiosis.

Cell-cycle analysis

The cell-cycle distribution was measured by two-step immunofluorescent detection of incorporated (100 μM, 1 h) bromo-2'-deoxyuridine (BrdU) and staining the nuclei with propidium iodide [3]. Cell-cycle analyses were performed by flow cytometric analyses of red and green fluorescence of 10000 cells.

Nucleotide determination

SK-N-BE(2)-C cells were cultured in CDM supplemented with 1 μM CPEC for 24 h. Medium was removed and the SK-N-BE(2)-C cells were washed twice with ice-cold phosphate-buffered saline (PBS; 140 mM NaCl, 9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, pH 7.4). After removal of PBS, ice-cold perchloric acid (0.4 M) was added to the flask and the flask was

incubated for 10 min on ice with intermittent scraping of the culture surface with a cell scraper (Costar Corp.). The suspension was removed and centrifuged at 10000 *g* at 4°C for 3 min. The supernatant was neutralised with K_2CO_3 and used for analysis of the ribonucleotides. Analysis of the ribonucleotides was performed by anion-exchange HPLC [10, 17]. Protein content of the remaining cell pellet was determined after dissolving the protein with 0.2 M NaOH [15].

RESULTS

Cell survival of non-synchronised SK-N-BE(2)-C cells exposed for 72 h to various CPEC concentrations in CDM was determined and compared to control cells, using an assay based on the reduction of MTT by viable cells. The IC_{50} value of CPEC determined under these conditions was approximately 100 nM.

CPEC (1 μM) caused a rapid depletion of CTP to undetectable levels within 3 h of the start of the incubation with a concomitant increase in CPE-CTP. CPE-CTP was present at a concentration of 3.3 ± 0.5 pmol/μg protein (mean ± S.D., n = 3). The CTP-pool in untreated SK-N-BE(2)-C was 3.8 ± 1.0 pmol/μg protein (mean ± S.D., n = 3) (Figure 3). The size of the UTP pool remained unchanged under these conditions. After 1 day of incubation in 1 μM CPEC and 10 μM cytidine, the concentration of CPE-CTP in the SK-N-BE(2)-C cells was 4.6 ± 0.8 pmol/μg whereas at 0.1 μM CPEC with the same cytidine concentration, the concentration of CPE-CTP was 2.1 ± 0.5 pmol/μg. Therefore, the CPE-CTP concentration in the cells decreased to a lesser extent than expected based on the change of the concentration ratio of CPEC to cytidine. In the presence of 1.0 μM CPEC and 10 μM cytidine, the CTP concentration was less than 10% of the concentration observed in controls, while the number of cells was only reduced by 15% in comparison to controls.

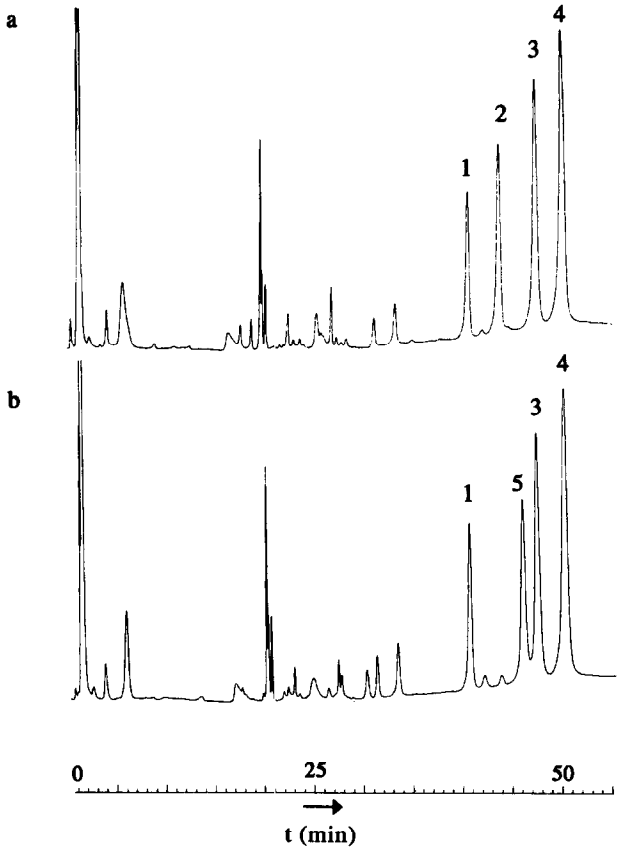

Figure 3. HPLC elution profiles of nucleotides extracted from (a) control SK-N-BE(2)-C cells and (b) SK-N-BE(2)-C cells which were exposed for 24 h to 1 μM CPEC. 1 = UTP, retention time (RT) = 40.8 min; 2 = CTP, RT = 43.6 min; 3 = ATP, RT = 47.6 min; 4 = GTP, RT = 49.9 min; 5 = CPE-CTP, RT = 45.6 min.

Exposure of SK-N-BE(2)-C cells for 1 day to 1 μM CPEC and 10 μM cytidine was less cytostatic compared to the incubation with only CPEC. The number of cells in the presence of CPEC and cytidine was 85% ± 6% (mean ± S.D., n = 3) that of control cells (viability ≥97%), while in the presence of CPEC alone 62% ± 7% (mean ± S.D., n = 3) of the number of control cells was observed (viability ≥88%). Therefore, cytidine partially prevented the cytostatic effect of CPEC for SK-N-BE(2)-C cells.

The cell-cycle distribution of SK-N-BE(2)-C cells after 24 h incubation with 1 μM CPEC was compared to that of controls. Most of the remaining population was arrested in the G_0/G_1-phase of the cell-cycle (Figure 4). Approximately 40% of the population showed an increased amount of incorporated BrdU compared to normal G_0/G_1 cells. However, a change in the DNA content of these cells was not observed.

CPEC (1 μM) tested on synchronised S-phase cells revealed an increased cytostatic effect in comparison to non-synchronised cells. Only less than 10% of the cells in the S-phase survived an incubation period of 3 days, whereas in non-synchronised cells 30% of the cells were viable. None of the synchronised S-phase cells were viable after 6 days.

Co-administration of 1 μM CPEC with 10 μM deoxycytidine to SK-N-BE(2)-C cells revealed that 15% of the cell population traversed through one cell-cycle within 3 days (normal doubling-time of untreated cells is 35 h). At day 3, the viability of these cells was slightly higher (65%) compared to cells treated with CPEC in the absence of deoxycytidine (50%). No increase in the number of cells treated with CPEC and deoxycytidine was observed beyond day 3.

After treatment with CPEC (1 μM), no cells were observed with signs of swelling, a marker of necrosis. However, a 4-fold enrichment of the cytoplasm of SK-N-BE(2)-C cells with mono- and oligonucleosomes after 72 h exposure to 1 μM CPEC was observed in comparison to that observed in control cells. The percentage of enrichment was in agreement with the number of cells showing zeiosis, shrinking and/or apoptotic bodies (60 of 150 cells in the presence of CPEC versus 33 of 390 cells in the controls).

DISCUSSION

Cyclopentenyl cytosine (CPEC) is an inhibitor of CTP-synthetase which is a "key" enzyme in the pyrimidine ribonucleotide pathway in cancer cells with an increased activity. CPEC had an IC_{50} value of approximately 100 nM for human neuroblastoma SK-N-BE(2)-C cells cultured in CDM. CPEC caused a depletion of CTP in these SK-N-BE(2)-C cells. Cytidine prevented to a large extent the cytostatic effect of CPEC. This is in line with the fact that uridine, cytidine and CPEC are metabolised in a competitive manner by the same uridine/cytidine kinase [18]. Deoxycytidine, using a specific kinase for its phosphorylation into dCMP, caused a partial rescue, presumably by permitting S-phase cells to complete the cell-cycle traverse after which they become arrested into the G_0/G_1-phase. These results suggest that CPEC influences RNA synthesis as well as DNA synthesis in neuroblastoma cells.

Recently, a gene encoding cytidine deaminase has been located

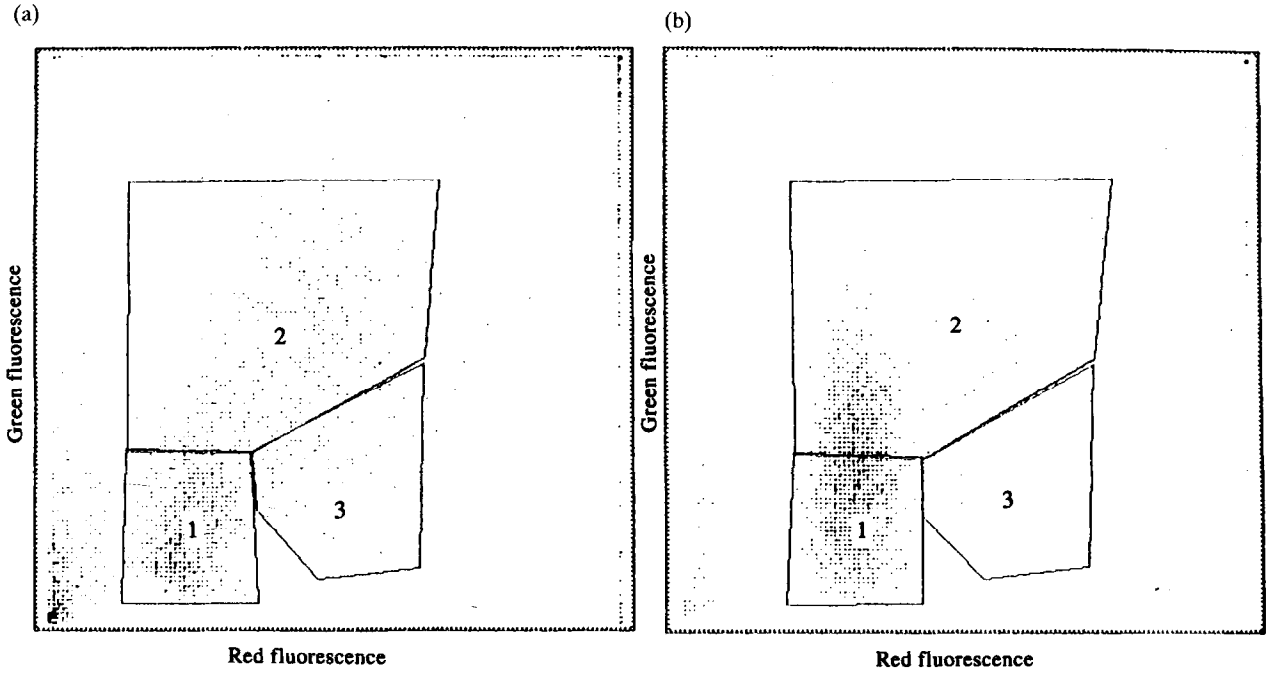

Figure 4. Cell-cycle distribution of SK-N-BE(2)-C cells in the absence of CPEC (a) and with CPEC (b). The vertical axis represents the amount of green fluorescence caused by a GAM-FITC anti-BrdU antibody staining nuclei which have actively synthesised DNA during the BrdU-labelling period. On the horizontal axis is the amount of red fluorescence caused by intercalation of propidium iodide into DNA reflecting the amount of DNA. 1, G_0/G_1-phase; 2, S-phase; 3, G_2/M-phase.

on chromosome 1p35-1p36.2 with a peak staining at chromosome 1p36.1 [19]. Cytidine deaminase can convert CPEC into a less toxic compound named cyclopentenyluridine, an analogue of uridine [18]. The smallest overlapping deletion noticed in neuroblastoma is at 1p36.1–36.2 [20]. A possible diminished deaminase activity in neuroblastoma cells with a loss of heterozygosity of 1p in comparison to normal neural crest tissues may increase the selectivity of CPEC towards these neuroblastoma cells. A further upregulation of the activity of CTP synthetase may decrease the effect of CPEC on neuroblastoma cells as has been noticed for other cell types [11].

The results with CPEC showed that the CTP pool in SK-N-BE(2)-C cells has almost completely disappeared in the presence of CPEC. The combination of CPEC with cytidine decreases the number of cells to 85% of that observed in controls after 1 day of incubation. However, the size of the CTP pool is decreased to 10% of that observed in control cells. This might suggest that the amount of CTP left is still sufficient to allow the proper synthesis of RNA or that the turnover of this CTP pool is increased.

CPEC arrested SK-N-BE(2)-C cells mainly in the G_0/G_1-phase or early S-phase of the cell cycle. Part of the cells showed an increased incorporation of BrdU in comparison to G_0/G_1-phase cells of the controls. However, a change in the DNA content was not observed. These cells may have just entered the S-phase and accumulated BrdU in their DNA, replacing dTTP as well as dCTP due to the abundance of BrdU and the shortage of dCTP induced by CPEC. These cells traverse very slowly through the cell cycle. A part of these cells may also represent G_0/G_1-phase cells with damaged DNA which is undergoing repair during the BrdU labelling. The shortage of dCTP leads to erroneous DNA strand formation and DNA strand breaks by misincorporation of other deoxyribonucleotides during DNA synthesis and DNA repair, without the incorporation of CPEC into DNA [21, 22]. This may explain the mono- and oligonucleosomes observed in SK-N-BE(2)-C cells during treatment with CPEC. In this respect, inhibitors of the ligation step of DNA repair processes may enhance the effect of CPEC.

CPEC reduced the increased activity of the "key" enzyme of the pyrimidine pathway CTP synthetase in neuroblastoma and induced a depletion of CTP, with a concomitant reduction of the proliferative potential, and upregulation of phenomena associated with apoptosis. Whether CPEC can also induce terminal differentiation in these neuroblastoma cells, as has been observed in other cell types, may be dependent on the duration of exposure, concentration and phase of the cell cycle of the cells.

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In Vivo Targeting of Human Neuroblastoma Xenograft by Anti-G_{D2}/Anti-FcγRI (CD64) Bispecific Antibody

J. Michon, B. Perdereau, F. Brixy, S. Moutel, W.-H. Fridman and J.-L. Teillaud

Antidisialoganglioside (G_{D2}) monoclonal antibodies can target *in vitro* and *in vivo* neuroblastoma cells. However, their *in vivo* use is limited by the presence of high levels of circulating IgG which hamper the recruitment of effector cells through the high affinity FcγRI (CD64). A bispecific Fab′ × Fab′ antiG_{D2}/antiFcγRI antibody (7A4 bis 22), which binds outside the IgG binding site of FcγRI, was therefore developed. This antibody binds both human G_{D2+} neuroblastoma and FcγRI$^+$ activated macrophages *in vitro*. It can localise a G_{D2} positive neuroblastoma xenografted on Nu/Nu mice. Scintigraphy tumour/muscle ratios showed that targeting with this antibody has an excellent selectivity for the tumour over normal tissues. Furthermore, although its whole body clearance is more rapid than that of the 7A4 parental antibody over the first 48 h, its selective tumour uptake is similar, as shown by immunoscintigraphy imaging. Thus, such a bispecific antibody may represent an efficient tool for *in vivo* therapy of neuroblastoma through its ability to recruit FcγRI$^+$ effector cells even in presence of circulating IgG and to bind concomitantly G_{D2+} tumour cells.

Key words: disialoganglioside, FcγRI, bispecific antibody, neuroblastoma, xenograft, scintigraphy
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