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The induction of myeloma cell death and DNA damage by tetrac, a thyroid hormone derivative

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Abstract

Multiple myeloma (MM) is a plasma cell malignancy in which involvement of the thyroid hormone-integrin $\alpha\beta 3$ pathway was shown, and pharmacologic inhibition of this pathway is a rational approach to disease management. A thyroid hormone derivative, tetraiodothyroacetic acid (tetrac), which inhibits L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) binding to $\alpha\beta 3$ integrin, was studied in five MM cell lines and primary bone marrow (BM) MM cells. Tetrac inhibited MM cell proliferation (absolute cell number/viability) and induced caspase-dependent apoptosis (annexin-V/PI and cell cycle). Activation of caspase-9 and caspase-3 was further demonstrated. Moreover, DNA damage markers, ataxia-telangiectasia-mutated (ATM) kinase, poly ADP-ribose polymerase (PARP-1) and histone γ H2AX were induced by tetrac. The various tetrac-initiated effects were attenuated by Arg-Gly-Asp (RGD) peptide, suggesting integrin involvement. Primary BM mononuclear cells were harvested from MM patients ($n=39$) at various disease stages. Tetrac-induced apoptosis (12/17 samples) and sensitized the cytotoxic action of bortezomib (6/9 samples). Lastly, expression of plasma membrane integrin $\alpha\beta 3$ was shown not only in the malignant plasma clone, but also in other cell populations within the BM samples ($n=25$). Tetrac is anti-proliferative and pro-apoptotic in MM and cells may offer a therapeutic approach for this disease.

Key Words

- ▶ integrin
- ▶ myeloma
- ▶ thyroid hormone derivatives

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Introduction

A large number of epidemiological and human studies have demonstrated an association between thyroid hormone and cancer. In patients with elevations of serum thyroid hormone, an increased risk of cancer compared with euthyroid patients has been reported (Ness *et al.* 2000, Hellevik *et al.* 2009). An association between thyroid dysfunction and the risk of development of multiple myeloma (MM), a plasma cell (PC) malignancy accounting

for more than 13% of hematological malignancies, has also been suggested (Dalamaga *et al.* 2008). Another body of epidemiological and clinical evidence suggests improved survival in individuals with a variety of cancers who also have hypothyroidism (Cristofanilli *et al.* 2005, Nelson *et al.* 2006, Illouz *et al.* 2009, Baldazzi *et al.* 2010, Hercbergs *et al.* 2010, Schmidinger *et al.* 2010). A few years ago, a binding site for the thyroid hormones

L-thyroxine (T_4) and 3,5,3'-triiodo-L-thyronine (T_3) was described on $\alpha\beta 3$ integrin (Bergh *et al.* 2005). This integrin is overexpressed in an array of cancer types (Desgrosellier & Cheresh 2010), including MM (Vacca *et al.* 2001, Ria *et al.* 2002, Tucci *et al.* 2009). The affinity of the receptor is higher for T_4 than for T_3 (Bergh *et al.* 2005), and binding of T_4 occurs at physiological free T_4 levels. The cell surface receptor-binding site for thyroid hormones on integrin $\alpha\beta 3$ is located in close proximity to the well-investigated Arg-Gly-Asp (RGD) recognition site. This region of $\alpha\beta 3$ contains receptors for other small molecules in addition to thyroid hormone that do not contain an RGD sequence and thus are 'non-RGD' binding sites. This thyroid hormone-binding site has been studied by crystallography and mathematical modeling of the thyroid hormone-binding kinetics (Aghajanova *et al.* 2011). The site has no structural homologies with the nuclear thyroid hormone receptors (TRs) that mediate genomic actions of the hormone. The description of a cell surface receptor binding site that is unrelated to conventional nuclear TRs has served to define the contributions of non-genomic actions of thyroid hormones to cancer. Upon binding of either hormone to the integrin, numerous effects are exerted by activating several signaling pathways (Davis *et al.* 2011). Although the thyroid hormone-integrin axis has been shown to be present in an array of tumor types, our group suggested a role for this pathway in a hematological malignancy, MM. This was confirmed by showing an increase in cell proliferation and activation of MAPK by T_3/T_4 via the $\alpha\beta 3$ integrin (Cohen *et al.* 2011). In this current work, we have characterized the effects of an antagonist at the hormone receptor site on the integrin, tetraiodothyroacetic acid (tetrac). Tetrac is a naturally occurring thyroid hormone derivative with low-potency thyromimetic activity inside the cell at TRs, but that selectively blocks binding of T_4 and T_3 to the thyroid hormone receptor site on $\alpha\beta 3$ (Davis *et al.* 2011, 2016). This agent was shown by *in vitro* and animal models to reduce cancer cell proliferation, cell migration and angiogenesis (Mousa *et al.* 2008, 2012, Rebbaa *et al.* 2008, Yalcin *et al.* 2009), to induce apoptosis in tumor cells (Glinskii *et al.* 2009, Yalcin *et al.* 2010) and to foster DNA double-strand breaks (Hercbergs *et al.* 2011). Tetrac has also been shown to chemosensitize several cancer cells that express chemoresistance (Rebbaa *et al.* 2008). Despite this extensive research in solid tumor models, tetrac has never been studied as a potential therapeutic agent in MM.

Methods and materials

Cell lines and primary BM cells

MM cell lines: CAG and ARK (established at the Arkansas Cancer Research Center), RPMI-8226 (CCL 155, ATCC), U266 (TIB-196, ATCC) and ARP-1. All cell lines were negative for mycoplasma contamination. Bone marrow (BM) aspirates were obtained upon written consent from a total of 40 patients at various disease stages (clinical data are presented in [Supplementary Table 1](#), see section on [supplementary data](#) given at the end of this article). Additionally, one normal BM sample from healthy subject was collected. All BM aspirates were obtained from patients treated at the Meir Medical Center after approval by an Institutional Review Board (IRB number #180-2010). BM mononuclear cells were isolated by Ficoll-Paque gradient centrifugation according to the manufacturer's instructions (Sigma-Aldrich). The different mononuclear cell populations were characterized and immunophenotyping was performed by flow-cytometry. Cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, penicillin and streptomycin antibiotics and the passage number for the experiments described was up to 20.

Reagents and chemicals

T_3 , T_4 and tetrac (Sigma-Aldrich) were dissolved in DMSO to 100mM and further dissolved in 1 mM KOH-propylene glycol, which was also used as a vehicle control (final concentration of 0.04N KOH with 0.4% polyethylene glycol (vol/vol)). RGD and Arg-Gly-Glu (RGE) peptides (Sigma-Aldrich) were dissolved at 100mM in PBS. Bortezomib (dissolved in saline to 2.6 μ M) was obtained from the oncology pharmacy at Meir Medical Center. The volume of solvent added to each well has been kept constant for all conditions. Pan-caspase inhibitor, Z-VAD-FMK was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Monoclonal antibody against $\alpha\beta 3$ integrin (LM609, PE/FITC-conjugated) was from Chemicon International. APC-CD138 antibodies were from Miltenyi Biotec (Bergisch Gladbach, Germany), FITC-CD45 and PE-CD38 antibodies were from BioLegend, San Diego, CA, USA. Proliferating cell nuclear antigen (PCNA) antibody was from Santa Cruz Biotechnology. Antibodies against total and cleaved caspase-9, caspase-3, PARP-1 and tubulin were from Cell Signaling. Anti-AIF was from EMD Millipore. Anti-pATM (phospho-serine 1981) was from Epitomics (Burlingame, CA, USA) and anti-pyH2AX

(phospho-serine 139) from Chemicon International. All antibodies were appropriately validated.

WST-1 proliferation assay

WST-1 reagent (Roche; 10% final concentration) was incubated according to the manufacturer's instructions as previously described (Cohen *et al.* 2015).

Flow-cytometry

Absolute cell number: cells were harvested in a fixed volume and counted. Cell cycle: following permeabilization and fixation by incubation with 70% ethanol (20 min, -20°C), the cells were stained with DNA propidium iodide (PI) (50 $\mu\text{g}/\text{mL}$) for 15 min at room temperature, along with RNase A (10 $\mu\text{g}/\text{mL}$) (Sigma-Aldrich) and cell cycle analysis was carried out by flow-cytometry (MACSQuant, Miltenyi Biotec). Apoptosis/necrosis: apoptotic cell death was measured by annexin-V/PI assay (BioVision) as previously described (Shinderman-Maman *et al.* 2016). Immunophenotype: immunophenotyping was performed in BM-derived mononuclear cells by using FITC-CD45, PE-CD38 (data not shown) and APC-CD138 antibodies. $\alpha\text{v}\beta 3$ expression: cell lines and primary BM cells were harvested in RPMI-1640 medium and labeled with 10 $\mu\text{g}/\text{mL}$ FITC- $\alpha\text{v}\beta 3$ or PE- $\alpha\text{v}\beta 3$ antibody (LM609).

RNA extraction and cDNA synthesis

RNA was extracted using a NucleoSpine RNA II kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions and eluted in 40 μL RNase-free water, as previously described (Cohen *et al.* 2015). RNA concentration and purity were measured using a NanoDrop 1000 Spectrophotometer (Thermo Scientific). 200 ng RNA were reverse transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's instructions.

Real-time PCR

Complementary DNA (cDNA) was analyzed for the following apoptotic genes: Apoptotic protease activating factor-1 (*apaf1*, accession number Nm_013229), *caspase-3* (accession number Nm_004346), p53-upregulated modulator of apoptosis (*PUMA*, accession number Nm_014417), *NOXA* (accession number Nm_021127) and *FAS* (accession number Nm_000043), by 7500 Fast Real-Time PCR system (Applied Biosystems), using Applied Biosystems Fast Sybr Green Mix (cat # 4385612).

Results, normalized to beta actin (accession number Nm_001101), were calculated as -fold change, using the comparative threshold cycle method ($2^{-\Delta\Delta\text{CT}}$) relative to control cells (i.e., controls were assigned a value of 1 by definition). Primers (Hylabs, Rehovot, Israel) were designed as published before (Cohen *et al.* 2011) in different exons in order to eliminate DNA contamination, using Primer-Express software (Applied Biosystems). Different bioinformatics tools, including BLAST and alignment PCR analysis, were used to design primers suitable for the amplification of fragments from the candidate genes. Primers sets were *Apaf1*: F TCGCTGCTCTGCCTTCT, R: CCATGGGTAGCAGCTCCTTCT. *Caspase 3*: F: CAGACAGTGGTGTGATGATGAC, R: TCGCCAAGAATAATAACCAGGTG. *Puma*: F: GAAGAGCAAATGAGCCAAACG, R: GGAGCAACCGGCAAACG. *Noxa*: F: CGGAGATGCCTGGGAAGAA, R: CCAAATCTCCTGAGTTGAGTAGCA. *Fas*: F: CCCTCCTACCTCTGGTTCTTACG, R: TGATGTCAGTCACTTGGGCATT. *Beta actin*: F: CCTGGCACCCAGCACAAT, R: GCCGATCCACACGGAGTACT.

Western blotting

Equal numbers of cells (10^6 cells/6-well plate) were used for total protein extraction quantified by BCA Protein assay (Thermo Scientific). The proteins (30 μg) were separated on 10–12.5% polyacrylamide gels, transferred to PVDF membranes and analyzed by Western blot, using primary antibodies and appropriate second HRP-conjugated antibody (Jackson Immuno-Research Laboratories). Immunoreactive proteins were detected by chemiluminescence reagents (Biological Industries, Beit Haemek, Israel). Alpha tubulin or beta actin was used for normalization. Band intensity was visualized using LAS-3000 (FujiFilm, Tokyo, Japan) and quantified by Multi-gauge, v3.0 software and normalized to protein amount.

Inhibition of caspase-dependent apoptosis

MM cells were treated in the presence/absence of 50 μM pan-caspase inhibitor, Z-VAD-FMK, dissolved in DMSO.

Statistical analysis

Mean $\pm$ s.d. was calculated for each value. In order to compare the means for pairs of groups (treatment vs control), Student's unpaired *t*-test was used. A value of $P < .05$ was considered significant.

Results

Tetrac inhibits MM cell proliferation and viability and induces apoptosis

We have previously demonstrated in MM cell lines that the activation of pERK by the thyroid hormones is effectively inhibited by tetrac (Cohen *et al.* 2011). Additional experiments indicated that the reduction of apoptotic genes, mainly by T₄, was reversed in the presence of tetrac (Supplementary Fig. 1). These anti-mitogenic actions were further explored in four MM cell lines (U266, ARK, RPMI-8226 and CAG). The cells were seeded (100,000/96-well plate) and treated daily with increasing concentrations of tetrac (10, 50 and 100 µM) for 24–96 h. At each time point, cell proliferation was estimated by WST-1 proliferation assay (Fig. 1A) and cell counts (Fig. 1B). Tetrac reduced MM cell proliferation by 50–75% in all cell lines tested, in a time/dose manner that was cell specific. In parallel, a reduction in the absolute cell number, starting at 48 h, was documented in all of the cell types.

Apoptosis analysis (annexin-V/PI, Fig. 1C) further indicated a time-dependent reduction in cell survival (An-/PI-) with a maximal effect after 72 h of 50 µM tetrac (35–63%) and 100 µM tetrac (76–90%). In parallel, an increase in apoptosis in response to 50–100 µM tetrac for 24–96 h was shown (Fig. 1D). The percentage of cells in early apoptosis (An+/PI-) and late apoptosis/necrosis (An+/PI+, An-/PI+) are presented as % of apoptotic cells. Representative annexin-V/PI results for RPMI-8226 cells and CAG are depicted in Supplementary Fig. 2A and B, respectively. Induction in SubG1, indicative of apoptosis, was further shown by cell cycle analysis (Fig. 1E). A representative cell cycle histogram for RPMI-8226 cells is presented in Supplementary Fig. 2C.

Tetrac activates caspases, but not AIF, in MM cell lines

To assess whether the induction in apoptosis involved the activation of caspases, RPMI-8226 cells (10⁶ cells/6-well plate) were treated with increasing tetrac concentrations (1–50 µM) for 1–8 h. Doxorubicin (1 µM) served as a positive control. Cleavage of caspase-3, caspase-9 and PARP-1, normalized to the non-cleaved form, as well as AIF levels, were analyzed by Western blots. In details, representative blots (Fig. 2A) indicate that cleavage of caspase-9 was induced following 1–4 h of incubation with 1 µM tetrac, as well as after 8 h in the presence of 50 µM tetrac concentration. Caspase-3 cleavage was completely absent at 1–2 h, while following an incubation of 4 h with 1 µM tetrac, an induction was observed. This effect

was sustained at 8 h of treatment at all concentrations examined. No significant effect on PARP-1 level was observed. Results in additional four MM cell lines are presented in Supplementary Fig. 3. The levels of apoptosis-inducing factor (AIF), a caspase-independent apoptosis marker, were unchanged (Fig. 2B). The calculated results following tetrac incubations are presented in Fig. 2C.

Characterizing involvement of p53 and caspases in action of tetrac in MM

After observing that caspases were induced by tetrac, we determined whether tetrac activities were caspase dependent, using a pan-caspase inhibitor, Z-VAD-FMK. In addition, p53 involvement was studied using RPMI-8226 p53-temperature-sensitive cells, which express wild-type p53 at permissive (32°C) and mutant p53 (E285K) at restrictive (37°C) temperatures (Teoh *et al.* 2000, Hu *et al.* 2015). The cells were treated with tetrac (50 µM) in the presence/absence of Z-VAD-FMK (50 µM) at 32°C and 37°C for 96 h and were first assessed for cell proliferation by WST-1 (Fig. 3A) and cell counting (Fig. 3B). At restrictive temperature (37°C, mutated p53), tetrac reduced cell proliferation (Fig. 3A, black bars) and cell number (Fig. 3B, black bars) by about 0.5-fold. This effect was not reversed by Z-VAD-FMK. Similar results (Fig. 3A and B, gray bars) were observed under permissive temperature (32°C, wild-type p53). When comparing the effect of tetrac on cell proliferation under both p53 conditions it appeared that tetrac's effect was more significant in the absence of wild-type p53 (Fig. 3A, marked by #). This effect was not reversed by Z-VAD-FMK in both permissive (32°C, wild-type p53) and restrictive (37°C, mutant p53) temperatures.

Next, cell death was determined by performing annexin-V/PI and cell cycle assays (flow-cytometry). Results (Fig. 3C) indicated that in cells bearing mutant p53 (37°C, black bars), as well as in cells containing wild-type p53 (32°C, gray bars), tetrac induced about a 3-fold increase in apoptotic cell death. A comparison of the apoptotic effect of tetrac between the two temperatures, depicted a greater significant effect in p53-independent cells (Fig. 3C, marked by #). Induction of cell death was partially inhibited by Z-VAD-FMK. Representative annexin-V/PI results for RPMI-8226 cells are depicted in Supplementary Fig. 4A.

Cell cycle analysis indicate that in cells lacking wild-type p53 (37°C, Fig. 3D), a potent increase in SubG1 was obtained with tetrac and was reversed by Z-VAD-FMK. However, in cells containing wild-type p53 (32°C, Fig. 3E), a parallel increase in S-G2M arrest was

Figure 1

Tetrac inhibits MM cell proliferation and viability and induces apoptosis. Four MM cell lines were treated with increasing concentrations of tetrac (10–100 μ M) for 24–96 h and evaluated for (A) Cell proliferation (WST-1, ELISA), (B) cell number (flow-cytometry), (C and D) apoptosis (Annexin-V/PI) and (E) cell cycle, by flow-cytometry. Experiments were repeated three times in triplicate. * $P \leq 0.05$, ** $P \leq 0.01$.

shown. Representative cell cycle results are depicted in [Supplementary Fig. 4B](#).

Tetrac increases DNA damage response markers in MM cells

Many cytotoxic antagonists lead to cell death via their effect on DNA damage response (DDR). We evaluated

by Western blotting the effect of tetrac on levels of three central DDR protein markers, phosphorylated ataxia-telangiectasia mutated (pATM), PARP-1 and histone γ H2AX, in RPMI-8226 cells treated with increasing concentrations of tetrac (1–50 μ M). Doxorubicin served as a positive control. Representative blots ([Fig. 4](#)) indicate a rapid and dose-dependent activation of pATM following 1 and 4 h of treatment, while at 2 h or 8 h, this effect was absent or greatly

Figure 2

Tetrac activates caspases and PARP-1 cleavage, but not AIF in MM. RPMI-8226 cells were incubated with tetrac (1–50 μ M) for 1–8 h, total protein was extracted and Western blots were carried out for (A) caspase-9, caspase-3, PARP-1 and (B) AIF. Shown is a representative experiment of two performed (C) Representative quantification of band intensity of the corresponding Western blots is presented as ratio of cleaved target protein over total protein intensity. Values are means $\pm$ s.d. Densitometry is expressed as percentage compared with the vehicle-treated group (considered as 100%, marked by a dashed line). Experiments were repeated twice. * $P \leq 0.05$, ** $P \leq 0.01$.

attenuated. PARP-1 was induced by all tetrac concentrations after 4 h of treatment, while no significant effect was observed at the remaining times. Regarding γ H2AX, activation was evident after 1 h of treatment with 10 and 50 μ M tetrac. At 2 h of treatment, an increase was observed for 1 and 10 μ M tetrac concentrations. γ H2AX activation by 1 μ M tetrac was sustained after 4 h of treatment. A second peak in γ H2AX phosphorylation was observed after 8 h of treatment, with 10 and 50 μ M tetrac concentrations. The calculated results following tetrac incubations are presented in Fig. 4B. Supplementary Figure 5A, B and C depicts results in three additional MM cell lines, presenting cell-specific time- and dose-dependent response to tetrac.

The effects of tetrac on proliferation, viability, apoptosis and DNA damage repair in MM cells are blocked by RGD

Tetrac-binding site on integrin $\alpha v \beta 3$ is at the thyroid hormone receptor site, near the RGD-recognition site (Cody *et al.* 2007, Freindorf *et al.* 2012, Davis *et al.* 2016). To support integrin involvement, RGD peptide was studied for its effects on tetrac actions. CAG cells were seeded (100,000/96-well plate) and treated in the presence/absence

of increasing concentrations of RGD peptide (50, 100 or 200 μ M) and tetrac (25–100 μ M) overnight (24 h of treatment). Representative cell viability experiment indicates that high concentrations of RGD, alone (Fig. 5A, gray line), did not affect cell proliferation, while tetrac, alone (Fig. 5A, black line), reduced cell proliferation in a concentration-dependent manner. This effect was allosterically interfered by increasing RGD concentrations (Fig. 5A, dashed gray lines). No effect was observed with the negative control RGE peptide (Fig. 5B). Comparable viability results were obtained in RPMI-8226 cells (Supplementary Fig. 4D). In addition, cell death (annexin-V/PI) was explored in CAG cells (100,000/96-well plate) treated overnight with high tetrac concentration (100 μ M) in the presence or absence of RGD (1–500 μ M). Representative results (Fig. 5C) demonstrate that tetrac reduced the surviving fraction (An-/PI-) by about 50%, and this effect was hindered by RGD. In accord with the proliferation results, RGD alone had no effect. The increase in apoptotic cell death in response to 100 μ M tetrac was effectively inhibited by RGD (Fig. 5D). Representative annexin-V/PI results are depicted in Supplementary Fig. 4C.

We next examined whether RGD interfered with the action of tetrac on DNA damage/repair. CAG cells

Figure 3

Involvement of p53 and caspases in action of tetrac on MM cells. Temperature-sensitive RPMI-8226 cells were treated with tetrac±Z-VAD-FMK and grown at 32°C or 37°C and evaluated for (A) cell proliferation (WST-1), (B) cell number (flow-cytometry), (C) apoptosis (Annexin-V/PI) and (D and E) cell cycle, flow-cytometry. Experiments were repeated three times at triplicate. * $P \leq 0.05$, ** $P \leq 0.01$. Significance between tetrac's effect under 32°C and 37°C is marked by #.

were incubated for 24h with increasing concentrations (0.1–100µM) of tetrac and RGD, together and alone (Fig. 5E). A representative blot indicates that tetrac significantly increased the level of γ H2AX phosphorylation by 2.8–4.67 fold, while RGD alone had no effect. When 1µM tetrac was combined with RGD (0.1–100µM), the induction in γ H2AX phosphorylation by tetrac was completely prevented (Fig. 5E, gray bars). Thus, the cancer cell-killing actions of tetrac are initiated at the integrin and the RGD recognition site and the tetrac-thyroid hormone-binding site on $\alpha v \beta 3$ are proximal to one another.

Tetrac induces apoptosis in primary BM cells from MM patients

After establishing the cytotoxic effects of tetrac in MM cell lines, we examined the effect of the drug on primary

BM cells. Mononuclear cells were isolated from the bone marrow of a myeloma patient (BM #1). The cells were seeded (100,000 cells/96 wells), treated in triplicates with tetrac (100nM or 1µM) for 96h and tested by annexin-V/PI assay for the presence of early apoptosis (an+/PI–) and for late apoptosis/necrosis (an+/PI+, an–/PI+) by flow-cytometry. Samples of the same cells treated with vehicle served as controls. Results demonstrate the induction of apoptosis by tetrac in whole mononuclear cell population (Fig. 6A) and in gated CD-138+ plasma cells (Fig. 6B), in comparison to untreated cells. Following this observation, the experiment was repeated with tetrac for 24–96h in an additional sixteen mononuclear BM samples (BM #1-17; clinical features are presented in Supplementary Table 1). The results from the entire study cohort (a total of seventeen samples) are presented in Supplementary Table 2. An induction in apoptotic cell death, in response

Figure 4

Tetrac increases DNA damage response markers in MM cells. RPMI-8226 cells were treated with tetrac (1–50 μM) for 1–8 h and were evaluated for pATM, PARP-1 and pγH2AX level and activation by Western blot. Experiments were repeated twice. Representative quantification of band intensity of the corresponding Western blots is presented as ratio of target protein to tubulin intensity. Values are means ± s.d. Densitometry is expressed as percentage compared with the vehicle-treated group (considered as 100%, marked by a dashed line). * $P \leq 0.05$, ** $P \leq 0.01$.

to low tetrac concentrations, was observed in twelve samples in comparison to samples of the same subject treated with vehicle (considered as 100%), in which the mean rate of basal % of apoptosis was 15 ± 5 .

Tetrac sensitizes MM cell lines and primary BM cells to the cytotoxic effect of bortezomib

Bortezomib is the first proteasome inhibitor in clinical use and is a leading treatment agent in MM (Chen *et al.* 2011). Based on the observation that thyroid hormone can antagonize bortezomib's action (Cohen *et al.* 2015), together with the anticancer activity of tetrac, led us to explore the potential of combined tetrac-bortezomib treatment. RPMI-8226 and CAG cells were seeded (100,000/96-well plate), treated overnight with tetrac (100 μM) with or without bortezomib (25 nM). Results indicate that bortezomib alone significantly decreased cell proliferation in RPMI-8226 cells by 68% and in CAG cells by 80%; tetrac results were by 60% and 72% reductions, respectively (Fig. 7A). The combined treatment lowered the proliferation rate by 80% in RPMI-8226 and by 90% in CAG cells. Cell survival (annexin-V/PI, Fig. 7B) was reduced by bortezomib in RPMI-8226 by 35% and in CAG cells by 70% while with tetrac by 35% and 50%, respectively. The combination of the two agents resulted in 55% and 85% reductions in cell

survival in the two cell lines, respectively. We then evaluated this treatment approach in primary BM mononuclear cells from a representative MM patient (BM#1) treated with bortezomib (25 nM) with/without tetrac (100 nM–1 μM). Tetrac sensitized the cells to the cytotoxic effect of bortezomib in the mononuclear cell population (Fig. 7C), and this sensitization was also demonstrated in CD138+ plasma cells (Fig. 7D). A representative annexin-V/PI assay is presented for whole BM cells (Fig. 7E, upper panel) and for CD138+ cells (Fig. 7E, lower panel). The same experiment was conducted on a total of eight additional BM samples, with comparable results obtained in five samples (BM#3–4, 7, 10 and 11), while in three samples (BM#2, 8 and 9), no sensitization was achieved (data not shown).

Primary BM cells from MM patients express αvβ3 integrin

The pleiotropic actions of thyroid hormone have been shown to be αvβ3 integrin mediated in myeloma cell lines (Cohen *et al.* 2011) and in mononuclear cells from the BM of myeloma patients (Cohen *et al.* 2015). αvβ3 integrin expression in myeloma cell lines, composed of 100% plasma cells (CD138+), is depicted in Supplementary Fig. 6. Comparable expression was shown on sorted plasma cells from BM of myeloma

Figure 5

Induction of cell death in MM cells is blocked by RGD. CAG cells were treated for an overnight with various concentrations of tetrac and examined for cell viability (WST-1) in combination with (A) RGD or (B) RGE. Experiments were repeated five times in triplicate. (C) Surviving cells (An-/PI-) and (D) apoptotic cells (An+/PI- and An+/PI+) were evaluated by flow-cytometry. Experiments were repeated three times in triplicate. (E) p7H2AX protein level and activation (Western blots). The experiment was repeated twice. Representative quantification of band intensity of the corresponding Western blots is presented as ratio of target protein to tubulin intensity. Values are means $\pm$ s.d. Densitometry is expressed as percentage compared with the vehicle-treated group (considered as 100%, marked by a dashed line). * $P \leq 0.05$, ** $P \leq 0.01$.

patients (Ria *et al.* 2002, Tucci *et al.* 2009, 2016). Our experimental model utilizes unsorted primary BM plasma cells, allowing study of the malignant clone within its natural surrounding cellular niche. We aimed to establish the integrin expression in this experimental cellular platform.

Mononuclear cells from the BM of a myeloma patient (BM#17, relevant clinical data is depicted in Supplementary Table 1) were stained with a FITC-labeled $\alpha\beta3$ antibody. Flow-cytometry analysis (Fig. 8A) depicts a high expression of $\alpha\beta3$ integrin in 82% of the cells. We then determined whether the high integrin expression is exclusively contributed by the malignant plasma cell clone. For that, the cells were stained with a plasma cell marker (CD138-APC) and a general mononuclear cells marker (CD45-FITC). Figure 8B depicts the malignant plasma clone (CD138+/CD45-, pink color) and the non-malignant mononuclear cells (CD138-/CD45+, green color). Next, the cells were stained with $\alpha\beta3$ -FITC antibody and gated for the previously described cell populations. Figure 8C indicates that the malignant

plasma cells (pink color) highly express this integrin, but this expression is also evident in non-malignant mononuclear cells (in green). Forward scatter (FSC) and side scatter (SSC) plot of the various cell populations is presented in Fig. 8D, showing malignant plasma cells (blue), monocytes (yellow), promyelocytes (purple) and lymphocytes (green). The relative $\alpha\beta3$ integrin expression in the different cell populations is shown in Fig. 8E. The same gating strategy was evaluated in a total of 25 MM BM samples (BM#5-6 and 17-39; relevant clinical data is depicted in Supplementary Table 1). In the majority of BM samples examined (19/25 samples, Supplementary Fig. 7A), positive integrin expression was documented, while the remaining samples were negative (a representative example is shown in Supplementary Fig. 7B). An additional normal BM sample was completely negative for the integrin expression (Control #1, Supplementary Fig. 7A). Although the highest integrin expression was frequently exhibited by the malignant plasma clone, in some cases, it was more pronounced in other mononuclear cells

Figure 6

Tetrac induces apoptosis in primary BM cells from MM patients. (A) Mononuclear cells from a MM BM sample (BM#1) were separated by Ficoll gradient, treated in triplicates with tetrac (100 nM–1 μ M) for 96 h and then analyzed for cell death (Annexin-V/PI, flow-cytometry). (A) Representative results for whole BM cells and (B) CD138+ cells. Samples of the same subject treated with vehicle served as controls. A full color version of this figure is available at <https://doi.org/10.1530/ERC-17-0246>.

(a representative example is shown in [Supplementary Fig. 7C](#)). Taken together, these collective results indicate that $\alpha\text{v}\beta 3$ integrin is expressed not only on the malignant myeloma clone, but also on the surrounding BM microenvironment cells.

Discussion

Tetrac is a naturally occurring deaminated analog of T_4 that binds the $\alpha\text{v}\beta 3$ integrin in two orientations and competitively displace both T_3 and T_4 , eliminating their non-genomic activities ([Bergh *et al.* 2005](#), [Davis *et al.* 2011](#), [Freindorf *et al.* 2012](#)). Tetrac possesses low hormone activity because of shortening of the side chain on the inner ring (removal of a carbon and amine), resulting in the conversion of propionic acid (thyroid hormone) to acetic acid (tetrac/triac). This transforms the compounds from thyroid agonist at the thyroid hormone receptors, to antagonist ([Davis *et al.* 2011](#)). These antagonistic effects were extensively studied *in vitro* and in animal cancer models (reviewed in [Davis *et al.* 2016](#)), but never examined in MM. In the current work, we have focused on blocking the thyroid hormone- $\alpha\text{v}\beta 3$ axis using tetrac in $\alpha\text{v}\beta 3$ integrin expressing MM cells. By a number of complementary methods in several myeloma cell lines, tetrac produced anti-proliferative effects that were not affected by caspase inhibition and pro-apoptotic effects that

were caspase-9 and caspase-3-dependent. No involvement of AIF, a caspase-independent apoptosis marker, was recognized. The peak antitumor action of unmodified tetrac nongenomically initiated at the thyroid hormone-tetrac receptor site on integrin $\alpha\text{v}\beta 3$ is at 10^{-7} – 10^{-6} M ([Lin *et al.* 2011](#)). The antagonistic effects were observed by tetrac in the myeloma cell models mainly at high molar concentrations. However, no toxicity was detected in mice treated with up to 60 mg/kg tetrac (equivalent to 250 μ M tetrac concentration) ([Rebbaa *et al.* 2008](#)). At the indicated concentrations, tetrac was well tolerated, and no significant toxicity was noticed, including unchanged animal weights over the course of the study. To note, the effects produced by tetrac were not always concentration dependent. Similar non-linear integrin-ligand association with other ligands (activators/inhibitors) was reported ([Legler *et al.* 2001](#), [Li & Springer 2017](#)). This may be attributed to the fact that integrins are altered following binding of ligands ([Campbell & Humphries 2011](#)). Such changes in the integrin configuration may alter the accessibility of ligands at different concentrations to various receptor sites on the integrin. Tetrac binding may therefore alter the integrin conformation, producing a non-linear effect.

To study the role of p53, a central regulator of apoptosis, in tetrac's pro-apoptotic action, temperature-sensitive RPMI-8226 cells that express mutant p53

Figure 7

Tetrac sensitizes MM cell lines and primary cells to bortezomib. RPMI-8226 and CAG cells were treated with bortezomib (25 nM) with/without tetrac (100 μ M) and were analyzed for (A) cell proliferation (WST-1, ELISA) and (B) survival (annexin-V/PI, flow-cytometry). Experiments were repeated at least 3 times, in triplicate. Primary BM cells from MM patients were treated with bortezomib (25 nM) with/without tetrac (100 nM–1 μ M) and were analyzed for (C and D) apoptosis (annexin-V/PI, flow-cytometry). (E) Representative annexin-V/PI results from a BM sample (BM#1) in whole BM cells (upper panel) and gated CD138+ plasma cells (lower panel). * $P \leq 0.05$, ** $P \leq 0.01$. A full color version of this figure is available at <https://doi.org/10.1530/ERC-17-0246>.

(E285K) at 37°C and wild type at 32°C, were used (Teoh *et al.* 2000, Hu *et al.* 2015). In the absence of active p53, tetrac induced caspase-dependent apoptosis, while in the presence of wild-type p53, the induction of caspase-dependent cell death was accompanied by cell cycle arrest that correlates with the well-established role of p53 as a cell cycle regulator (Giono & Manfredi 2006).

Key proteins that coordinate the recognition of DNA damage, ATM, PARP-1 and histone γ H2AX were rapidly induced by tetrac. In cell-free assays (unpublished data), we have demonstrated that tetrac does not directly bind to DNA and induce breaks, but rather inhibits DNA

repair mechanisms. This observation is supported by experiments in which this drug did not induce direct DNA damage but led to a potent reduction in repair mechanisms (Herbergs *et al.* 2011, Leith *et al.* 2017). Additional experiments, such as ChIPSeq, should be performed to reveal the mechanism by which tetrac inhibits DNA repair. To note, unmodified tetrac may also interfere with the classical hormones action at other levels, such as the cellular entry of T_4 and other non-genomic effects that do not involve $\alpha\beta 3$ signaling. Therefore, additional intracellular effects by unmodified tetrac cannot be ruled out.

Figure 8

Primary bone marrow cells from MM patients highly express $\alpha v \beta 3$ integrin. Flow-cytometry analysis of cells incubated with (A) $\alpha v \beta 3$ antibody and (B) CD138-APC and CD45-FITC antibodies. Malignant plasma cell clone is depicted in pink; mononuclear cells in green. (C) Analysis of expression of integrin in CD138+ (pink) vs CD138-cells (green). (D) Forward scatter (FSC) and side scatter (SSC) plot of the various cell populations: plasma cells (blue), promyelocytes (purple), monocytes (yellow) and lymphocytes (green). (E) Relative $\alpha v \beta 3$ expression in the various cell populations. Isotype control is depicted in black.

Three-dimensional crystallographic modeling of the $\alpha v \beta 3$ integrin confirmed that tetrac-binding site, similar to that of the thyroid hormones, is located near the RGD-recognition site (Cody *et al.* 2007, Freindorf *et al.* 2012, Davis *et al.* 2016). Our results show that various tetrac-initiated effects were attenuated in the presence of RGD. Moreover, at equimolar concentrations, it appeared that tetrac was far more potent than RGD, which had no effect whatsoever, even at high micromolar concentrations. These results appear to be consistent with preclinical and clinical experience with RGD antagonists which report that high millimolar concentrations are required to produce an inhibitory effect in cancer (Weis *et al.* 2009).

Experiments performed on primary BM cells from MM patients often involve the enrichment of the samples with CD138+ plasma cells. However, this approach overlooks other mononuclear cells that are present in the BM. Given the recently acknowledged importance of the microenvironment in supporting MM (Rossi *et al.* 2013), there might be an advantage in studying the malignant clone within its natural surrounding niche. Such studies on heterogeneous cell populations in the myeloma BM have been reported (Lichtenstein *et al.* 1989, Liu *et al.* 2005, Noll *et al.* 2014, Chen *et al.* 2017, Gao *et al.* 2017). In this work, tetrac at low concentrations induced apoptosis in primary BM mononuclear MM cells at various disease stages. This apoptosis was evident in whole BM mononuclear cells and more profoundly in the plasma cell clone. Although no correlation between the response to tetrac and disease stage was documented, this should be examined in a larger scale study. Of note, in myeloma cell lines, the concentrations of tetrac needed to produce an apoptotic effect were generally higher. This may suggest that the mononuclear cellular milieu susceptible to tetrac indirectly assists in MM cell death.

We have reported that thyroid hormone antagonizes the cytotoxic effect of bortezomib (Cohen *et al.* 2015). Given that tetrac is a selective inhibitor of the T_3/T_4 - $\alpha v \beta 3$ axis, we hypothesized that there would be an advantage in combining tetrac with bortezomib. Our results provided support for a sensitizing effect for most actions in the combined treatment. Tetrac has been shown before to increase the sensitivity of resistant cancer cells to chemotherapy (Rebbaa *et al.* 2008, Davis *et al.* 2011, 2014), via the plasma membrane multi-drug resistance (MDR) pumps.

Finally, the expression of $\alpha v \beta 3$ integrin in whole BM mononuclear cells was revealed not only on the malignant plasma clone, as previously reported (Ria *et al.* 2002, Tucci *et al.* 2009), but also on heterogeneous cell populations within the BM (monocytes, lymphocytes and promyelocytes). The expression of $\alpha v \beta 3$ on peripheral blood monocytes and granulocytes has recently been reported (Shojaei *et al.* 2012). To the best of our knowledge, our results are the first to indicate $\alpha v \beta 3$ abundance on monocytes, granulocyte precursors (promyelocytes) and lymphocytes in the MM BM. These results point out that the thyroid hormone- $\alpha v \beta 3$ axis may be relevant not only to the malignant clone, but also to the surrounding BM mononuclear cells. Such an assumption was recently supported by another study showing the relevance of the thyroid hormone- $\alpha v \beta 3$ axis to the crosstalk between the BM microenvironment and other malignant cells (Schmohl *et al.* 2015, Davis *et al.* 2016). This further suggests that it may be important to study primary MM cells in the context of their stromal cell milieu.

In conclusion, tetrac generated anti-proliferative and pro-apoptotic responses and increased the sensitivity to combined tetrac-bortezomib treatment in MM cells. This, together with our observation of $\alpha v \beta 3$ integrin

expression in primary MM and stromal cells, highlights the relevance of this integrin in this disease and suggests that pharmacologic inhibition of the thyroid hormone-integrin $\alpha v \beta 3$ pathway may serve as a potential approach to disease management.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/ERC-17-0246>.

Declaration of interest

Co-author P J D is stockholder and officer in a company developing modified forms of tetrac as anticancer agents. The other authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

K C designed, preformed, analyzed and interpreted the experimental data. U A and M E assisted in the selection of patients included in the study and the collection of the BM samples. O A F designed, analyzed and interpreted the experimental data. K C, P J D, A H, M E and O A F wrote the manuscript. All authors read and approved the final manuscript.

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