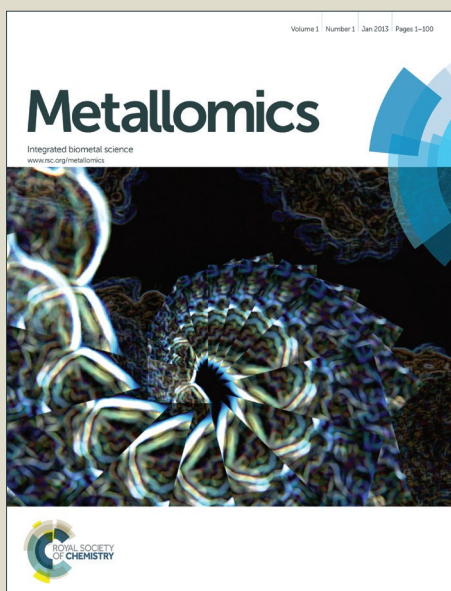


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ARTICLE

Strong induction of iodothyronine deiodinases by chemotherapeutic selenocompounds

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The biological activity of thyroid hormones (TH) is regulated by selenoenzymes of the iodothyronine deiodinase (DIO) family catalysing TH activating and inactivating reactions. Besides TH metabolism, several studies indicate an important role of DIO isoenzymes in tumorigenesis and cancer growth. It is therefore of therapeutic importance to identify modulators of DIO expression. We have synthesized and studied a series of selenocompounds containing a methyl- or benzyl-imidoselenocarbamate backbone. One of these novel compounds had chemotherapeutic activities in a murine xenograft tumour model by an unknown mechanism. Therefore, we tested their effects on DIO expression *in vitro*. In HepG2 hepatocarcinoma cells, DIO1 activity was strongly (up to 10-fold) increased by the methyl- but not by the corresponding benzyl-imidoselenocarbamates. Steady-state mRNA levels remained unaltered under these conditions indicating a post-transcriptional mode of action. The effects were further characterized in HEK293 cells stably expressing DIO1, DIO2 or DIO3. Even within the artificial genetic context of the expression vectors, all three DIO isoenzymes were up-regulated by the methyl- and to a lesser extent by the benzyl-imidoselenocarbamates. Consistent stimulating effects were observed with methyl-N,N'-di(quinolin-3-ylcarbonyl)-imidoselenocarbamate (EI201), a selenocompound known for its anti-tumour activity. DIO inducing effects were unrelated to the intracellular accumulation of selenium, yet the precise mode of action remains elusive. Collectively, our data highlight that these selenocompounds may constitute interesting pharmacological compounds for modifying DIO expression potentially affecting the balance between cell differentiation and proliferation.

Introduction

The family of iodothyronine deiodinases (DIO) contains three selenoenzymes involved in thyroid hormone (TH) metabolism. All DIO isoenzymes contain selenocysteine as an essential residue in their active site needed for biological activity^{1,2}. A well-coordinated and balanced regulation of DIO expression is critical for adapting TH activities to the actual cellular needs in TH target tissues. The DIO2 isoenzyme is capable of 5'-deiodination thereby activating the prohormone T4 to the active TH metabolite T3, whereas DIO3 is specialized in degradation of T4 to rT3 and T3 to T2, respectively, thereby limiting TH activity³⁻⁵. DIO1 is able to catalyze both of these reactions. Details on the control mechanisms affecting its catalytic direction are, however, poorly characterized.

The appropriate control of TH activity is essential for the coordinated development in vertebrate species, regular energy metabolism and many other biochemical and endocrine pathways⁶⁻⁹. Besides the developmental and metabolic effects, recent studies suggest an involvement of DIO in tumorigenesis¹⁰. Although their precise role is not yet understood, isoenzyme-

specific expression changes have been reported in a variety of tumors¹¹⁻¹³. DIO3 expression becomes reactivated in human neoplasia, such as in hepatic haemangiomas¹⁴, malignant brain tumors¹⁵ and malignant basal cell carcinoma¹². In tumor xenograft models, elevated DIO3 and loss of DIO2 expression resulted in increased levels of cyclin D1 and accelerated cell proliferation. Accordingly, the targeted knockdown of DIO3 led to a fivefold reduction in tumor growth¹². Collectively, these studies support the concept that control of TH activity balances cell differentiation versus tumor cell proliferation. We have synthesized a series of selenocompounds with a methyl- or benzyl-imidoselenocarbamate backbone affecting selenoprotein expression *in vitro* by inducing biosynthesis of selenoprotein P and glutathione peroxidase 1 while in parallel suppressing activity of thioredoxin reductase 1^{16,17}. The net effect on cell growth may involve a combination of effects on the selenoproteome in tumor cells. As one of these selenocompounds proved as a potent chemotherapeutic agent in a murine tumor model *in vivo*¹⁸, we now specifically tested their impact on DIO biosynthesis, and report on remarkable effects on DIO expression.

Experimental

Materials

All chemicals were obtained from Sigma-Aldrich (München, Germany), Abcam (Cambridge, UK), Thermo Fisher Scientific (Schwerte, Germany), PEQLAB (Erlangen, Germany) or Merck (Darmstadt, Germany). Cell culture materials were from Sarstedt (Nümbrecht, Germany), Life Technologies (Karlsruhe, Germany) or Biochrom AG (Berlin, Germany).

Chemistry

The synthesis of these compounds was carried out starting from Se-methyl selenourea (EI201, EI204, EI206) or Se-benzyl selenourea (EI601, EI604, EI606) as hydrohalides, and the corresponding heteroaryl acyl chloride, as described earlier¹⁶.

Cell culture

The cancer cell lines HepG2 and HEK293 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). HepG2 cells were cultured in DMEM/F12 (Life Technologies) and HEK293 cells in DMEM medium (Biochrom AG) containing 10% fetal bovine serum (FBS) in a humidified incubator at 37°C in 5% CO₂.

HEK293 cells were transfected with pCDH expression plasmids (Systems Biosciences, Mountain View, CA) carrying the complete open reading frames of human DIO2 or DIO3 in front of and in frame to a FLAG-tag along with a common SECIS element for facilitating protein quantification and ensuring detection of full-length proteins only. Stable lines were generated by puromycin treatment, isolated and propagated.

Treatments

Prior to treatments, cells were counted and seeded at a concentration of 2.5×10^5 cells per well in 6-well plates. After 24 h of incubation, medium was removed and cells were washed twice with PBS. Cells were stimulated with the imidoselenocarbamates at 1.0 μM f.c. for 6 h and 48 h, and with 0.1 μM sodium selenite, respectively. These different concentrations were chosen because of the relative toxicities of the compounds, as the LD₅₀ of sodium selenite in HepG2 is 10-100 times lower than of the selenocarbamates (1-2 μM vs. 88 μM)^{16, 19}. HepG2 cells were stimulated in serum-free medium. HEK293 cells were stimulated in the presence of 2.5% FBS as the cells were not viable under serum-free conditions.

Se determination

Cells were isolated, washed in PBS and homogenized in homogenization buffer (250 mM sucrose, 20 mM HEPES, 1 mM EDTA, pH 7.4). Aliquots of the homogenates were digested in 65% HNO₃ for 1 h at 70°C, and a gallium standard was added as internal control. A benchtop TXRF (total reflection X-ray fluorescence) spectrophotometer (Picofox TM S2, Bruker) was

used to determine Se concentrations. Samples were analyzed as described previously¹⁹.

Deiodinase Activity Assays

Cells were lysed in homogenization buffer containing 1 mM DTT. DIO activities were measured by a non-radioactive assay as described previously²⁰. Briefly, the non-radioactive assay relies on the release of iodide from the preferred substrate, its subsequent separation and detection by a photometric method first described by Sandell and Kolthoff in 1937²¹. The iodinated substrate used for DIO1 measurements was reverse-T3 (rT3), DIO2 activity was determined with L-thyroxine (T4) and DIO3 was tested with L-3,3',5-triiodothyronine (T3). The radioactive assay was essentially as described²².

Western Blot analysis

Cells were lysed in RIPA buffer containing 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS. After 30 min on ice, cell lysates were clarified by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentrations were assessed by Bradford reagent (Bio-Rad Laboratories). Proteins were separated by SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semi-dry transfer. The following antibodies were used; anti-FLAG (1:2000, Sigma), anti-DIO1 (1:2000, kind gift of Prof. V. Gladyshev, Boston, U.S.A.), anti-rabbit IgG (1:2000, Dako) in combination with HRP-conjugated anti-mouse IgG (1:5000, GE Healthcare). Signals were recorded after incubation with ChemiGlow West Chemiluminescence Substrate Kit (Protein Simple, Santa Clara, USA) by a digital imaging system (Alpha Innotech FluorChem FC2 Imager, R&D Systems, Wiesbaden, Germany).

Quantitative reverse transcription PCR

Total RNA was isolated from HepG2 and HEK293 cells using TriFast™ reagent (PEQLAB, Erlangen, Germany). Per reaction, 1 μg RNA was reverse transcribed using iScript cDNA Synthesis Kit (BioRad, München, Germany) according to the manufacturer's instructions. Quantitative realtime PCR (qRT-PCR) was performed using the FastStart Universal SYBR Green PCR Master Mix (Roche, Stockholm, Sweden) in combination with the Bio-Rad iCycler iQ5 System (BioRad). The results were normalized using HPRT as reference gene. Primer sequences are listed in the supplement (Table S1). Efficiency of amplification was determined by linear dilution using the REST algorithm²³. Purity of RNA preparations and comparable extraction efficiencies from cultured cells were assessed by analytical gel electrophoresis. Specificity of amplifications was verified by melting curve analyses.

Cell Viability Assessment

Compound-specific cytotoxic effects were studied by exposing HepG2 and HEK293 cells in culture to sodium selenite and serial dilutions of the imidoselenocarbamates (to f.c. of 0.0;

0.01; 0.1, 1.0 and 10 μM). Viability was determined using CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) according to the manufacturer's instructions. Relative light units (RLU) corresponding to the ATP content/viability of cells were recorded and used to assess relative toxicities.

Statistical analysis

GraphPad Prism 5 and SPSS were used for data analysis. Normal distribution of data was assessed by a Kolmogorov-Smirnov test. Data are expressed as mean \pm SD and analyzed by Student's t-test or ONE-WAY-ANOVA followed by Dunnett's post hoc test. Statistical significance was defined as $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***).

Results

Induction of DIO1 by imidoselenocarbamates

Treatment of hepatocarcinoma HepG2 cells by the imidoselenocarbamates caused a strong increase in DIO1 enzymatic activity (Fig. 1).

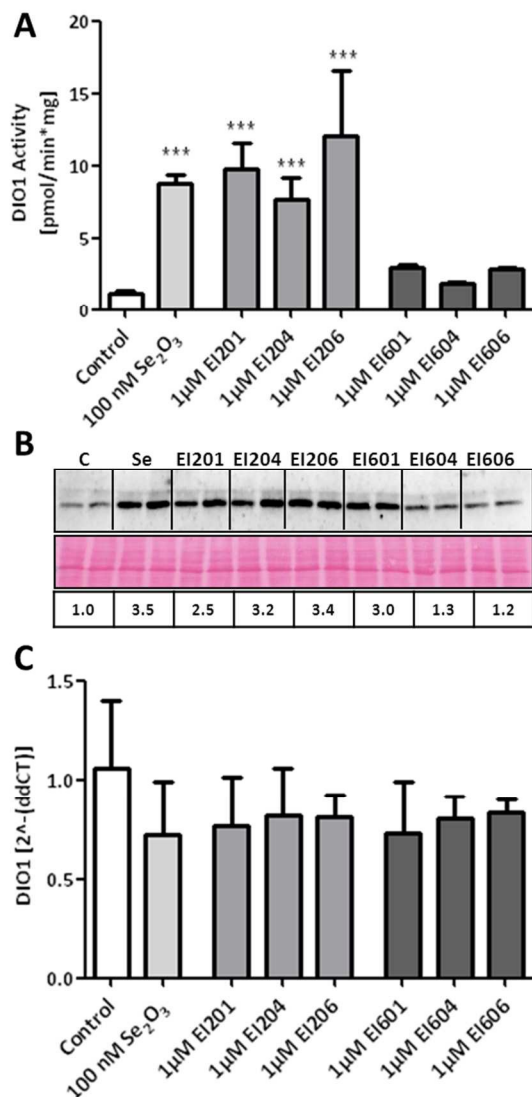


Fig. 1 Compound-specific effects of imidoselenocarbamates on DIO1 expression in HepG2 cells. (A) DIO1-activity is strongly up-regulated by sodium selenite (Se₂O₃) and the methyl-imidoselenocarbamates EI201, EI204, and EI206 in comparison to the benzyl-derivatives EI601, EI604, and EI606. (B) Western Blot analysis of DIO1 protein levels is in agreement with the activity data. Ponceau Red staining of the membrane verifies equal loadings and uniform transfer. Relative expression levels are expressed as ratio over control. (C) qRT-PCR analysis indicates that DIO1 mRNA levels remained unaffected by the treatments. The imidoselenocarbamates were applied at a f. c. of 1 μM for 48 h. Data represent mean \pm SD; n=3.

The effects were compound- and backbone- specific. While the methyl-imidoselenocarbamates (EI201, EI204, and EI206) caused 8- to 12-times increased activity, the respective benzyl-imidoselenocarbamates (EI601, EI604, and EI606) were less effective (Fig. 1A).

Western blot analyses of DIO1 protein concentrations are in agreement with these results (Fig. 1B), with the notable exception of the benzyl-imidoselenocarbamate EI601. The reason for this apparent discrepancy is currently unknown. Sodium selenite served as positive control for DIO1 induction in HepG2 cells. In order to better characterize the underlying mode of regulation, qRT-PCR analyses were conducted (Fig. 1C). DIO1 mRNA expression levels remained constant in response to the different selenocompounds used, indicating that the effects were exerted at the post-transcriptional level.

Similar effects on FLAG-tagged DIO1 were observed in HEK293 cells stably transfected with a DIO1 expression vector. The imidoselenocarbamates EI201, EI204, EI206 and EI601 caused strongly increased DIO1 enzymatic activities, while EI604 and EI606 were ineffective (not shown).

Effects of imidoselenocarbamates on DIO2 expression in stably transfected HEK293 cells

DIO2 is the major isoenzyme responsible for intracellular TH activation of thyroxine (T₄) to the active hormone T₃. The recombinant DIO2 is undetectable by Western blot analysis under control conditions as its biosynthesis to immunologically detectable amounts appears to stringently require a minimum of available Se. The imidoselenocarbamates were again strong stimulators of expression and increased DIO2 activities in the stably transfected HEK293 cells (Fig. 2A).

The incubations with sodium selenite, EI201, EI204, EI206 and EI601 elicited the strongest effects increasing DIO2 activity by 5- to 7-times above control. These effects were reflected in the Western blot analysis of recombinant FLAG-tagged DIO2 (Fig. 2B). Notably, again the benzyl-carbamate EI601 was peculiar in showing some isoenzyme-specificity and eliciting strong effects on DIO2 activity, as compared to the moderate effects on DIO1 (Fig. 1) and DIO3 (below), respectively. A parallel analysis on transcript level revealed only moderate effects of the compounds as modulators of DIO2 mRNA steady-state levels pointing again to post-transcriptional mechanisms as the major regulatory pathways (Supplementary Figure S1).

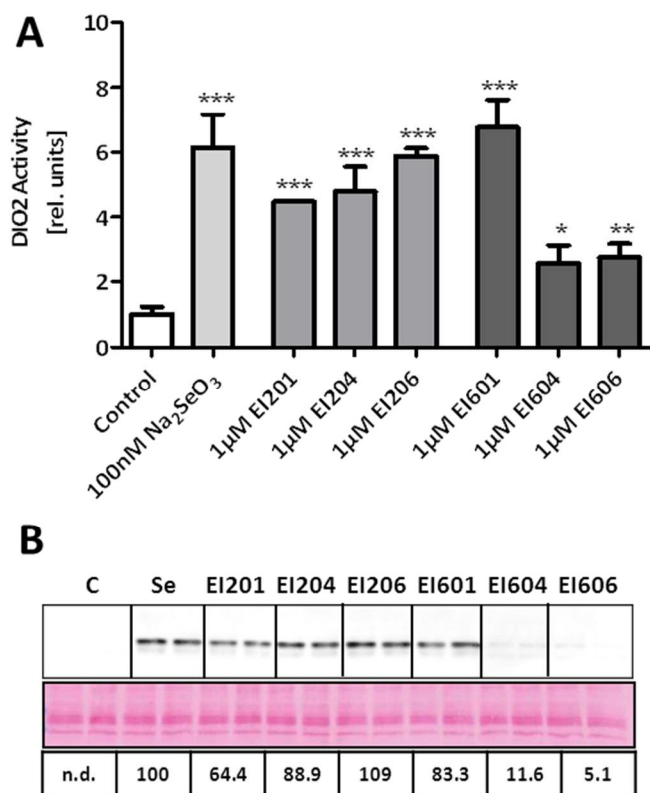


Fig. 2 Effects of imidoselenocarbamates on DIO2 expression in stably transfected HEK293 cells. (A) Some of the imidoselenocarbamates induced DIO2 activity to a similar extent as the positive control selenocompound sodium selenite (Se) at 100 nM f.c.. (B) Western Blot analysis verifies strongly induced DIO2 protein levels by selenite, EI201, EI204, EI206, and EI601. Ponceau Red staining of the membrane verifies equal loadings and uniform transfer. Relative expression levels are expressed as ratio over control (A) or as percentage of the signals obtained with sodium selenite (Se) (B). The imidoselenocarbamates were applied at f. c. of 1 µM for 48 h. Data represent mean + SD, n=3.

Effects of imidoselenocarbamates on DIO3 expression

Finally, the effects on the third biological relevant DIO isoenzyme were studied. DIO3 is the degradation-specific family member limiting TH activities by removing an iodine atom from the inner ring. HEK293 cells stably expressing FLAG-tagged DIO3 were established and tested as described above. The different imidoselenocarbamates were again strong inducers of DIO3 enzymatic activity (Fig. 3A). These effects were reflected in increased DIO3 protein amounts as observed by Western blot analysis (Fig. 3B). The qRT-PCR analyses verified again that the effects were largely independent of altered DIO3 transcript concentrations (Supplementary Figure S1).

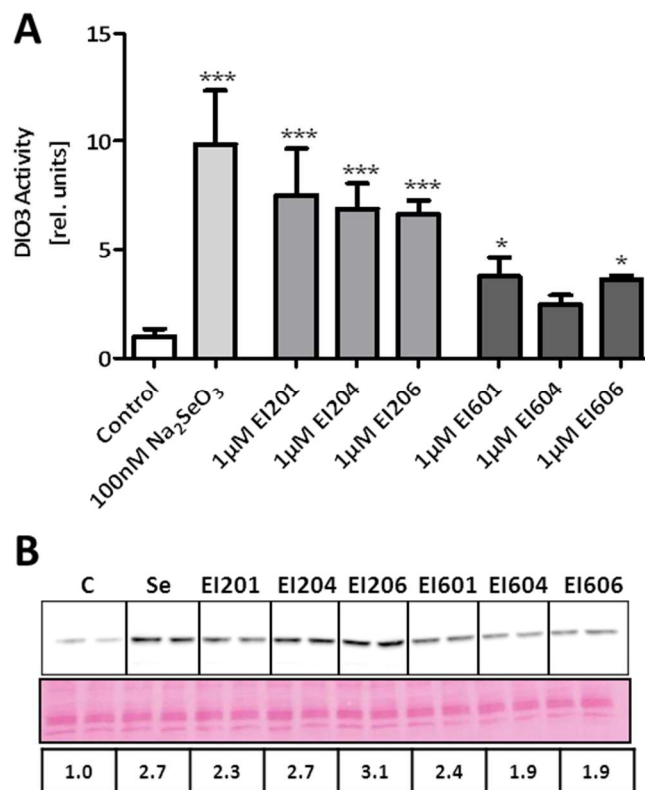


Fig. 3 Effects of imidoselenocarbamates on DIO3 expression in stably transfected HEK293 cells. (A) All imidoselenocarbamates along with sodium selenite induced DIO3 activity, especially EI201, EI204, and EI206. (B) Western Blot analyses verify that selenite and especially the methyl-imidoselenocarbamates (EI201, EI204, EI206) induce DIO3 protein levels strongly. The imidoselenocarbamates were applied at f. c. of 1 µM for 48 h. Data represent mean + SD, n=3.

Se accumulation in response to selenocarbamate treatments

Selenocompounds are characterized by varying degrees of stability, toxicity, nutritional value, association with cells and cellular uptake¹⁹. In order to better characterize the different imidoselenocarbamates, cellular selenium concentrations were determined after incubation of the cells with the selenocompounds (Fig. 4).

As expected, the different imidoselenocarbamates elicited compound-specific effects on the cellular Se concentrations causing high Se accumulation. Notably, the effects differed when between the two cell lines analyzed, i.e., EI606 was specifically effective in HepG2 cells (Fig. 4A) in comparison to the HEK293 cells expressing recombinant DIO2 (Fig. 4B) or DIO3 (Fig. 4C), where EI204 along with the whole set of benzyl-imidoselenocarbamates showed similar effects, far more intense than the positive standard compound selenite.

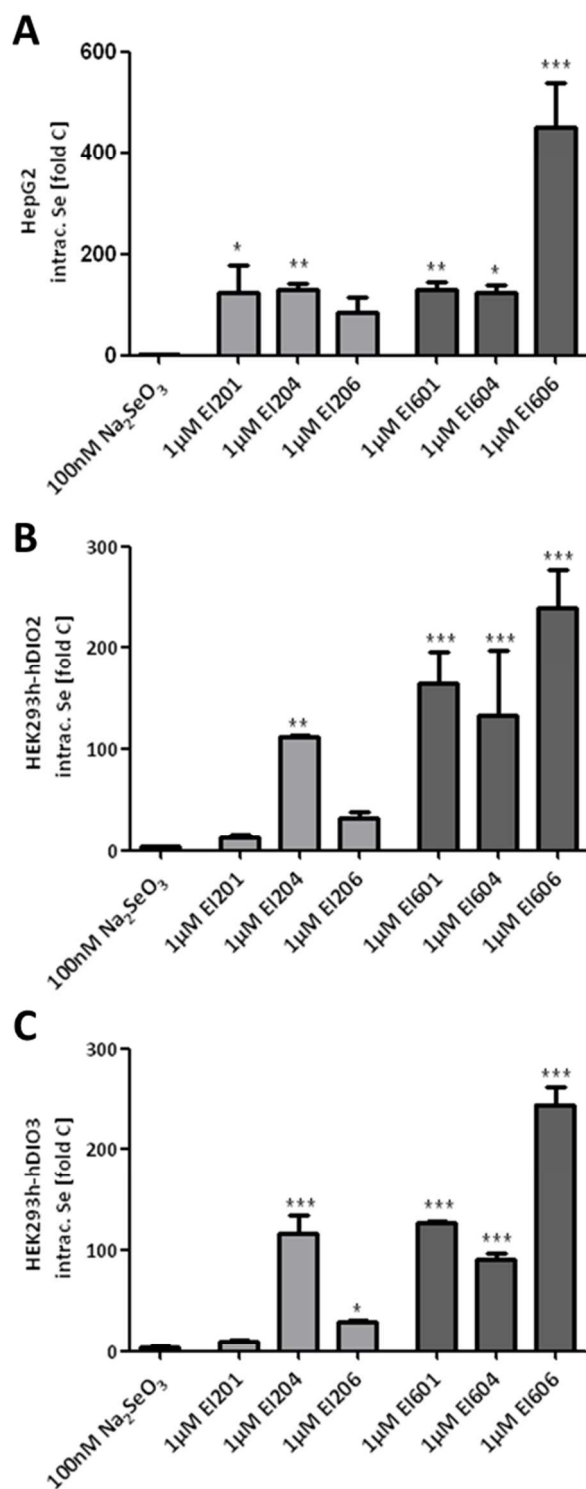


Fig. 4 Effects of imidoselenocarbamates on cellular Se concentrations. (A) All imidoselenocarbamates increased cellular Se concentrations in HepG2 cells strongly, far above the effects of the positive control substance sodium selenite. A similar picture is observed in (B) HEK293 cells stably transfected with FLAG-tagged DIO2 or (C) stably expressing FLAG-tagged DIO3. Notably, the benzyl-series of imidoselenocarbamates, especially EI606, was most efficient in raising cellular Se concentrations. Data represent mean + SD, n=3.

Test for deiodinating activity of the imidoselenocarbamates

Selenocompounds may act as DIO mimics having direct deiodinating activities²⁴. To control whether the imidoselenocarbamates are directly catalyzing the liberation of iodide in the DIO reactions, we selected one representative substance of the methyl- and one of the benzyl-series (Fig. 5A).

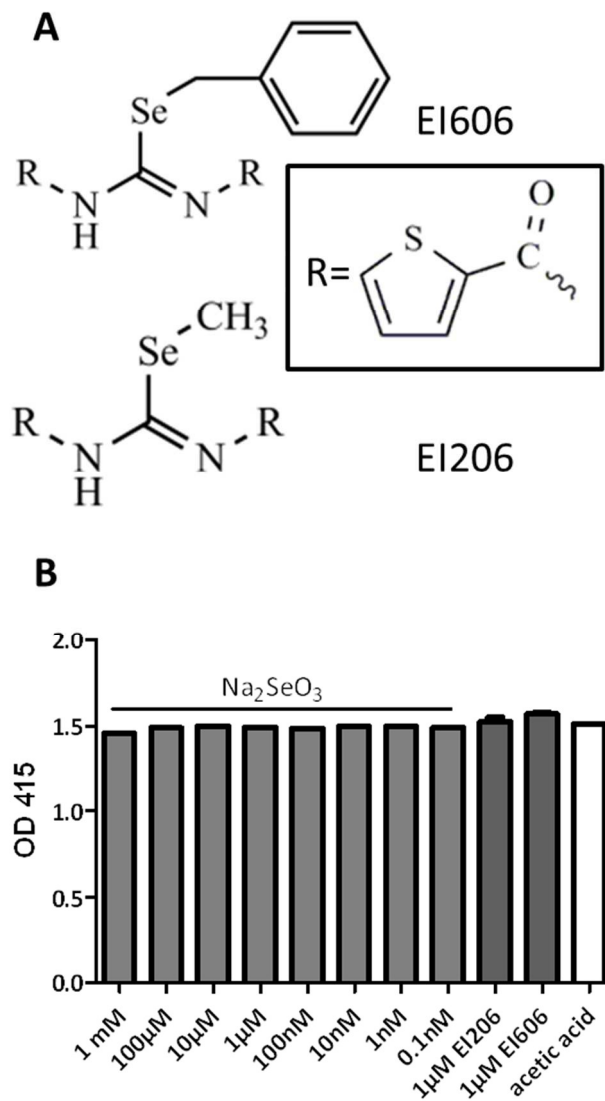


Fig. 5 Test of the imidoselenocarbamates for direct deiodinating activities in vitro. Two imidoselenocarbamates were selected and tested for their direct deiodinating activity. (A) Schematic structures of the benzyl- (top) and methyl- (bottom) series of imidoselenocarbamates. As an example, EI606 and EI206 carry as side chain (R) the indicated thiophenyl-group. An overview on all the structures used in the studies is found in a previous publication¹⁷. (B) Neither selenite nor the imido-selenocarbamates or the solvent (acetic acid) shows deiodinase activity (OD at 415 nm) when tested by the DIO assay in vitro. Data represent mean + SD; n=3.

When tested with the substrates under enzyme assay conditions, neither selenite or acetic acid nor EI206 or EI606 was able to catalyze iodide liberation from the substrate in a cell-free context (Fig. 5B).

As a second test for potential direct effects of the imidoselenocarbamates on endogenous DIO activity, we incubated murine liver homogenates as a natural source of Dio1 with the different imidoselenocarbamates for 1 h under enzyme assay conditions. In these experiments, Dio1-activity was determined using the classical radioactive deiodination assay, as it has a higher degree of sensitivity²². Again, the imidoselenocarbamates did not affect DIO1-activity under cell-free conditions (Fig. 6A).

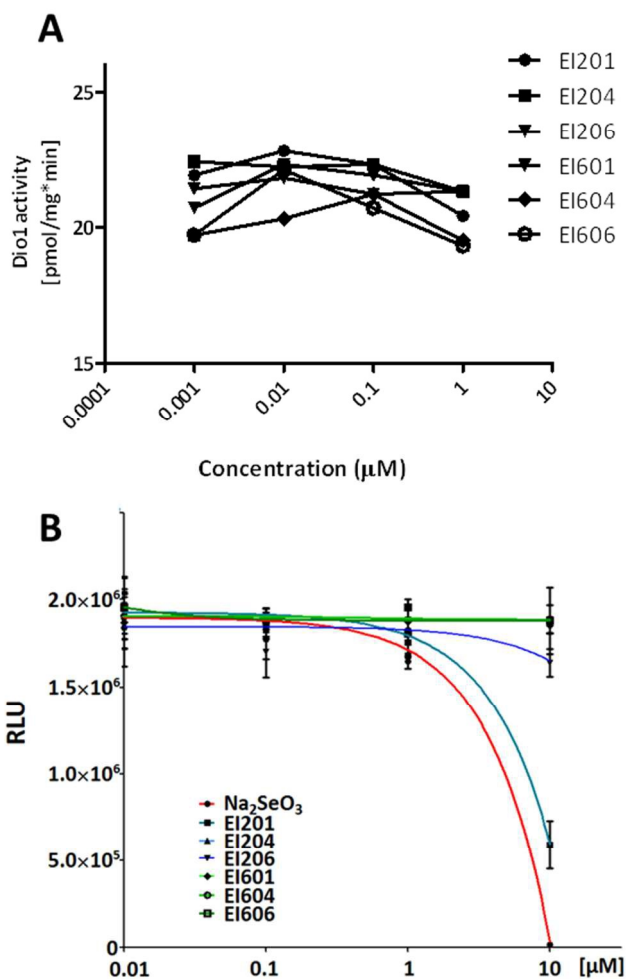


Fig. 6 Test of the imidoselenocarbamates for directly affecting hepatic Dio1 activity and for cytotoxicity in liver cells in vitro. (A) Incubation of mouse liver homogenates with the imidoselenocarbamates for 1 h in different concentrations does not affect DIO1 activity as determined by the classical radioactive DIO assay. (B) Comparison of relative cytotoxicities in HepG2 cells indicates relatively little cytotoxicity of the imidoselenocarbamates in contrast to sodium selenite. Data represent mean + SD; n=4.

Comparison of the relative cytotoxicities

To compare potential compound-specific cytotoxic effects, serial dilutions of the different imidoselenocarbamates to final concentrations of 0.0; 0.01; 0.1; 1.0 and 10 μM were applied to HepG2 cells in culture (Fig. 6B). Even up to the highest concentration of 10 μM , little toxicity was observed for the

majority of compounds with the exception of EI201. In comparison, toxicity of sodium selenite as positive control was already elicited at 1.0 μM . These data confirm that the imidoselenocarbamates are well tolerated by living cells.

Discussion

Thyroid hormones (TH) are central endocrine messengers that control virtually all major organs and metabolic pathways. The intracellular TH activity is regulated by the uptake and DIO-dependent metabolism of TH as well as by the availability of functional TH receptors. Gene mutations may cause a resistance to TH phenotype²⁵. The importance of TH transport across cell membranes has finally been established by the identification of inherited mutations in monocarboxylate transporter 8 (MCT8) in children with the Allan-Herndon-Dudley syndrome²⁶. Inherited mutations in any of the three DIO genes have not yet been identified.

Our current knowledge on their physiological importance is mainly derived from transgenic mouse models²⁷ and genetic association studies²⁸. The general picture that emerged highlights an importance of DIO2 for TH activation during central nervous system and bone development^{29, 30}, DIO1 for iodide recycling and TH metabolism³¹, and DIO3 for protection from inadequate high TH stimulation, e.g. during cochlear or cone development^{32, 33}. Further insights into the role of DIO for TH metabolism came from the characterization of children with inherited mutations in selenoprotein biosynthesis displaying reduced DIO expression along with an abnormal TH pattern in blood³⁴. Moreover, a deranged DIO expression is held responsible for the development of the low-T3 syndrome in critical disease and under certain pathophysiological conditions³⁵.

The major therapeutic strategy for dealing with increased TH concentrations in patients, i.e. hyperthyroidism, involves removing the thyroid gland or destroying iodide accumulating cells by radiotherapy, reducing TH biosynthesis by inhibitors of iodide uptake and organification or by impairing the activation of T4 to active T3 by DIO inhibitors like propylthiouracil^{36, 37}.

The treatment of hypothyroidism, i.e., a relative lack of functional TH, involves the substitution of the TH prohormone T4 to restore euthyroidism³⁸. It is assumed that the TH target cells adapt the intracellular T3 concentration according to the actual needs by DIO-mediated T4 activation to T3 or inactivation to rT3³. Experimentally, DIO1 expression can be up-regulated by TH in liver or pituitary^{39, 40}, DIO2 can be induced by the cAMP pathway⁴¹, and all DIO isoenzymes are induced by Se supplementation in severely Se-deficient subjects⁴². This interaction may explain some of the positive health benefits of Se supplementation in thyroid diseases⁴³.

A more specific and better controllable pharmacological regulation of DIO expression is highly desirable as TH are of central importance for carbohydrate metabolism and diabetes⁴⁴, critical illness³⁵ and cancer⁴⁴. The imidoselenocarbamates described in this study became highly enriched by the cells, elicited compound- and cell-specific effects on DIO expression

and as such constitute a promising class of novel TH modulators (Fig. 7).

Especially the methyl-selenocarbamates may prove as suitable lead structures as studies with mice already indicated their positive risk-benefit profile¹⁸.

In addition, the extraordinarily strong effects of the benzyl-series on cellular Se concentrations may also prove of therapeutic value if some targeting of the compounds could be achieved⁴⁵. The cell-specific effects on Se concentrations in HepG2 and HEK293 cells support this idea as a potentially promising strategy. In how far these novel imido-selenocarbamates are metabolized and broken down within the cells to serve as Se donors, become deposited as available Se reservoir by an association with cellular membranes or elicit trans-activities on the selenoprotein biosynthesis machinery remains to be studied in future analyses.

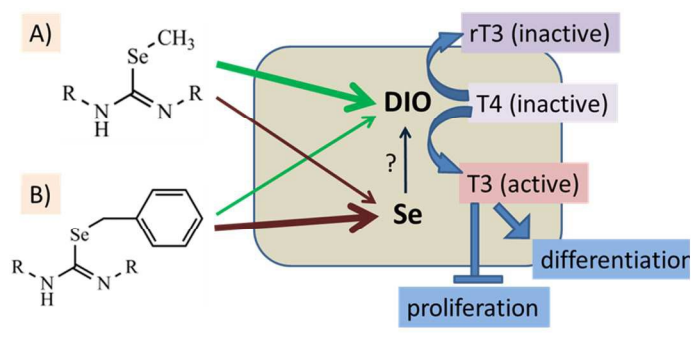


Fig. 7 Schematic picture highlighting the deduced biological effects of imidoselenocarbamates on Se concentrations, DIO expression and biological response of tumor cells. Imidoselenocarbamates increase intracellular Se levels and DIO activities. The effects are cell- and compound-specific partly depending on their methyl- (A) or benzyl- (B) backbone. DIO isoenzymes convert the thyroid prohormone T4 to active T3 or inactive reverse T3 (rT3). Strongly induced DIO activities will affect energy metabolism, differentiation and proliferation and may contribute to the chemotherapeutic effects of imidoselenocarbamates as observed in the *in vivo* model.

Conclusions

In the context of drug research, we conclude that the strong modulating effects of the methyl-imidoselenocarbamates on the expression of DIO isoenzymes as here observed *in vitro* may have contributed to their anticancer effects as observed in the murine tumor model *vivo*¹⁸. Further studies are needed to verify this notion. Collectively, our data confirm the qualification of the imidoselenocarbamates as interesting drug candidates for pharmacologically affecting TH metabolism.

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