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Evaluation of the anticancer properties of the predicted hBaxBH3-mimetic compound 2-hydroxy-3,5-dinitrobenzamide in a mammary carcinogenesis-induced rat model

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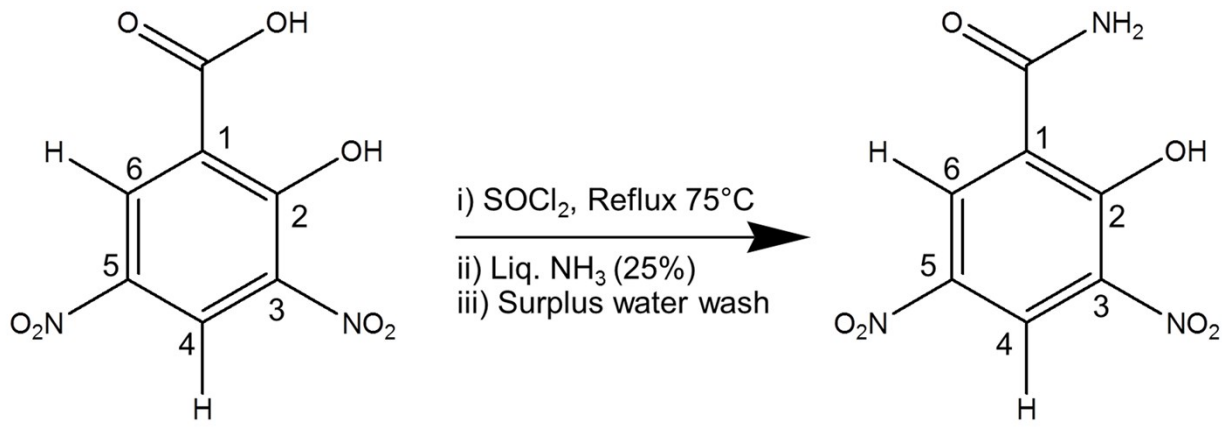

Fig. S1 Schematic diagram representing methodology of synthesis of 2-hydroxy-3,5-dinitrobenzamide (HDNB) from 3,5-dinitrosalicylic acid (DNSA).

Structural characterizations of the HDNB

The HDNB was not commercially available during course of the present studies and even till now. Owing to some scientific concerns, a crystal structure of the HDNB reported in the literature had been recently retracted and no other structural data on the compound were available in the literature to the best of our knowledge. Synthetic methodology of the HDNB is outlined in Fig. S1 and also described in the 'method' section. Though the synthetic procedure of the HDNB was straightforward to some extent, structural characterizations of the compound were challenging as discussed below herein. The HDNB was bright yellow in colour and water insoluble, whereas DNSA (3,5-dinitrosalicylic acid, the starting compound) was pale yellow in colour and water soluble. Figure S2A depicts difference absorption spectrum obtained by subtracting absorption spectrum of the DNSA from that of the HDNB measured at identical conditions and the data represented increased absorption intensity of the HDNB over the DNSA in the wavelength range of 425 – 500 nm (compounds showing absorption with these wavelengths appear yellow or orange in colours). The observed hyperchromic effect of the HDNB over DNSA could be attributed to the presence of $CONH_2$ group at 1st position of the HDNB, whereas COOH group is present in the counterpart of the DNSA. The electron-donating potential of the $CONH_2$ is relatively stronger than that of COOH moiety[1] and consequently increased electron drift from the $CONH_2$ (comparing from the COOH) to the electron-withdrawing groups of NO_2 (responsible chromophore for yellow colour of both the HDNB and DNSA) at 3rd and 5th positions through π-bonds of benzene ring could deepen the yellow colour of the HDNB over that of the DNSA due to probable reduction in π to π* energy levels. However, the data from the UV spectrometric studies on the HDNB could be only an indirect evidence to support for the presence of $CONH_2$ group in the compound. Thus, to determine

structure of the HDNB in an authentic manner, the compound was subjected to GC-MS analysis and the data obtained from the experiments are shown in Fig. S2B. Total ion chromatogram of the HDNB showed only two fragment species depicting molecular mass of 44 Da and 184 Da. Mass spectrum of molecular ion showing 184 Da is shown in the Fig. S2B and comprehensive analysis of various fragmented ions appeared in the spectrum suggested that the compound was 2,4-dinitro phenol as processed and screened against NIST 2005 library. Obviously, the 2,4-dinitro phenol is a common structural skeleton for both the HDNB and DNSA. Though the molecular fragment ion depicting mass of 44 Da is exactly matching with mass of $CONH_2$ group (as expected for the HDNB), we cannot rule out carboxylate ion (matching mass of 44 Da) that may be generated from COOH group of the DNSA. Though the GC-MS analyses were inconclusive to authentically elucidate 3D structure of the HDNB, combined analysis of data obtained from the UV and GC-MS experiments suggested that the HDNB was formed from the DNSA as shown in the Fig. S1. It is based on the following facts: (i) the absorption spectra and solubility of the HDNB and DNSA are different from each other; (ii) the HDNB was proven to have 2,4-dinitro phenol (common structural skeleton of the HDNB and DNSA) and a chemical moiety with molecular mass of 44 Da as suggested by the GC-MS.

Infrared (IR) spectra of the DNSA and HDNB are shown in Fig. S2. Presence of carboxylic acid groups in chemical molecules can be unambiguously identified through unique IR absorption bands for C=O and OH groups of COOH: strong C=O stretching between 1760 – 1660 cm^{-1} along with broad OH stretching band in the region of 3400 – 2500 cm^{-1} certainly represent presence of carboxylic acid group(s) in the compounds[2]. The IR spectrum of the DNSA showed a strong vibration band at 1677 cm^{-1} and broad band in the region of 3100 – 2500 cm^{-1} indicating the presence of a COOH group in the compound (Fig. S2C). In contrary, IR spectrum

of the HDNB showed absence of a broad band between 3100 - 2500 cm^{-1} and presence of a strong band at 1602 cm^{-1} suggesting that absence of COOH group and presence of a carbonyl moiety (Fig. S2D). The CO stretching frequency for the HDNB was observed by 75 cm^{-1} lower than that for the DNSA implying that carbonyl group of the HDNB may presumably linked with an electronegative group. Interestingly, IR vibration frequency for CO of the $CONH_2$ is obviously expected to be lower than that of the COOH due to resonance effects in amides, which increases CO bond length and consequently decreases CO bond strength comparing to carboxylic acid group. Evidently, IR spectrum of the HDNB also showed a characteristic strong NH stretching band at 3135 cm^{-1} implying that presence of an amide group in the compound. Characteristic IR absorption bands for other chemical moieties such as NO_2, phenolic OH, and CH of aromatic rings present in both the HDNB and DNSA are listed in Table S1.

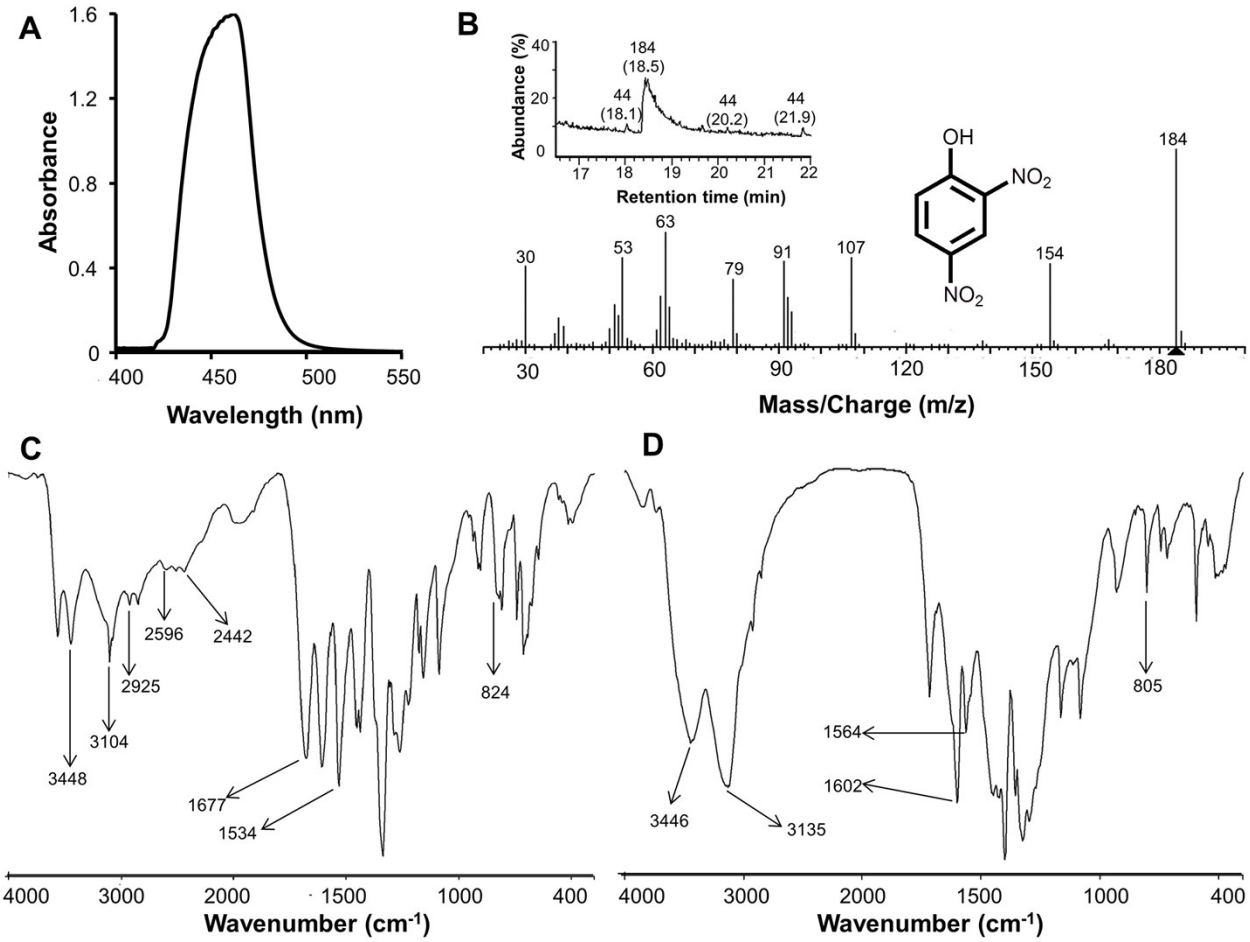

Fig. S2 Structural characterizations of the HDNB as studied by UV, GC-MS and FT-IR spectrometric techniques. (A) Difference absorption spectrum obtained by subtracting absorption spectrum of the DNSA from that of the HDNB. (B) Structure of a fragment molecule accounting various ions in the GC-MS mass spectrum of the HDNB is depicted. GC-MS total ion chromatogram for the HDNB is shown as inset figure and retention times of fragment ions depicting molecular mass of 184 Da and 44 Da are given in brackets. FT-IR spectra of (C) the DNSA and (D) HDNB are shown with labeled bands for certain characteristic vibrational frequencies of the compounds.

Table S1 Characteristic IR absorption frequencies for various functional groups present in the DNSA and HDNB.

	CO group stretching (cm^{-1})	**OH group of carboxylic acid stretching (cm^{-1})**	**NH stretching (cm^{-1})**	**Phenolic OH stretching (cm^{-1})**	**NO_2 group stretching (cm^{-1})**	**CH of aromatic ring bending (cm^{-1})**
DNSA	1677	3104, 2925, 2854, 2596, 2516, 2442	-	3448	1534	824
HDNB	1602	-	3135	3446	1564	805

References

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2. J. Coates, in Encyclopedia of analytical chemistry, ed. R.A. Meyers, John Wiley & Sons, Chichester, 2000, Interpretation of infrared spectra, a practical approach, 10815-10837.