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Partial agonistic activity of two irreversible β-adrenergic receptor ligands, bromoacetylated derivatives of alprenolol and pindolol

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Irreversible antagonists are required for classical pharmacological determinations of agonist affinity and the assessment of non-linear receptor–effector coupling (formerly referred to as "spare receptors") [1]. Irreversible antagonists have also been used in studies of receptor metabolism [2]. The assessment of non-linear receptor–effector coupling is especially important in studies of β-adrenergic receptors since agonist potency and signal amplification may vary by several orders of magnitude among model systems [1].

Many irreversible probes for β-adrenergic receptors have been utilized but most of them have low affinity for β-adrenergic receptors and non-specific interactions with other cellular proteins [3, 4]. Bromoacetylated derivatives of alprenolol (BAAM*) and pindolol (BIM) were introduced as high-affinity, irreversible antagonists for the β-adrenergic receptor [4–6]. Because alprenolol and pindolol have considerable partial agonistic activity [7, 8], it is conceivable that BAAM and BIM may also be partial agonists. Such partial agonism might undermine the utility of BAAM and BIM as irreversible probes of β-adrenergic receptors.

The present study was designed to determine whether BAAM and BIM possess partial agonistic activity. For this purpose we have employed a recently developed biochemical assay in established cell lines using forskolin to amplify the stimulation of cAMP accumulation by partial agonists [7, 8].

Materials and Methods

Wild-type (strain 24.3.2) S49 lymphoma cells were maintained in suspension culture as described [7]. BC_3H_1 smooth muscle-like cells were grown as monolayers as described [8], and experiments were conducted after 4–6 days of confluence (≈10^6 cells/35 mm dish).

To determine cAMP accumulation, S49 cells were incubated at 37° for 5 min with the indicated drugs in the presence or absence of 1 μM forskolin, and intracellular cAMP accumulation was assayed as described previously [7]. A similar procedure was used with BC_3H_1 cells, as described previously [8]. The generated cAMP was measured with a competitive protein binding assay as described [7].

Bromoacetylated derivatives of alprenolol (BAAM: N^8-(bromoacetyl)-N^1-[3-[(*o*-allylphenyl)oxy]-2-hydroxypropyl]-(Z)-1, 8-diamino-*p*-menthane) and pindolol (BIM: N^8-(bromoacetyl)-N^1-[3-(4-indolyloxy)-2-hydropropyl]-(Z)-1, 8-diamino-*p*-menthane) were provided by Dr. Josef Pitha (Gerontology Research Centre, National Institute of Aging, The Francis Scott Key Medical Center, Baltimore, MD).

The maximal stimulation of cAMP accumulation and the $-\log EC_{50}$ were calculated by fitting the pooled experimental data to a sigmoid curve using the InPlot program (GraphPAD Software, San Diego, CA). Calculating the parameters from the pooled data does not allow standard error determination for the fitted parameters, but readers can estimate the confidence intervals from the raw data given in the figures.

Results and Discussion

Similar to alprenolol, pindolol, and many other weak partial agonists at β-adrenergic receptors [7, 8], BAAM and BIM did not cause detectable stimulation of cAMP accumulation in S49 lymphoma cells ([2] and data not shown). In the presence of 1 μM forskolin, however, BAAM and BIM stimulated cAMP accumulation (Fig. 1). The maximal enhancement of cAMP accumulation was similar to that previously reported for the respective parent compounds and was approximately 5% of that for the full agonist isoproterenol [7, 8]. The $-\log EC_{50}$ values for BAAM and BIM were 8.1 and 8.8, respectively (mean of five experiments each). These values are in good agreement with the apparent affinity of these drugs at β_2-adrenergic receptors in binding studies [2, 6]. Similarly, we have previously observed a good correlation between affinity and $-\log EC_{50}$ for stimulating cAMP accumulation in the presence of forskolin for a wide variety of other partial agonists at β-adrenergic receptors [8].

The partial agonistic activities of BAAM and BIM were not restricted to S49 lymphoma cells but were also seen in BC_3H_1 smooth muscle cells (Fig. 2). In this cell line we also found that the agonistic effects of BAAM and BIM on cAMP accumulation were blocked by the β-adrenergic receptor antagonist propranolol. The stimulation of cAMP accumulation by pindolol in S49 cells is also blocked by propranolol [7]. The facts that the potency for stimulating cAMP accumulation match well the affinity for β-adrenergic binding sites and that propranolol blocks cAMP

* Abbreviations: BAAM, bromoacetyl alprenolol menthane; and BIM, bromoacetyl indolyloxy methane.

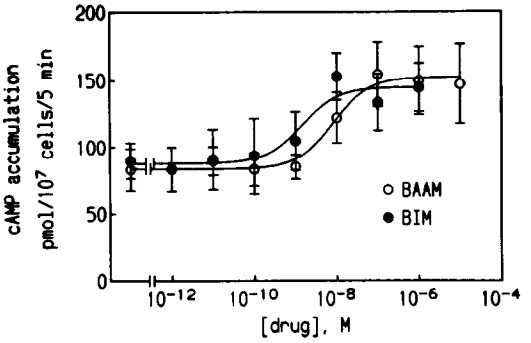

Fig. 1. BAAM and BIM concentration–response curves for stimulation of cAMP accumulation in S49 lymphoma cells in the presence of 1 μM forskolin. Data are means ± SEM of five experiments. Maximal cAMP accumulation values were 68.9 and 57.3 pmol cAMP formed/10^7 cells/5 min (83 and 65% over forskolin-stimulated cAMP accumulation). Compared to control values in the absence of drugs, the statistical significance of the enhanced response in the presence of BAAM was $P = 0.09$ at 10 nM and $P < 0.05$ at 100 nM and in the presence of BIM it was $P < 0.01$ at 10 nM.

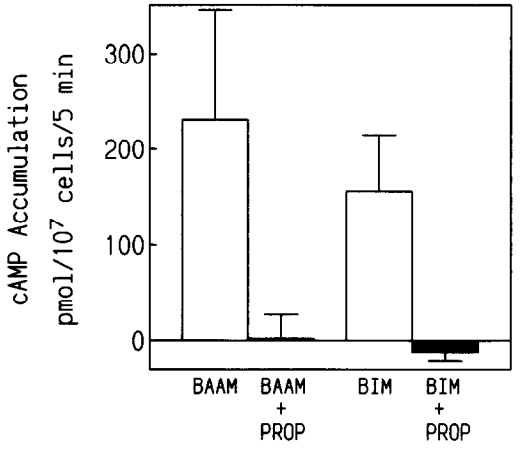

Fig. 2. BAAM (1 μM)- and BIM (1 μM)-stimulated cAMP accumulation in BC_3H_1 smooth muscle-like cells in the presence of 1 μM forskolin. Cyclic AMP accumulation by forskolin alone was 473 ± 101 pmol/10^7 cells/5 min (N = 10) and was subtracted from the values in the presence of BAAM or BIM. Data are means ± SEM; N = 8 for BAAM; N = 6 for BIM; N = 3 for either drug in the presence of 1 μM (−)-propranolol (PROP). For BAAM, one of nine experiments did not show an increase in cAMP accumulation and has been omitted. The increase in BAAM-stimulated cAMP in the presence of forskolin was marginally significant ($P = 0.099$), while that for BIM was very significant in these cells ($P = 0.002$).

accumulation demonstrate that the bromoacetylated derivatives of alprenolol and pindolol act via β-adrenergic receptors and not via an atypical site, as has been proposed for pindolol derivatives [9].

The agonistic efficacy of a partial agonist varies between tissues and species and depends largely on the presence and extent of amplification in the cellular signal transduction cascade. A weak partial agonist such as pindolol or prenalterol may appear as an almost pure antagonist in some systems and as a full agonist in others [10–12]. Since BAAM and BIM appear to have a similar intrinsic efficacy as does pindolol, it can be expected that they might demonstrate substantial agonistic activity in other systems beyond those that we studied. Thus, the partial agonistic activities of BAAM and BIM could affect the interpretation of experimental data obtained with these compounds in two ways. First, agonist properties of BAAM and BIM may promote down-regulation of β-adrenergic receptors in addition to the irreversible alkylation that these compounds produce. Whether or not agonist-mediated down-regulation of receptors has similar effects on cellular responses as does inactivation by irreversible antagonists is not known. Second, partial agonists might desensitize β-adrenergic and other cellular responses mediated by the stimulatory GTP-binding regulatory protein in addition to receptor down-regulation since heterologous desensitization can be achieved by prolonged low-level stimulation of β-adrenergic receptors [13]. At present it is not possible to predict the precise ramifications of these effects on estimates of agonist affinity, non-linear receptor–effector coupling or receptor metabolism. However, one can assume that the impact will be greatest in tissues with strong amplification mechanisms for β-adrenergic receptor signalling.

In summary, our data demonstrate that the irreversible β-adrenergic receptor probes BAAM and BIM are partial agonists. They should be used with caution until the impact of this finding on estimates of agonist affinity, non-linear receptor–effector coupling and receptor metabolism can be more precisely defined.

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Effects of benfluron and its two metabolites on respiratory processes in P388 murine leukemia and Ehrlich ascites cells

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Benfluron [5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo(*c*)fluorene*] has cytostatic potential [1]. In the course of pre-clinical tests of benfluron, biotransformation of the compound has been studied both *in vitro* and *in vivo* [2]. The metabolites were isolated; two of them are shown in Fig. 1.

In our previous paper the effect of benfluron metabolites on the glycolysis of both P388 and Ehrlich ascites cells was investigated [3]. A significant inhibition of aerobic glycolysis in the presence of the NOBF was found in P388 cells. Linker *et al.* [4] calculated that Ehrlich cells, grown in standard medium, produce 60% of ATP via oxidative pathways and 40% via glycolysis. Beckner *et al.* [5] showed that in the presence of glucose the motility of metastatic cells in the human melanoma line A 2058 depends primarily on energy from glycolysis. These findings suggest that the inhibition of glycolysis *in vivo* might reduce the tumor cells' ability to leave the primary tumor mass and metastasize to secondary sites.

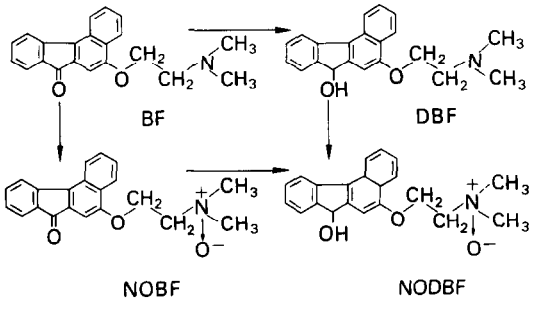

Fig. 1. The chemical structures of benfluron (BF) and its metabolites; DBF, NOBF and *N*-oxide of 7-dihydrobenfluron (NODBF).

* Abbreviations: benfluron, 5-(2-dimethylaminoethoxy)-7-oxo-7*H*-benzo(*c*)fluorene; NOBF, *N* oxide of benfluron; DBF, 7-dihydrobenfluron; DMSO, dimethyl sulfoxide.

Up to now, however, little has been known about the action of benzo(*c*)fluorene derivatives on respiratory processes in intact tumor cells. This paper describes the effects of benfluron and its two metabolites on the endogenous and exogenous respiration, in the presence of succinate as substrate, of both P388 murine leukemia and Ehrlich ascites carcinomal cells.

Materials and Methods

Sources of most materials and methods of tumor cell preparation, and incubation have been reported previously [3]. Mice with transplanted P388 cells were from Dr V. Ujhazy, Cancer Research Institute, Bratislava. The cells were washed in a saline phosphate medium [6] and suspended in the same medium as described in earlier papers [7, 8]. Oxygen uptake was measured with a Clark oxygen electrode. The effect of benfluron and its metabolites on the endogenous respiration of both tumour cells was determined on the basis of oxygen consumption in a saline phosphate medium [6]. Respiration with succinate as exogenous substrate was studied in the MES medium [9], pH 6.2. For the inhibition of endogenous respiration, rotenone was present at a concentration of 3 μmol/L.

Benfluron was dissolved in DMSO and DBF, and NOBF in ethanol shortly before use as stock solutions of different concentrations. In control experiments, DMSO and/or ethanol replaced the benfluron-metabolite solutions. The final concentration of DMSO was less than 1% which does not affect the metabolic processes studied [10].

Results and Discussion

The effects of benfluron itself and its two metabolites on the endogenous respiration of both Ehrlich ascites and P388 murine leukemia cells are shown in Fig. 2. Comparing the results, it is evident that P388 cells are less "sensitive" than Ehrlich ascites cells. NOBF (3) did not interfere with the respiratory processes in Ehrlich ascites cells, even at quite high concentrations. The modification of the chemical structure of the molecule of benzo(*c*)fluorene (introduction of $N^+ \rightarrow O^-$) resulted in changes in its physicochemical properties (lipophilicity) and thus in its distribution in the intracellular space. Possible explanations could be that the intramitochondrial NOBF concentration in intact tumor cells is less than in the surrounding medium, that NOBF does not easily enter into the cell at pH 7.4 or that it binds to other proteins. In P388 cells up to a concentration of