# FLUXAPYROXAD (addendum)

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## Explanation

Fluxapyroxad was evaluated in 2012 by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR), which concluded that high doses of fluxapyroxad caused hepatocellular adenomas and carcinomas and thyroid follicular cell adenomas and carcinomas in rats.

Following a request for additional maximum residue levels by the Codex Committee on Pesticide Residues, fluxapyroxad was placed on the agenda of the present Meeting, which assessed additional toxicological information on fluxapyroxad available since the last review.

Five in vitro studies were provided that investigated the potential of fluxapyroxad to activate nuclear hormone receptors and to stimulate cell proliferation using rat or human hepatocytes or rat liver microsomes to support the mode of action proposed for rodent liver tumours and its lack of relevance to humans.

## Evaluation for acceptable intake

### 1. Toxicological studies

### *1.1 Special studies*

#### *(a) Mode of action studies*

The potential of fluxapyroxad to activate nuclear hormone receptors – i.e. constitutive androstane receptor (CAR), pregnane X receptor (PXR), aryl hydrocarbon receptor (AhR) and peroxisome proliferator–activated receptor alpha (PPARα) – and to stimulate cell proliferation/ replicative DNA synthesis was investigated in five in vitro studies using rat hepatocytes, rat liver microsomes or human hepatocytes.

In the first study, designed as a range-finding study for the subsequent main study (Elcombe, 2016c), cultured primary hepatocytes isolated from male wild-type and CAR knockout Sprague Dawley rats were incubated either with fluxapyroxad (batch no. COD-001049; purity 99.2%) at concentrations ranging from 10 to 300 µmol/L or with vehicle control (0.1% volume per volume [v/v] dimethyl sulfoxide [DMSO]). Concentrations were based on a preliminary cytotoxicity evaluation. Phenobarbital (PB) (100 and 1000 µmol/L) and epidermal growth factor (EGF) (25 ng/mL) were used

as positive controls for the induction of the enzyme activities and replicative DNA synthesis, respectively. The medium, with fluxapyroxad or control substances, was replenished daily for a further 3 days.

Cell toxicity was assessed after 96 hours of culture by measurement of ATP depletion. Cellular ATP was determined by luminometry. CAR-mediated activation of cytochrome P450 2B (CYP2B) in hepatocytes was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (pentoxyresorufin *O*-depentylation [PROD]) or benzyloxyresorufin (benzyloxyresorufin *O*-debenzylation [BROD]). PXR-mediated activation of CYP2B and CYP3A in cultured hepatocytes was determined by BROD. PXR-mediated activation of CYP3A1 in cultured hepatocytes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from benzyloxyquinoline (benzyloxyquinoline *O*-debenzylation [BQ]). The number of cells undergoing replicative DNA synthesis (S-phase) was determined immunocytochemically following the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into hepatocyte nuclei over the last 3 days of culture. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes / total no. of hepatocytes) × 100].

The effects of fluxapyroxad and PB on cytochrome P450 activity in wild-type and CAR knockout hepatocytes are presented in Tables 1 and 2.

***Table 1. Induction of CYP2B and CYP3A activities in wild-type rat hepatocytes treated with fluxapyroxad or PB[a]***

| Treatment and concentration | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-hydroxyquinoline/min per milligram) |
|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 0.223 ± 0.040 | 0.643 ± 0.009 | 0.032 ± 0.001 |
| | (100.0 ± 17.9) | (100.0 ± 1.4) | (100.0 ± 3.0) |
| PB | | | |
| 100 µmol/L | 1.286 ± 0.168*** | 5.920 ± 0.415*** | 0.064 ± 0.005*** |
| | (576.7 ± 75.1) | (920.4 ± 64.4) | (201.5 ± 14.4) |
| 1 000 µmol/L | 1.827 ± 0.308*** | 9.496 ± 0.552*** | 0.158 ± 0.008*** |
| | (819.6 ± 138.3) | (1 476.4 ± 85.8) | (495.4 ± 23.6) |
| Fluxapyroxad | | | |
| 10 µmol/L | 1.612 ± 0.199*** | 7.494 ± 0.717*** | 0.130 ± 0.004*** |
| | (723.2 ± 89.1) | (1 165.1 ± 111.5) | (407.3 ± 12.6) |
| 30 µmol/L | 1.354 ± 0.169*** | 8.380 ± 0.114*** | 0.194 ± 0.018*** |
| | (607.3 ± 76.0) | (1 302.9 ± 17.7) | (607.4 ± 55.2) |
| 100 µmol/L | 0.800 ± 0.248* | 5.288 ± 0.315*** | 0.260 ± 0.010*** |
| | (359.1 ± 111.4) | (822.1 ± 49.0) | (811.8 ± 31.5) |
| 300 µmol/L | 0.356 ± 0.462 | 2.633 ± 2.369 | 0.104 ± 0.117 |
| | (159.8 ± 207.5) | (409.4 ± 368.3) | (326.2 ± 364.3) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-depentylation; v/v: volume per volume; *: $P < 0.05$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.
*Source*: Elcombe (2016a)

In wild-type rat hepatocytes, fluxapyroxad caused increases of up to 7.2-, 13- and 8.1-fold in PROD, BROD and BQ activities, respectively. PROD and BROD activities peaked at 10 and 30

µmol/L, respectively, declining at higher concentrations, whereas the CYP3A-mediated metabolism of BQ was highest at 100 µmol/L and decreased at 300 µmol/L (Table 1). The decrease in CYP2B activity at 100 µmol/L was attributed to an inhibition of CYP2B by fluxapyroxad, as was demonstrated in a separate study using rat liver microsomes (Elcombe, 2016b). The lower levels of induction of PROD, BROD and BQ at 300 µmol/L (Table 1) were probably the result of cytotoxicity, as ATP levels were reduced by about 99% at this dose.

***Table 2. Induction of CYP2B and CYP3A activities in CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]***

| Treatment and concentration | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-hydroxyquinoline/min per milligram) |
|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 0.212 ± 0.017 | 0.771 ± 0.097 | 0.030 ± 0.002 |
| | (100.0 ± 8.0) | (100.0 ± 12.6) | (100.0 ± 7.7) |
| PB | | | |
| 100 µmol/L | 0.220 ± 0.043 | 0.902 ± 0.074 | 0.027 ± 0.004 |
| | (103.6 ± 20.3) | (117.0 ± 9.6) | (89.7 ± 12.5) |
| 1 000 µmol/L | 0.363 ± 0.032** | 2.121 ± 0.058*** | 0.076 ± 0.002*** |
| | (171.0 ± 15.3) | (275.2 ± 7.5) | (252.7 ± 8.2) |
| Fluxapyroxad | | | |
| 10 µmol/L | 0.350 ± 0.011*** | 2.278 ± 0.099*** | 0.089 ± 0.007*** |
| | (165.3 ± 5.3) | (295.6 ± 12.8) | (297.5 ± 21.8) |
| 30 µmol/L | 0.434 ± 0.062** | 2.659 ± 0.333*** | 0.143 ± 0.003*** |
| | (204.7 ± 29.3) | (345.0 ± 43.2) | (479.3 ± 11.1) |
| 100 µmol/L | 0.364 ± 0.012*** | 1.865 ± 0.412* | 0.206 ± 0.022*** |
| | (171.8 ± 5.7) | (242.0 ± 53.5) | (688.9 ± 73.3) |
| 300 µmol/L | 0.428 ± 0.026*** | 1.156 ± 0.031** | 0.166 ± 0.036** |
| | (202.1 ± 12.3) | (150.0 ± 4.0) | (554.4 ± 119.5) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-dependentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.
*Source*: Elcombe (2016a)

Fluxapyroxad treatment of CAR knockout rat hepatocytes resulted in a non-dose-dependent increase of up to only 2.0-fold in CYP2B activity when measured as PROD. BROD levels showed up to a 3.5-fold increase at 30 µmol/L. The higher induction was considered to be the result of a co-metabolism of benzyloxyresorufin by CYP3A, as the induction of this cytochrome is PXR mediated, which is not knocked out in the rat model used. The lower levels of induction of BROD and BQ at 300 µmol/L (Table 2) were probably the result of cytotoxicity, as ATP levels were reduced by about 99% at this dose.

PB treatment of wild-type and knockout hepatocytes resulted in dose-dependent increases in CYP2B and CYP3A enzyme activities, as indicated by increases of up to 8.2-, 15- and 5.0-fold in PROD, BROD and BQ activities (Tables 1 and 2).

Fluxapyroxad treatment of wild-type rat hepatocytes resulted in an increase of up to 1.5-fold in replicating cells (Table 3). Similar to the induction of PROD activity, the greatest induction of cell proliferation was noted at 10 µmol/L, with a gradual decrease in the S-phase labelling index with

increasing concentrations. At 300 µmol/L, cytotoxicity was noted, which was visually evident by fewer and less confluent hepatocytes. PB and EGF caused 1.8- and 4.1-fold increases in the S-phase labelling index, respectively, in wild-type hepatocytes.

***Table 3. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in wild-type and CAR knockout rat hepatocytes[a]***

| | S-phase labelling index (%) | |
|---|---|---|
| **Test item and concentration** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 10.88 ± 1.48<br>(100.0 ± 13.6) | 4.68 ± 0.56<br>(100.0 ± 11.9) |
| PB | | |
| 100 µmol/L | 19.80 ± 2.24***<br>(182.1 ± 20.6) | 4.59 ± 0.43<br>(98.1 ± 9.2) |
| 1 000 µmol/L | 19.59 ± 1.65***<br>(180.1 ± 15.1) | 5.14 ± 0.56<br>(109.9 ± 11.9) |
| Fluxapyroxad | | |
| 10 µmol/L | 15.81 ± 1.07***<br>(145.3 ± 9.8) | 4.87 ± 0.80<br>(104.0 ± 17.1) |
| 30 µmol/L | 15.16 ± 1.39**<br>(139.4 ± 12.8) | 4.07 ± 0.90<br>(87.0 ± 19.2) |
| 100 µmol/L | 11.92 ± 1.29<br>(109.6 ± 11.8) | 1.60 ± 0.39***<br>(34.2 ± 8.4) |
| 300 µmol/L | 9.31 ± 4.30[b]<br>(85.6 ± 39.5) | Significant cytotoxicity[b] |
| EGF | | |
| 25 ng/mL | 44.17 ± 3.51***<br>(406.1 ± 32.3) | 20.32 ± 4.54***<br>(434.2 ± 97.0) |

CAR: constitutive androstane receptor; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 5$ per group.

[b] Cytotoxicity at this concentration.

*Source*: Elcombe (2016a)

Treatment of CAR knockout rat hepatocytes with fluxapyroxad resulted in a dose-dependent decrease in cell proliferation. Severe cytotoxicity was observed at 300 µmol/L. Phenobarbital did not affect cell proliferation. EGF led to a 4.3-fold increase in the labelling index in CAR knockout hepatocytes (Table 3).

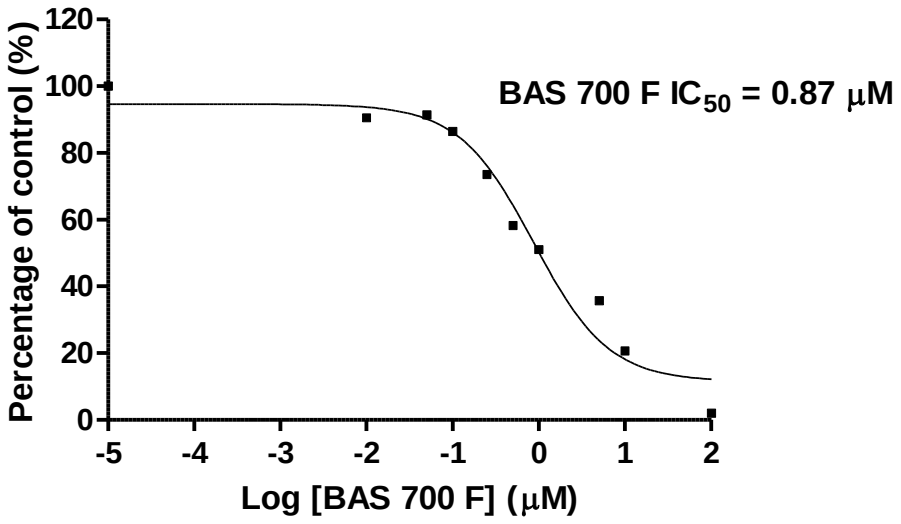
This study indicates that fluxapyroxad induces S-phase (replicative DNA synthesis) and CYP2B activity through CAR activation. Additionally, the experiment with CAR knockout hepatocytes showed that fluxapyroxad can activate PXR and subsequently induce CYP3A (Elcombe, 2016a).

In the second study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to inhibit CYP2B and CYP3A activities was assessed in Sprague Dawley rat liver microsomes. The activity of CYP2B in PB-induced rat liver microsomes was determined spectrofluorometrically by the

formation of resorufin from pentoxyresorufin (PROD). PROD activity was measured in PB-induced rat liver microsomes following incubation with fluxapyroxad at a concentration of 0, 0.01, 0.05, 0.1, 0.25, 0.5, 1, 5, 10 or 100 µmol/L. The activity of CYP3A in 5-pregnen-3β-ol-20-one 16α-carbonitrile–induced rat liver microsomes was determined spectrofluorometrically by the formation of 7-hydroxyquinoline from BQ. BQ activity was measured following incubation with fluxapyroxad at a concentration of 100 µmol/L.

Fluxapyroxad inhibited PROD (CYP2B) activity, with a median inhibitory concentration ($IC_{50}$) of 0.87 µmol/L (95% confidence limits: 0.42–1.79), decreasing activity to 2.0% of the control value at the top concentration of 100 µmol/L (Fig. 1).

***Fig. 1. PROD (CYP2B) inhibition by fluxapyroxad***




Note: The −5 log (µmol/L) concentration is the full activity of the control and not the actual concentration. This data point was included to aid with the curve fit, as recommended by the Prism software.

Fluxapyroxad at 100 µmol/L decreased BQ (CYP3A) activity to 69% of control levels.

This study shows that fluxapyroxad potently inhibits PROD activity and only mildly reduces BQ activity at a high concentration (Elcombe, 2016b).

In the third study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate AhR, CAR, PXR and PPARα and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary hepatocytes isolated from male wild-type and CAR knockout Sprague Dawley rats at fluxapyroxad concentrations ranging from 1 to 16 µmol/L. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. No positive controls for the induction of AhR or PPARα were included. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

AhR-mediated activation of CYP1A in hepatocytes was determined spectrofluorometrically by the formation of resorufin from ethoxyresorufin (ethoxyresorufin *O*-deethylation [EROD]), as well as by measurement of CYP1A1 and CYP1A2 mRNA levels. CAR-mediated activation of CYP2B was determined spectrofluorometrically by the formation of resorufin from pentoxyresorufin (PROD) or benzyloxyresorufin (BROD), as well as by measurement of CYP2B1 and CYP2B2 mRNA levels. PXR-mediated activation of CYP2B and CYP3A was determined spectrofluorometrically by the formation of resorufin from benzyloxyresorufin (BROD) and the formation of 7-hydroxyquinoline

from BQ, as well as by measurement of the CYP3A1 mRNA level. PPARα-mediated activation of CYP4A was measured as the formation of 12-hydroxylauric acid from lauric acid (lauric acid ω-hydroxylase [LAH]) using liquid chromatography with tandem mass spectrometry. In addition, the activity of PPARα-induced peroxisome proliferation was determined by the peroxisomal acyl-coenzyme A (CoA) oxidase (Acox1)–mediated desaturation of acyl-CoA using palmitoyl CoA as substrate. In addition, CYP4A1 mRNA levels and Acox1 mRNA levels were measured. Cell toxicity was assessed after 96 hours of culture by measurement of ATP depletion. Cellular ATP was determined by luminometry. The number of cells undergoing replicative DNA synthesis (S-phase) was determined immunocytochemically following the incorporation of BrdU into hepatocyte nuclei over the last 3 days of culture. The number of hepatocytes undergoing DNA synthesis was recorded as the labelling index [(no. of labelled hepatocytes / total no. of hepatocytes) × 100].

The effects of fluxapyroxad on cytochrome P450 activity in wild-type and CAR knockout hepatocytes are presented in Tables 4 and 5.

In wild-type hepatocytes incubated with fluxapyroxad, dose-dependent increases in CYP1A, CYP2B and CYP3A enzyme activities were noted, as indicated by 3.8-, 3.8-, 10- and 4.0-fold increases in EROD, PROD, BROD and BQ activities, respectively (Tables 4 and 5). The effects of fluxapyroxad and PB on enzyme activities were comparable. Compared with classical AhR activators such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and 2,3,7,8-tetrachlorodibenzofuran (TCDF), which induce about 18- and 35-fold increases in CYP1A enzyme activity, respectively (Budinsky et al., 2010), the increase in CYP1A activity induced by fluxapyroxad and PB was small.

The dose-dependent induction of CYP1A activity (EROD) in CAR knockout hepatocytes by fluxapyroxad was similar to that in wild-type hepatocytes. In CAR knockout hepatocytes treated with fluxapyroxad, the induction of PROD metabolism by CYP2B was much lower than in wild-type hepatocytes, reaching a maximum 1.5-fold increase at a fluxapyroxad concentration of 16 µmol/L. The increase was comparable with the 1.3-fold increase seen with 500 µmol/L PB. Also, the BROD metabolism (maximum 3.5-fold at 16 µmol/L) was considerably lower in CAR knockout hepatocytes than in wild-type hepatocytes (10-fold). The higher metabolism for BROD compared with PROD is considered to be partly due to the co-metabolism of BROD by CYP3A. CYP3A enzyme activity (BQ) was not affected in CAR knockout hepatocytes (maximum 5.8-fold increase) compared with wild-type hepatocytes (maximum 4.0-fold increase), as CYP3A activity is regulated via the PXR receptor, which is not knocked out in the CAR knockout hepatocytes (Tables 4 and 5).

In wild-type rat hepatocytes, fluxapyroxad induced a slight, non-dose-dependent increase in CYP4A-catalysed lauric acid 12-hydroxylation (maximum 1.8-fold). PB (500 µmol/L) induced a similar increase. The peroxisomal acyl-CoA oxidase (PCoA) activity was slightly reduced by fluxapyroxad at concentrations at and above 8 µmol/L, whereas PB had no effect on PCoA activity. PCoA activity in CAR knockout hepatocytes tended to increase (maximum 2.3-fold) at and above 4 µmol/L.

The effects of fluxapyroxad on cytochrome P450 mRNA levels in wild-type and CAR knockout hepatocytes are presented in Tables 6 and 7.

Fluxapyroxad induced a slight increase in CYP1A1 and CYP1A2 mRNA in wild-type (up to 2.5-fold) and CAR knockout hepatocytes (up to 1.9-fold). PB induced similar increases in mRNA. Dose-dependent inductions of CYP2B1 (up to about 200-fold) and CYP2B2 mRNA (up to about 300-fold) were observed in rat wild-type hepatocytes. Induction by 500 µmol/L PB was about 400-fold for CYP2B1 and 670-fold for CYP2B2 mRNA. In CAR knockout hepatocytes, the mRNA induction by fluxapyroxad was only up to 6-fold for CYP2B1 and up to 2.8-fold for CYP2B2.

Fluxapyroxad dose-dependently increased CYP3A1 mRNA levels in wild-type and CAR knockout rat hepatocytes, up to about 50-fold. It is noted that the PXR receptor is functional in both types of hepatocytes. CYP3A1 mRNA induction was 32- and 8.5-fold after treatment of wild-type and CAR knockout hepatocytes, respectively, with 500 µmol/L PB. Fluxapyroxad induced no increase in CYP4A1 mRNA levels in wild-type or CAR knockout hepatocytes. In fact, CYP4A1 mRNA levels tended to decrease with increasing fluxapyroxad levels. A 2.3-fold increase was noted in wild-type

***Table 4. Induction of CYP1A, CYP2B and CYP3A activities in wild-type and CAR knockout Sprague Dawley rat hepatocytes treated with fluxapyroxad or PB[a]***

| Test item and concentration | EROD (pmol resorufin/min per milligram) | | PROD (pmol resorufin/min per milligram) | | BROD (pmol resorufin/min per milligram) | |
|---|---|---|---|---|---|---|
| | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 14.07 ± 0.83<br>(100.0 ± 5.9) | 9.72 ± 0.65<br>(100.0 ± 6.7) | 0.320 ± 0.059<br>(100.0 ± 18.4) | 0.343 ± 0.044<br>(100.0 ± 12.9) | 0.939 ± 0.140<br>(100.0 ± 14.9) | 0.814 ± 0.125<br>(100.0 ± 15.3) |
| Fluxapyroxad | | | | | | |
| 1 µmol/L | 22.60 ± 0.28***<br>(160.6 ± 2.0) | 20.70 ± 0.63***<br>(213.0 ± 6.5) | 0.548 ± 0.032**<br>(171.5 ± 10.1) | 0.346 ± 0.016<br>(100.7 ± 4.5) | 3.931 ± 0.134***<br>(418.6 ± 14.3) | 1.273 ± 0.048**<br>(156.4 ± 5.9) |
| 2 µmol/L | 29.26 ± 0.36***<br>(208.0 ± 2.5) | 30.14 ± 0.27***<br>(310.2 ± 2.8) | 0.835 ± 0.058***<br>(261.1 ± 18.2) | 0.384 ± 0.013<br>(111.8 ± 3.7) | 5.739 ± 0.226***<br>(611.0 ± 24.0) | 1.565 ± 0.070***<br>(192.2 ± 8.6) |
| 4 µmol/L | 35.87 ± 2.05***<br>(254.9 ± 14.6) | 38.76 ± 3.12***<br>(398.9 ± 32.1) | 0.943 ± 0.057***<br>(295.1 ± 17.8) | 0.494 ± 0.031**<br>(143.9 ± 9.1) | 7.206 ± 0.335***<br>(767.2 ± 35.7) | 2.106 ± 0.056***<br>(258.7 ± 6.9) |
| 8 µmol/L | 45.19 ± 1.66***<br>(321.2 ± 11.8) | 47.32 ± 3.846***<br>(487.0 ± 39.6) | 1.109 ± 0.103***<br>(347.1 ± 32.1) | 0.475 ± 0.011**<br>(138.3 ± 3.1) | 8.677 ± 0.860***<br>(923.8 ± 91.5) | 2.423 ± 0.154***<br>(297.6 ± 18.9) |
| 16 µmol/L | 53.85 ± 3.34***<br>(382.8 ± 23.7) | 56.75 ± 3.62***<br>(584.1 ± 37.3) | 1.208 ± 0.015***<br>(377.8 ± 4.7) | 0.502 ± 0.071*<br>(146.2 ± 20.6) | 9.540 ± 0.767***<br>(1 015.8 ± 81.6) | 2.876 ± 0.069***<br>(353.3 ± 8.5) |
| PB | | | | | | |
| 500 µmol/L | 39.72 ± 2.15***<br>(282.3 ± 15.3) | 29.91 ± 1.14***<br>(307.8 ± 11.7) | 1.437 ± 0.080***<br>(449.7 ± 24.9) | 0.440 ± 0.030*<br>(128.0 ± 8.7) | 9.927 ± 0.461***<br>(1 056.9 ± 49.1) | 1.699 ± 0.078***<br>(208.7 ± 9.5) |

BROD: benzyloxyresorufin *O*-debenzylation; CAR: constitutive androstane receptor; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; EROD: ethoxyresorufin *O*-deethylation; PB: phenobarbital; PROD: pentoxyresorufin *O*-dependentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % of control ± standard deviation; $n = 3$ per group.

*Source*: Elcombe (2016c)

***Table 5. Induction of CYP3A and CYP4A activities and PPARα-induced peroxisomal acyl-CoA oxidase in wild-type and CAR knockout Sprague Dawley rat hepatocytes treated with fluxapyroxad or PB***

| | BQ (nmol 7-OH quinoline/min per milligram) | | LAH (nmol 12-OH lauric acid/h per milligram) | | PCoA (nmol NADH/min per milligram) | |
|---|---|---|---|---|---|---|
| **Test item and concentration** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 0.055 ± 0.002<br>(100.0 ± 4.4) | 0.025 ± 0.002<br>(100.0 ± 7.9) | 0.540 ± 0.111<br>(100.0 ± 20.5) | 0.702 ± 0.019<br>(100.0 ± 2.7) | 0.427 ± 0.095<br>(100.0 ± 22.2) | 0.176 ± 0.035<br>(100.0 ± 20.2) |
| Fluxapyroxad | | | | | | |
| 1 µmol/L | 0.075 ± 0.007*<br>(137.2 ± 13.6) | 0.038 ± 0.001***<br>(151.2 ± 2.8) | 0.751 ± 0.084*<br>(139.0 ± 15.6) | 1.049 ± 0.363<br>(149.4 ± 51.7) | 0.363 ± 0.144<br>(85.0 ± 33.8) | 0.177 ± 0.041<br>(100.7 ± 23.4) |
| 2 µmol/L | 0.089 ± 0.006***<br>(162.8 ± 10.7) | 0.041 ± 0.001***<br>(162.6 ± 5.7) | 0.907 ± 0.062**<br>(167.9 ± 11.5) | 1.193 ± 0.086***<br>(170.0 ± 12.3) | 0.392 ± 0.146<br>(91.8 ± 34.2) | 0.251 ± 0.141<br>(142.6 ± 80.4) |
| 4 µmol/L | 0.113 ± 0.009***<br>(206.1 ± 16.1) | 0.060 ± 0.005***<br>(238.1 ± 18.4) | 0.881 ± 0.146**<br>(163.2 ± 27.0) | 1.146 ± 0.004***<br>(163.3 ± 0.6) | 0.357 ± 0.135<br>(83.7 ± 31.7) | 0.393 ± 0.101*<br>(224.0 ± 57.7) |
| 8 µmol/L | 0.156 ± 0.008***<br>(284.0 ± 15.0) | 0.093 ± 0.007***<br>(370.1 ± 26.4) | 0.717 ± 0.057*<br>(132.8 ± 10.6) | 1.116 ± 0.023***<br>(159.1 ± 3.3) | 0.220 ± 0.116<br>(51.4 ± 27.1) | 0.413 ± 0.059**<br>(235.0 ± 33.6) |
| 16 µmol/L | 0.219 ± 0.011***<br>(399.3 ± 20.5) | 0.146 ± 0.002***<br>(577.6 ± 8.7) | 0.950 ± 0.049**<br>(176.0 ± 9.2) | 1.074 ± 0.027***<br>(153.1 ± 3.8) | 0.200 ± 0.110<br>(46.9 ± 25.8) | 0.405 ± 0.006***<br>(230.4 ± 3.5) |
| PB | | | | | | |
| 500 µmol/L | 0.152 ± 0.001***<br>(278.0 ± 2.3) | 0.043 ± 0.003***<br>(172.5 ± 10.7) | 0.893 ± 0.154**<br>(165.4 ± 28.6) | 0.898 ± 0.033***<br>(128.0 ± 4.8) | 0.567 ± 0.170<br>(132.8 ± 39.9) | 0.229 ± 0.030<br>(130.2 ± 17.3) |

BQ: benzyloxyquinoline *O*-debenzylation; CAR: constitutive androstane receptor; CoA: coenzyme A; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; LAH: lauric acid ω-hydroxylase; NADH: nicotinamide adenine dinucleotide (reduced); PB: phenobarbital; PCoA: peroxisomal acyl-coenzyme A oxidase; PPARα: peroxisome proliferator–activated receptor alpha; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation. Values in parentheses are mean % control ± standard deviation; $n = 3$ per group.

*Source*: Elcombe (2016c)

***Table 6. Induction of CYP1A1/1A2 and CYP2B1/2B2 mRNA in wild-type and CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]***

| | CYP1A1 mRNA | | CYP1A2 mRNA | | CYP2B1 mRNA | | CYP2B2 mRNA | |
|---|---|---|---|---|---|---|---|---|
| **Test item and concentration** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 1.00 ± 0.00 | 1.00 ± 0.17 | 1.00 ± 0.11 | 1.00 ± 0.36 | 1.00 ± 0.76 | 1.00 ± 0.54 | 1.00 ± 0.17 | 1.00 ± 0.20 |
| Fluxapyroxad | | | | | | | | |
| 1 µmol/L | 1.29 ± 0.21 | 1.26 ± 0.20 | 1.00 ± 0.17 | 1.01 ± 0.24 | 48.65 ± 25.56* | 1.42 ± 0.86 | 44.38 ± 30.34 | 1.26 ± 1.15 |
| 2 µmol/L | 0.82 ± 0.17 | 1.21 ± 0.04 | 1.39 ± 0.23 | 1.01 ± 0.10 | 69.56 ± 16.64** | 2.49 ± 2.61 | 58.80 ± 14.55** | 1.33 ± 0.62 |
| 4 µmol/L | 0.83 ± 0. 04** | 1.28 ± 0.02* | 1.57 ± 0.22* | 1.20 ± 0.08 | 79.69 ± 7.61*** | 3.23 ± 4.05 | 113.16 ± 30.74** | 1.36 ± 0.59 |
| 8 µmol/L | 1.12 ± 0.09 | 1.67 ± 0.05** | 1.43 ± 0.70 | 1.32 ± 0.21 | 150.41 ± 57.65* | 2.67 ± 1.09 | 296.33 ± 77.37** | 2.83 ± 1.00* |
| 16 µmol/L | 2.01 ± 0.30** | 1.25 ± 0.14 | 2.54 ± 0.53** | 1.91 ± 0.14* | 212.07 ± 33.99*** | 6.26 ± 3.79 | 287.06 ±105.16** | 2.10 ± 0.79 |
| PB | | | | | | | | |
| 500 µmol/L | 2.51 ± 0.25*** | 1.46 ± 0.31 | 1.88 ± 0.22** | 1.24 ± 0.37 | 395.71 ± 8.50*** | 1.49 ± 0.48 | 668.30 ± 66.47*** | 1.84 ± 0.22** |

CAR: constitutive androstane receptor; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

*Source*: Elcombe (2016c)

***Table 7. Induction of CYP3A1, CYP4A1 and Acox1 mRNA in wild-type and CAR knockout rat hepatocytes treated with fluxapyroxad or PB[a]***

| | CYP3A1 mRNA | | CYP4A1 mRNA | | Acox1 mRNA | |
|---|---|---|---|---|---|---|
| **Test item and concentration** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** | **Wild-type rat hepatocytes** | **CAR knockout rat hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 1.00 ± 0.01 | 1.00 ± 0.57 | 1.00 ± 0.27 | 1.00 ± 0.24 | 1.00 ± 0.10 | 1.00 ± 0.14 |
| Fluxapyroxad | | | | | | |
| 1 µmol/L | 4.38 ± 0.82** | 6.09 ± 4.49 | 0.82 ± 0.30 | 0.88 ± 0.25 | 1.03 ± 0.17 | 0.93 ± 0.05 |
| 2 µmol/L | 5.39 ± 0.81*** | 5.53 ± 0.56*** | 0.49 ± 0.10* | 0.48 ± 0.10* | 0.72 ± 0.07* | 0.94 ± 0.09 |
| 4 µmol/L | 11.14 ± 1.35*** | 14.35 ± 0.49*** | 0.53 ± 0.08* | 0.42 ± 0.10* | 0.76 ± 0.09* | 0.95 ± 0.03 |
| 8 µmol/L | 21.51 ± 3.02*** | 33.73 ± 6.25*** | 0.57 ± 0.34 | 0.31 ± 0.09* | 0.86 ± 0.11 | 1.04 ± 0.05 |
| 16 µmol/L | 49.26 ± 4.44*** | 52.76 ± 3.51*** | 0.76 ± 0.11 | 0.15 ± 0.04** | 1.59 ± 0.21* | 0.89 ± 0.11 |
| PB | | | | | | |
| 500 µmol/L | 32.18 ± 1.73*** | 8.48 ± 4.24* | 2.28 ± 0.17** | 0.88 ± 0.20 | 1.51 ± 0.05** | 0.99 ± 0.04 |

Acox1: acyl-coenzyme A oxidase 1; CAR: constitutive androstane receptor; CYP: cytochrome P450; mRNA: messenger ribonucleic acid; PB: phenobarbital; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

*Source*: Elcombe (2016c)

hepatocytes treated with 500 µmol/L PB. In addition, there was no indication for PPARα-induced peroxisome proliferation, as no biologically relevant changes in Acox1 mRNA levels were observed after fluxapyroxad or PB treatment in wild-type or CAR knockout hepatocytes.

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in rat hepatocytes are presented in Table 8.

***Table 8. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in wild-type and CAR knockout rat hepatocytes[a]***

| | S-phase labelling index (%) | |
|---|---|---|
| **Test item and concentration** | **Wild-type hepatocytes** | **CAR knockout hepatocytes** |
| Vehicle control (0.1% [v/v] DMSO) | 8.15 ± 0.60<br>(100.0 ± 7.4) | 5.63 ± 0.66<br>(100.0 ± 11.7) |
| Fluxapyroxad | | |
| 1 µmol/L | 9.89 ± 1.55*<br>(121.4 ± 19.0) | 5.56 ± 0.85<br>(98.9 ± 15.2) |
| 2 µmol/L | 13.18 ± 1.28***<br>(161.7 ± 15.7) | 6.03 ± 0.72<br>(107.1 ± 12.7) |
| 4 µmol/L | 15.65 ± 2.00***<br>(192.1 ± 24.6) | 5.96 ± 0.67<br>(106.0 ± 12.0) |
| 8 µmol/L | 16.53 ± 0.64***<br>(202.8 ± 7.8) | 5.97 ± 0.68<br>(106.1 ± 12.1) |
| 16 µmol/L | 15.53 ± 1.49***<br>(190.5 ± 18.2) | 5.58 ± 0.47<br>(99.3 ± 8.3) |
| PB | | |
| 500 µmol/L | 11.32 ± 0.52***<br>(138.9 ± 6.3) | 5.63 ± 0.49<br>(100.0 ± 8.7) |
| EGF | | |
| 25 ng/mL | 40.16 ± 3.52***<br>(492.7 ± 43.2) | 23.43 ± 1.84***<br>(416.3 ± 32.8) |

CAR: constitutive androstane receptor; DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n$ = 5 per group). Values in parentheses are mean % of control ± standard deviation.

*Source*: Elcombe (2016c)

Fluxapyroxad dose-dependently increased replicative DNA synthesis up to 2.0-fold, as indicated by an increased percentage of cells in replication (S-phase labelling index). PB (500 µmol/L) induced a 1.4-fold increase in replicative DNA synthesis, whereas EGF induced a 4.9-fold increase in S-phase labelling index. Fluxapyroxad and PB treatment did not affect replicative DNA synthesis in CAR knockout rat hepatocytes, indicating that induction of cell proliferation by fluxapyroxad and PB is mediated by the CAR receptor. EGF induced a 4.2-fold increase in replicative DNA synthesis in CAR knockout rat hepatocytes.

This study shows that fluxapyroxad increases S-phase (replicative DNA synthesis) and CYP2B activity through CAR activation. The induction profile of fluxapyroxad was similar to that of PB. Fluxapyroxad and PB also activated PXR and subsequently induced CYP3A, as BQ activities were still elevated in the CAR knockout rat hepatocytes. There was only a slight activation of AhR, as

shown by a small induction of CYP1A mRNA expression and enzyme activity. There was no evidence of PPARα-mediated activation, as CYP4A and peroxisome proliferation markers were generally unchanged (Elcombe, 2016c).

As the rat carcinogenicity study was conducted in Wistar rats, a fourth study was conducted to demonstrate similar CAR-mediated effects in this rat strain. In this study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate AhR, CAR, PXR and PPARα and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary hepatocytes isolated from male Wistar rats at fluxapyroxad concentrations ranging from 1 to 100 µmol/L. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. No positive controls for the induction of AhR or PPARα were included. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

The methods to assess activation of AhR, CAR, PXR and PPARα and stimulation of cell proliferation/replicative DNA synthesis were the same as described in Elcombe (2016c) above.

The effects of fluxapyroxad on cytochrome P450 activity in Wistar rat hepatocytes are presented in Table 9.

Fluxapyroxad induced a dose-dependent increase in CYP1A enzyme activity (EROD), up to about 6.5-fold, in Wistar rat hepatocytes. PB treatment at 500 µmol/L resulted in a 2.7-fold increase in CYP1A enzyme activity. CYP2B enzyme activity measured as PROD and BROD peaked at 10 µmol/L fluxapyroxad (13- and 12-fold increases, respectively), but declined at 30 and 100 µmol/L to about a 5.0-fold increase. This decrease was probably due to an inhibition of CYP2B by fluxapyroxad ($IC_{50}$ = 0.87 µmol/L), as was demonstrated by Elcombe (2016b). PB at 500 µmol/L induced similar increases in PROD (12-fold) and BROD (9.5-fold) (Table 9).

Fluxapyroxad induced a dose-dependent increase in CYP3A enzyme activity (BQ), up to 8.6-fold. PB treatment at 500 µmol/L resulted in a 2.5-fold increase in CYP3A activity (Table 9).

The induction of CYP4A1 enzyme activity, measured by 12-hydroxylation of lauric acid (LAH), was marginally affected, as indicated by a maximum 2.8-fold increase. Treatment with PB resulted in a 1.7-fold increase in lauric acid hydroxylation (Table 9).

PPARα-induced peroxisomal proliferation, as measured by palmitoyl-CoA oxidation, was not affected by fluxapyroxad or PB.

The effects of fluxapyroxad on cytochrome P450 mRNA levels in Wistar rat hepatocytes are presented in Table 10.

Fluxapyroxad induced a small increase in CYP1A1 (up to 2.5-fold) and CYP1A2 mRNA (up to 4.6-fold) in Wistar rat hepatocytes. PB induced 1.8- and 1.9-fold increases in CYP1A1 and CYP1A2 mRNA, respectively (Table 10).

Dose-dependent inductions of CYP2B1 (up to about 46-fold) and CYP2B2 mRNA (up to about 19-fold) were observed in wild-type rat hepatocytes at fluxapyroxad concentrations up to 10 µmol/L. At higher fluxapyroxad concentrations, induction of CYP2B1 and CYP2B2 mRNA decreased to 11- and 7.2-fold at 100 µmol/L, respectively. Induction by 500 µmol/L PB was 100-fold for CYP2B1 and 56-fold for CYP2B2 mRNA (Table 10).

Fluxapyroxad dose-dependently increased CYP3A1 mRNA levels in Wistar rat hepatocytes up to about 70-fold. It is noted that the PXR receptor is functional in both types of hepatocytes. CYP3A1 mRNA induction was 23-fold after treatment with 500 µmol/L PB (Table 10).

Fluxapyroxad and PB induced no increase in CYP4A1 and Acox1 mRNA levels in Wistar rat hepatocytes. In fact, CYP4A1 mRNA levels tended to decrease with increasing fluxapyroxad levels (Table 10).

***Table 9. Induction of CYP1A, CYP2B, CYP3A and CYP4A as well as of PPARα-induced PCoA in Wistar rat hepatocytes treated with fluxapyroxad or PB[a]***

| Test item and concentration | EROD (pmol resorufin/min per milligram) | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-OH quinoline/min per milligram) | LAH (nmol 12-OH lauric acid/h per milligram) | PCoA (nmol NADH/min per milligram) |
|---|---|---|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 10.73 ± 0.43<br>(100.0 ± 4.0) | 0.143 ± 0.025<br>(100.0 ± 17.6) | 1.21 ± 0.09<br>(100.0 ± 7.3) | 0.062 ± 0.003<br>(100.0 ± 4.5) | 0.411 ± 0.035<br>(100.0 ± 8.6) | 0.563 ± 0.038<br>(100.0 ± 6.8) |
| Fluxapyroxad | | | | | | |
| 1 µmol/L | 15.33 ± 3.01<br>(142.9 ± 28.0) | 0.759 ± 0.075***<br>(532.0 ± 52.2) | 5.48 ± 0.44***<br>(454.2 ± 36.8) | 0.100 ± 0.002***<br>(162.6 ± 3.4) | 0.660 ± 0.210<br>(160.7 ± 51.2) | 0.427 ± 0.078<br>(75.9 ± 13.9) |
| 3 µmol/L | 27.17 ± 2.22***<br>(253.2 ± 20.7) | 1.408 ± 0.203***<br>(986.7 ± 141.9) | 10.09 ± 0.66***<br>(835.5 ± 55.0) | 0.149 ± 0.008***<br>(241.5 ± 12.4) | 0.845 ± 0.121**<br>(205.6 ± 29.4) | 0.493 ± 0.133<br>(87.6 ± 23.7) |
| 10 µmol/L | 41.74 ± 6.99**<br>(389.0 ± 65.1) | 1.897 ± 0.082***<br>(1 329.2 ± 57.2) | 14.40 ± 0.23***<br>(1 192.7 ± 19.3) | 0.305 ± 0.017***<br>(492.8 ± 27.4) | 0.915 ± 0.091***<br>(222.7 ± 22.2) | 0.478 ± 0.194<br>(84.9 ± 34.4) |
| 30 µmol/L | 57.27 ± 5.12***<br>(533.9 ± 47.7) | 1.649 ± 0.243***<br>(1 155.5 ± 170.3) | 13.17 ± 0.30***<br>(1 090.7 ± 25.0) | 0.533 ± 0.030***<br>(861.8 ± 49.0) | 1.136 ± 0.217**<br>(276.4 ± 52.9) | 0.371 ± 0.003***<br>(65.8 ± 0.5) |
| 100 µmol/L | 70.12 ± 4.89***<br>(653.6 ± 45.6) | 0.715 ± 0.030***<br>(501.1 ± 21.2) | 6.12 ± 0.27***<br>(507.2 ± 22.7) | 0.491 ± 0.013***<br>(794.3 ± 20.6) | 1.076 ± 0.091***<br>(261.9 ± 22.1) | 0.483 ± 0.137<br>(85.8 ± 24.4) |
| PB | | | | | | |
| 500 µmol/L | 29.29 ± 2.72***<br>(273.0 ± 25.4) | 1.664 ± 0.043***<br>(1 165.8 ± 30.3) | 11.49 ± 0.35***<br>(951.9 ± 28.8) | 0.157 ± 0.004***<br>(253.3 ± 6.6) | 0.695 ± 0.045***<br>(169.3 ± 10.9) | 0.444 ± 0.136<br>(78.8 ± 24.2) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; EROD: ethoxyresorufin *O*-deethylation; LAH: 12-hydroxylation of lauric acid; NADH: nicotinamide adenine dinucleotide (reduced); PB: phenobarbital; PCoA: peroxisomal acyl-coenzyme A oxidase; PPARα: peroxisome proliferator–activated receptor alpha; PROD: pentoxyresorufin *O*-dependentylation; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Values in parentheses are mean % control ± standard deviation.

*Source*: Elcombe (2016d)

***Table 10. Induction of CYP1A1/1A2, CYP2B1/2B2, CYP3A1, CYP4A1 and Acox1 mRNA in Wistar rat hepatocytes treated with fluxapyroxad or PB[a]***

| Test item and concentration | CYP1A1 mRNA | CYP1A2 mRNA | CYP2B1 mRNA | CYP2B2 mRNA | CYP3A1 mRNA | CYP4A1 mRNA | Acox1 mRNA |
|---|---|---|---|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 1.00 ± 0.10 | 1.00 ± 0.03 | 1.00 ± 0.41 | 1.00 ± 0.36 | 1.00 ± 0.26 | 1.00 ± 0.23 | 1.00 ± 0.13 |
| Fluxapyroxad | | | | | | | |
| 1 µmol/L | 1.12 ± 0.05 | 1.16 ± 0.10 | 31.30 ± 3.40*** | 15.00 ± 1.03*** | 5.77 ± 0.22*** | 0.87 ± 0.14 | 0.98 ± 0.05 |
| 3 µmol/L | 1.05 ± 0.07 | 1.85 ± 0.39* | 44.89 ± 20.48* | 18.02 ± 3.31*** | 12.63 ± 0.67*** | 0.47 ± 0.14* | 0.88 ± 0.05 |
| 10 µmol/L | 1.11 ± 0.15 | 2.08 ± 0.30** | 46.44 ± 4.98*** | 18.61 ± 2.26*** | 33.57 ± 3.93*** | 0.40 ± 0.05* | 0.94 ± 0.03 |
| 30 µmol/L | 1.34 ± 0.29 | 2.52 ± 0.39** | 27.30 ± 13.59* | 12.71 ± 2.85** | 70.26 ± 6.91*** | 0.25 ± 0.12** | 1.00 ± 0.17 |
| 100 µmol/L | 2.46 ± 0.11*** | 4.59 ± 0.68*** | 10.79 ± 2.15** | 7.23 ± 2.54* | 69.89 ± 2.10*** | 0.27 ± 0.03** | 1.41 ± 0.15* |
| PB | | | | | | | |
| 500 µmol/L | 1.82 ± 0.16** | 1.85 ± 0.06*** | 99.82 ± 6.37*** | 56.23 ± 2.31*** | 23.00 ± 1.61*** | 1.13 ± 0.10 | 1.06 ± 0.10 |

Acox1: acyl-coenzyme A oxidase 1; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

*Source*: Elcombe (2016d)

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in rat hepatocytes are presented in Table 11.

***Table 11. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in Wistar rat hepatocytes[a]***

| Test item and concentration | S-phase labelling index (%) |
|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 10.40 ± 1.23<br>(100.0 ± 11.9) |
| Fluxapyroxad | |
| 1 µmol/L | 16.51 ± 1.44***<br>(158.8 ± 13.8) |
| 3 µmol/L | 24.45 ± 0.78***<br>(235.2 ± 7.5) |
| 10 µmol/L | 27.11 ± 2.15***<br>(260.7 ± 20.7) |
| 30 µmol/L | 26.83 ± 1.75***<br>(258.0 ± 16.9) |
| 100 µmol/L | 15.15 ± 2.62**<br>(145.7 ± 25.2) |
| PB | |
| 500 µmol/L | 16.09 ± 2.11***<br>(154.7 ± 20.3) |
| EGF | |
| 25 ng/mL | 52.37 ± 6.68***<br>(503.6 ± 64.2) |

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 5$ per group). Values in parentheses are mean % of control ± standard deviation.

*Source*: Elcombe (2016d)

Fluxapyroxad treatment of Wistar rat hepatocytes induced a dose-dependent, up to 2.6-fold increase in replicative DNA synthesis. Replicative DNA synthesis peaked at 10 and 30 µmol/L and decreased at 100 µmol/L, which was considered by the study author as probably being due to cytotoxicity at the highest dose. PB at 500 µmol/L induced a 1.5-fold increase in replicative DNA synthesis. EGF induced a 5.0-fold increase in replicative cell proliferation (Table 11).

This study showed that fluxapyroxad activates the nuclear hormone receptors CAR and PXR, inducing both S-phase (replicative DNA synthesis) and CYP2B/CYP3A mRNA levels and activity in Wistar rat primary hepatocytes. Inhibition of CYP2B was evident. A slight activation of AhR was observed, as shown by a small induction of CYP1A mRNA expression and enzyme activity. There was no evidence of PPARα activation by fluxapyroxad (Elcombe, 2016d).

In the fifth study, the potential of fluxapyroxad (batch no. COD-001049; purity 99.2%) to activate CAR and PXR and stimulate cell proliferation/replicative DNA synthesis was investigated in cultured primary human hepatocytes from two male donors. Donor A had no history of smoking, drug use or medication, whereas donor B was a smoker with a history of drug abuse and medication. The

effects of fluxapyroxad at concentrations ranging from 1 to 100 µmol/L were investigated. PB (500 µmol/L) was tested in parallel for comparison with fluxapyroxad. EGF (25 ng/mL) was used as a positive control for the induction of replicative DNA synthesis. Cultured hepatocytes were exposed to fluxapyroxad, PB or EGF for 3 days.

Activation of CAR was detected by induction of PROD and BROD (CYP2B) activities and CYP2B6 mRNA levels. Activation of PXR was detected by induction of BQ (CYP3A) activity and CYP3A4 mRNA levels. Cell proliferation was determined by replicative DNA synthesis, whereas cytotoxicity was assayed via determination of the ATP content relative to the vehicle control, DMSO.

The effects of fluxapyroxad and PB on CYP2B and CYP3A activities in human hepatocytes are presented in Table 12 for donor A and Table 13 for donor B.

***Table 12. Induction of CYP2B and CYP3A activities in human hepatocytes treated with fluxapyroxad or PB: donor A[a]***

| Test item and concentration | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-hydroxyquinoline/min per milligram) |
|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 0.191 ± 0.022 | 1.490 ± 0.200 | 0.178 ± 0.009 |
| | (100.0 ± 11.8) | (100.0 ± 13.4) | (100.0 ± 5.1) |
| Fluxapyroxad | | | |
| 1 µmol/L | 0.220 ± 0.025 | 2.491 ± 0.190** | 0.260 ± 0.005*** |
| | (115.3 ± 13.1) | (167.2 ± 12.7) | (146.1 ± 3.0) |
| 3 µmol/L | 0.275 ± 0.011** | 3.218 ± 0.036*** | 0.314 ± 0.025*** |
| | (144.3 ± 5.9) | (216.0 ± 2.4) | (176.6 ± 13.9) |
| 10 µmol/L | 0.332 ± 0.027** | 3.488 ± 0.080*** | 0.455 ± 0.013*** |
| | (174.2 ± 14.0) | (234.1 ± 5.3) | (256.1 ± 7.3) |
| 30 µmol/L | 0.344 ± 0.041** | 4.380 ± 0.406*** | 0.399 ± 0.053** |
| | (180.4 ± 21.6) | (294.0 ± 27.2) | (224.6 ± 30.1) |
| 100 µmol/L | 0.230 ± 0.017 | 2.821 ± 0.087*** | 0.066 ± 0.018*** |
| | (120.5 ± 8.8) | (189.3 ± 5.8) | (37.3 ± 10.4) |
| PB | | | |
| 500 µmol/L | 0.463 ± 0.019*** | 8.158 ± 0.441*** | 0.939 ± 0.036*** |
| | (242.7 ± 10.2) | (547.5 ± 29.6) | (528.4 ± 20.2) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-dependentylation; v/v: volume per volume; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ replicate analyses at each concentration). Values in parentheses are mean % of control ± standard deviation.

*Source*: Elcombe (2016e)

***Table 13. Induction of CYP2B and CYP3A activities in human hepatocytes treated with fluxapyroxad or PB: donor B[a]***

| Test item and concentration | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-hydroxyquinoline/min per milligram) |
|---|---|---|---|
| Vehicle control (0.1% [v/v] DMSO) | 0.086 ± 0.009 | 0.579 ± 0.080 | 0.321 ± 0.034 |
| | (100.0 ± 10.8) | (100.0 ± 13.8) | (100.0 ± 10.5) |

| Test item and concentration | PROD (pmol resorufin/min per milligram) | BROD (pmol resorufin/min per milligram) | BQ (nmol 7-hydroxyquinoline/min per milligram) |
|---|---|---|---|
| Fluxapyroxad | | | |
| 1 µmol/L | 0.049 ± 0.014* | 0.670 ± 0.104 | 0.404 ± 0.005 |
| | (57.4 ± 16.6) | (115.7 ± 18.0) | (125.8 ± 1.4) |
| 3 µmol/L | 0.042 ± 0.006** | 0.679 ± 0.044 | 0.508 ± 0.025** |
| | (49.1 ± 7.0) | (117.2 ± 7.7) | (158.4 ± 7.8) |
| 10 µmol/L | 0.051 ± 0.010* | 0.908 ± 0.147* | 0.743 ± 0.019*** |
| | (60.1 ± 11.3) | (156.7 ± 25.3) | (231.8 ± 6.0) |
| 30 µmol/L | 0.057 ± 0.009* | 0.959 ± 0.130* | 0.796 ± 0.027*** |
| | (66.5 ± 10.2) | (165.6 ± 22.4) | (248.1 ± 8.5) |
| 100 µmol/L | 0.105 ± 0.032 | 1.019 ± 0.203* | 0.107 ± 0.017*** |
| | (122.2 ± 37.4) | (176.0 ± 35.1) | (33.3 ± 5.5) |
| PB | | | |
| 500 µmol/L | 0.111 ± 0.008* | 1.789 ± 0.091*** | 1.236 ± 0.117*** |
| | (129.3 ± 9.7) | (309.0 ± 15.6) | (385.3 ± 36.4) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; DMSO: dimethyl sulfoxide; PB: phenobarbital; PROD: pentoxyresorufin *O*-dependentylation; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ replicate analyses at each concentration). Values in parentheses are mean % of control ± standard deviation.

*Source*: Elcombe (2016e)

In hepatocytes from donor A (Table 12), CYP2B activity was increased by fluxapyroxad, as shown by increased PROD (1.8-fold) and BROD (2.9-fold) activities at 30 µmol/L. CYP3A (BQ) activity peaked at 10 µmol/L, with a 2.6-fold increase. The lower enzyme activities at 100 µmol/L may be due to cytotoxicity or cytochrome P450 inhibition. Basal activity of PROD was average, whereas those of BROD and BQ were at the upper end of the historical control ranges observed in the laboratory (Table 14).

***Table 14. Historical control data for PROD, BROD and BQ activities in rat and human hepatocytes***

| | PROD | BROD | BQ |
|---|---|---|---|
| Rat ($n = 18$) | | | |
| Vehicle[a] | 0.30 (0.04–1.33) | 1.11 (0.27–2.53) | 0.05 (0.03–0.08) |
| 500 µmol/L PB[b] | 6.2 (2.2–11.7) | 10.3 (6.7–14.3) | 2.5 (2.0–2.8) |
| Human ($n = 19$) | | | |
| Vehicle[a] | 0.19 (0.05–0.52) | 0.84 (0.27–1.91) | 0.11 (0.02–0.24) |
| 500 µmol/L PB[b] | 1.5 (0.9–2.4) | 4.4 (2.9–6.0) | 5.3 (3.9–8.5) |

BQ: benzyloxyquinoline *O*-debenzylation; BROD: benzyloxyresorufin *O*-debenzylation; *n*: number of studies conducted between 2010 and 2016; PB: phenobarbital; PROD: pentoxyresorufin *O*-dependentylation

[a] Basal activity in pmol substrate/minute per milligram protein; data are mean, with minimum–maximum in parentheses.

[b] Fold induction above controls; data are mean, with minimum–maximum in parentheses.

*Source*: Elcombe (2016e)

In hepatocytes from donor B (Table 13), PROD was slightly increased only at the highest concentration (1.2-fold), whereas BROD was dose-dependently increased from 10 µmol/L onwards,

with a maximum 1.8-fold increase at 100 µmol/L. The study author noted that BROD activity is considered a superior marker of CYP2B activity in human hepatocytes compared with PROD (Mudra & Parkinson, 2004). Furthermore, the observed CYP2B6 activity induction is consistent with the induction of CYP2B6 mRNA (see below). CYP3A4 activity as determined by BQ peaked at 30 µmol/L, with a 2.5-fold increase, and substantially declined to one third of the control activity at 100 µmol/L. Basal activities of PROD and BROD in hepatocytes of donor B were at the lower end of the historical control ranges, whereas the BQ turnover was almost 1.5-fold higher than the maximum observed in previous studies (Table 14).

PB treatment resulted in 2.4-, 5.5- and 5.3-fold increases in PROD and BROD activities and BQ turnover in donor A (Table 12) and 1.3-, 3.1- and 3.9-fold increases in donor B (Table 13), respectively. Generally, the magnitude of all responses to PB treatment was within the historical control range.

The effects of fluxapyroxad and PB on CYP2B6 and CYP3A4 mRNA levels in hepatocytes of donors A and B are presented in Table 15.

***Table 15. Induction of CYP2B6 and CYP3A4 mRNA in human hepatocytes treated with fluxapyroxad or PB[a]***

| | Donor A | | Donor B | |
|---|---|---|---|---|
| **Test item and concentration** | **CYP2B6 mRNA** | **CYP3A4 mRNA** | **CYP2B6 mRNA** | **CYP3A4 mRNA** |
| Basal Ct[b] | 24.73 ± 0.59 | 21.75 ± 0.65 | 25.88 ± 0.19 | 19.57 ± 0.42 |
| Vehicle control (0.1% [v/v] DMSO) | 1.00 ± 0.20 | 1.00 ± 0.20 | 1.00 ± 0.41 | 1.00 ± 0.37 |
| Fluxapyroxad | | | | |
| 1 µmol/L | 1.57 ± 0.03** | 1.23 ± 0.11 | 1.27 ± 0.13 | 1.27 ± 0.27 |
| 3 µmol/L | 3.39 ± 2.41 | 1.66 ± 0.86 | 0.87 ± 0.22 | 0.98 ± 0.27 |
| 10 µmol/L | 3.49 ± 0.32*** | 3.70 ± 0.93** | 2.00 ± 0.84 | 1.58 ± 0.09 |
| 30 µmol/L | 4.41 ± 0.95** | 3.26 ± 1.37* | 3.33 ± 0.54** | 1.75 ± 0.42 |
| 100 µmol/L | 3.57 ± 0.12*** | 1.18 ± 0.09 | 1.20 ± 0.11 | 0.23 ± 0.06* |
| PB | | | | |
| 500 µmol/L | 7.84 ± 0.27*** | 7.52 ± 1.31** | 8.18 ± 3.74* | 5.86 ± 0.23*** |

Ct: cycle threshold; CYP: cytochrome P450; DMSO: dimethyl sulfoxide; mRNA: messenger ribonucleic acid; PB: phenobarbital; v/v: volume per volume; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 3$ per group). Results are expressed as fold change relative to controls, where control values were normalized to 1.00. Rat β-actin was employed as the internal control.

[b] Calculated from individual Taqman® mRNA data.

*Source*: Elcombe (2016e)

For donor A, basal levels of CYP2B6 mRNA were about average, whereas CYP3A4 mRNA levels were in the upper third of the historical range (Table 16; the lower the Ct value, the higher the mRNA concentration). For donor B, the CYP3A4 basal levels in particular were at the uppermost end of the historical control range, whereas CYP2B6 mRNA basal levels tended towards the lower third of the historical control range. Induction of CYP2B6 and CYP3A4 by 500 µmol/L PB was average compared with the historical control range. Induction of CYP3A4 mRNA in donor B by 500 µmol/L PB was at the low end of the historical control range. It seems that the already high basal level of CYP3A4 mRNA was somehow difficult to increase further.

***Table 16. Historical control data for basal Ct values and fold induction values for CYP2B6 and CYP3A4 in human hepatocytes after treatment with 500 µmol/L PB[a]***

| | CYP2B6 | | CYP3A4 | |
|---|---|---|---|---|
| | **Basal Ct** | **500 µmol/L PB** | **Basal Ct** | **500 µmol/L PB** |
| Human (*n* = 10) | 24.72 (23.0–26.9) | 8.33 (7.67–9.62)[b] | 23.24 (19.6–27.5) | 7.85 (5.86–41.7)[b] |

Ct: cycle threshold; CYP: cytochrome P450; PB: phenobarbital; *n*: number of studies performed between 2010 and 2016; the data were provided by CXR Biosciences Ltd on request

[a] Data are mean, with minimum–maximum in parentheses.

[b] Fold induction above control.

*Source*: Elcombe (2016e)

The 400- to 700-fold induction of CYP2B mRNA in wild-type hepatocytes of Sprague Dawley rats by 500 µmol/L PB (Elcombe, 2016c) was considerably higher than in human hepatocytes. However, it is noted that the maximum mRNA induction by fluxapyroxad was about half that of PB in rat hepatocytes as well as in human hepatocytes. Thus, with respect to CYP2B induction, human hepatocytes of both donors responded as expected.

The effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in human hepatocytes are presented in Table 17.

***Table 17. Effects of fluxapyroxad, PB and EGF on replicative DNA synthesis in human hepatocytes[a]***

| | S-phase labelling index (%) | |
|---|---|---|
| **Test item and concentration** | **Donor A** | **Donor B** |
| Vehicle control (0.1% [v/v] DMSO) | 0.33 ± 0.09<br>(100.0 ± 28.0) | 0.48 ± 0.07<br>(100.0 ± 15.5) |
| Fluxapyroxad | | |
| 1 µmol/L | 0.34 ± 0.11<br>(102.1 ± 32.8) | 0.47 ± 0.19<br>(97.9 ± 38.5) |
| 3 µmol/L | 0.38 ± 0.16<br>(114.1 ± 49.5) | 0.50 ± 0.21<br>(102.8 ± 43.4) |
| 10 µmol/L | 0.33 ± 0.06<br>(99.6 ± 18.7) | 0.55 ± 0.11<br>(113.6 ± 23.0) |
| 30 µmol/L | 0.30 ± 0.11<br>(90.0 ± 33.6) | 0.48 ± 0.17<br>(98.8 ± 36.3) |
| 100 µmol/L | 0.07 ± 0.07***[b]<br>(21.6 ± 19.7) | 0.13 ± 0.09***<br>(26.6 ± 18.9) |
| PB | | |
| 500 µmol/L | 0.41 ± 0.09<br>(122.1 ± 26.4) | 0.64 ± 0.19<br>(132.6 ± 40.1) |
| EGF | | |
| 25 ng/mL | 4.50 ± 0.89***<br>(1 352.2 ± 268.6) | 3.47 ± 0.38***<br>(719.7 ± 77.9) |

DMSO: dimethyl sulfoxide; DNA: deoxyribonucleic acid; EGF: epidermal growth factor; PB: phenobarbital; v/v: volume per volume; ***: $P < 0.001$ (Student's *t*-test, two-sided)

[a] Values are mean ± standard deviation ($n = 5$ per group). Values in parentheses are mean % control ± standard deviation.

[b] Cytotoxicity observed.

*Source*: Elcombe (2016e)

Fluxapyroxad did not increase the S-phase labelling index. Actually, at 100 µmol/L, a decreased labelling index was noted for both donors, which was considered to be due to cytotoxicity. At least for donor A, cytotoxicity was visually evident by fewer hepatocytes, resulting in poorer confluency. Furthermore, PB also did not increase replicative DNA synthesis, whereas EGF caused a 14- and 7.2-fold increase of the S-phase labelling index in hepatocytes from donors A and B, respectively, indicating that the human hepatocytes were able to proliferate.

The study indicates that fluxapyroxad and PB increase CAR/PXR-mediated CYP2B6 and CYP3A4 mRNA levels and enzyme activity in human hepatocytes. However, fluxapyroxad and PB did not induce proliferation of human hepatocytes, whereas human hepatocytes could be stimulated to proliferation by the positive control substance, EGF (Elcombe, 2016e).

## Comments

### Toxicological data

In vitro studies in CAR wild-type and knockout rat hepatocytes show that fluxapyroxad can activate the nuclear receptors CAR and PXR, leading to the induction of the P450 enzymes CYP2B and CYP3A and hepatocellular proliferation. Qualitatively, these effects of fluxapyroxad are similar to those of PB (Elcombe, 2016a,b,c,d).

An in vitro study using human hepatocytes showed that, similar to PB, fluxapyroxad can activate human CAR and PXR, but does not stimulate proliferation of these cells (Elcombe, 2016e).

## Toxicological evaluation

The new in vitro studies support the conclusion of the 2012 Meeting that high doses of fluxapyroxad cause hepatocellular adenomas and carcinomas in rats through a mitogenic mode of action associated with induction of CYP2B-type P450, and that this mode of action does not occur in humans.

The Meeting concluded that the new studies support the existing acceptable daily intake (ADI) of 0–0.02 mg/kg body weight (bw) and have no impact on the acute reference dose (ARfD) of 0.3 mg/kg bw established in 2012.

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